RESEARCH ARTICLE

Isolation of *Sphaerotilus–Leptothrix* strains from iron bacteria communities in Tierra del Fuego wetlands

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Abstract

Sheath-forming iron- and manganese-depositing bacteria belonging to the *Sphaerotilus–Leptothrix* group (SLG) are widespread in natural and artificial water systems. Known requirements for their growth include the presence of organic substrates and molecular oxygen. High concentrations of reduced iron or manganese, although not necessary for most species, make their growth a noticeable phenomenon. Such microbial communities have been studied mostly in the Northern Hemisphere. Here, we present descriptions of diverse ochre-depositing microbial communities in Tierra del Fuego, Argentina, using a combined approach of microscopical examination, clone library construction and cultivation focused on SLG bacteria. To date, only few SLG type strains are available. The present work increases the number and diversity of cultivated SLG bacteria by obtaining isolates from biofilms and sediment samples of wetlands in Tierra del Fuego. Thirty isolates were selected based on morphological features such as sheath formation and iron/manganese deposition. Five operational taxonomic units (OTUs) were deduced. Sequencing of 16S rRNA genes showed that one OTU is identical to the *Leptothrix mobilis* Feox-1^T-sequence while the four remaining OTUs show similarity values related to previously described type strains. Similarity values ranged from 96.5% to 98.8%, indicating possible new species and subspecies.

Introduction

The cold and temperate regions of the Northern Hemisphere harbor wetlands known for a long time to be populated by a broad diversity of iron-depositing bacteria (Naumann, 1921; Mulder & Van Veen, 1963; Haaijer *et al.*, 2008; Wang *et al.*, 2009). Due to the activity of large numbers of iron bacteria, visible ochreous deposits are formed in zones supplied with ferrous iron and at least low concentrations of oxygen. Those zones occur in numerous environments including aquatic systems, aerobic microhabitats in the rhizosphere of wetland plants (Emerson *et al.*, 1999; Neubauer *et al.*, 2007), and aerobic/anaerobic transition zones in sediments or soils where long-term enrichment of iron may even lead to formation of economically exploitable bog iron ore (Crerar *et al.*, 1979; Szewzyk *et al.*, 2011).

One of the most prominent groups of iron bacteria in these wetlands are sheath-forming bacteria which, based on their remarkable morphology, were classified in the genera *Leptothrix* and *Sphaerotilus* and gained attention since the early days of microbiology (Roth, 1797; Kützing, 1843; Winogradsky, 1888). The high diversity of these bacteria in natural samples led to descriptions of many species of the *Sphaerotilus–Leptothrix* group (*SLG)* based mainly on morphology. Studies of the group often based on enrichment cultures and only few axenic isolates are available. Unfortunately, some of those cultures have not been deposited in public collections and were lost before their phylogenetic position could be clarified by DNA sequencing (Spring, 2006). This situation led to confusion about matching experimental data to known species names, many of which are based primarily on morphology and are not valid using modern taxonomic requirements

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(Tindall *et al.*, 2010). A significant discrepancy between observable SLG diversity in nature and lack of available isolates limited further characterization of the group (Spring, 2006). Recent efforts led to descriptions of two additional species and one subspecies (Gridneva *et al.*, 2011), extending the formerly monotypic genus *Sphaerotilus*. Within the genus *Leptothrix,* type strains remain available for *L. mobilis* (Feox1) and *L. discophora* (SS1). For *L. cholodnii,* other strains (SP6 and CCM 1827) have been used as the assigned type strain LVMW 99 has been lost as has been the type strain, *L. lopholea* LVMW 124. *Leptothrix ochracea* dominates many iron-oxidizing freshwater communities and is considered the type species of *Leptothrix.* Although ribosomal sequence information has been gained by culture independent methods (Fleming *et al.*, 2011), *L. ochracea* remains uncultured.

This work presents a combined approach to study several newly found neutrophilic iron-depositing communities from Tierra del Fuego, Argentina.

L. ochracea is mentioned frequently in descriptions of iron-depositing microbial communities although the diversity of SLG in those habitats is much higher. While this finding was repeatedly described by microscopists (Naumann, 1921; Cholodny, 1926; Dorff, 1934), it seems not to be adequately reflected in cultivation-based or genetic diversity studies. The aim of this study was to learn more about the diversity of sheathed iron bacteria in nature. This was carried out by searching for uncharted morphologically diverse iron bacteria communities and applying cultivation on unusual solid media to make more SLG species available. Lepto-A medium was developed for isolation and preservation of 'wild-type' SLG-morphology. Also, culture independent methods were applied, namely clone libraries and microscopy to identify iron bacteria genetically or by comparing them to known morphotypes from the Northern Hemisphere.

Materials and methods

Bacterial strains

Reference strains for morphological and phylogenetic comparison with the isolates were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany), ATCC (American Type Culture Collection, USA) and CCM (Czech Collection of Microorganisms, Czech Republic). The following strains were used: *L. cholodnii* CCM 1827, *L. mobilis* DSM 10617^T, *L. discophora* ATCC 43182^T, *L. sp.* CCM 2812 (received under the name of *L. discophora*), *S. natans* DSM 6575^T, *S. montanus* DSM 565^T, and *S. hippei* DSM 566^T. Their 16S rRNA gene was sequenced to validate identity by comparing them to available sequences in public databases.

In addition to reference strains from public collections, a variety of strains belonging to the SLG was previously isolated from different sources by our working group. Five of them were included in phylogenetic comparisons to increase the diversity of known members of the SLG and support phylogenetic tree construction. Strains OT B 406 (JQ945994), OT B 418 (JQ945995), and OT B 441 (JQ945993) were isolated from iron-encrusted biofilm samples, and OT B 607 (JQ945996) was isolated from planktonic, iron bacteria aggregates. All were obtained from Bogengraben in the National Park Unteres Odertal, Germany. Strain W 153 (JQ946031) was isolated from an ochre sample taken from a drinking water well in Berlin, Germany (Thronicker & Szewzyk, 2011).

Sampling

During the Patagonian summers in 2009 and 2011, two expeditions were undertaken to the southern part of Tierra del Fuego Island, Argentina, to search for habitats harboring iron bacteria. Several springs, brooks, and ponds, containing macroscopically visible depositions of ferric iron were found, often scattered in extended wetlands. Sampling sites were named alphabetically, and their global position was recorded (Table 2). Measurements of ferrous iron, manganese, and water hardness were conducted via colorimetric test strips (Merckoquant, Merck, Darmstadt, Germany) in January 2009. The pH, dissolved O_2 and conductivity were recorded using a portable instrument for field measurement (U10, Horiba, Kyoto, Japan) in January 2011.

Different types of samples were taken, including water-saturated soil, sediment, parts of microbial mats, and biofilms grown on plant or stone surfaces. Samples were collected directly into sterile, 2-mL screw-cap tubes or 15-mL centrifuge tubes. Thin biofilm samples especially convenient for microscopic analysis and also used for isolation were obtained by placing glass slides in water bodies using cork floaters and nylon strings to suspend them at a defined depth and position. Additionally, glass slides were placed vertically into sediment layers. Once biofilm formation was visible, slides were transferred into sterile, 50-mL centrifuge tubes containing water from the sampling site. After transportation in cooling bags for about 1–4 h (depending on the distance from the sampling site), samples were stored as follows: at about 8 °C for microscopy and isolation and at about −10 °C for clone library construction. Samples for isolation were transported to Germany which required storage of about 4 days.

Microscopic observations

Environmental samples were microscopically examined to detect iron bacteria morphotypes at the CADIC Institute

Table 1. Sampling sites and isolates

Sampling site	Position (Latitude; Longitude)	Sample type	Isolate	OTU
G	S 54° 46.592 W 67° 41.944	Biofilm	FG 2	I
			FG 8	I
		Sediment	FG 62	I
			FG 67	I
			FG 68	II
C	S 54° 45.602 W 68° 11.746	Biofilm*	FC 121	V
		Sediment	FC 116	V
			FC 117	III
			FC 118	IV
			FC 119	III
			FC 125	III
			FC 126	III
			FC 127	IV
			FC 170	V
			FC 178	V
			FC 181	IV
			FC 183	V
H	S 54° 56.459 W 66° 54.017	Biofilm	FH 163	II
			FH 23	V
			FH 93	V
			FH 36	V
		Sediment	FH 15	V
			FH 10	V
			FH 11	V
			FH 14	V
J	S 54° 51.998 W 67° 16.767	Biofilm	FJ 128	V
			FJ 135	V
			FJ 136	V
			FJ 138	IV
			FJ 139	V
			FJ 189	III
E	S 54° 42.857 W 68° 03.134	Sediment	FE 140	V
			FE 141	V
			FE 142	V

*Biofilm harvested from submerged grass sample, all other biofilms originated from exposed glass slides.

in Ushuaia, Tierra del Fuego, using a Leica DM 2500 microscope, equipped with 10× up to 100× objectives and a reflex camera mounted on a trinocular tube. Examinations were performed using bright field (BF), dark field, and differential interference contrast (DIC).

Morphotypes of iron bacteria were identified using morphology described in the iron bacteria literature and the field guide, 'Schizomycetes' (Häusler, 1982). Pictures were processed including frame cropping, brightness, contrast, and white-point adjustment. Size bars were inserted based on photos of a microscale object slide.

Construction of clone libraries

Two samples of microbial mats from sites G and H were chosen for community analysis by clone library construction. Both samples were dominated by SLG morphotypes and thus considered promising to yield different SLG sequences. Sample G was taken from a cotton-like mat developing in a stagnating zone while sample H was a light ochre-colored, roundish pellet from a stream. DNA was extracted with FastDNA SPIN Kit for Soil and a FastPrep-instrument (MP Biomedicals, Eschwege, Germany) following the manufacturers protocol. PCR amplification of 16S rRNA gene was performed using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Weisburg *et al.*, 1991) and the following thermal cycling program: initial step 97 °C for 2 min, 35 cycles of 97 °C for 1 min, 58 °C for 45 s, and 72 °C for 2 min, final elongation step 72 °C for 7 min. Amplicons were purified using an AgaroseOut Gel-Extraction DNA Kit (Roboklon, Berlin, Germany) following the manufacturer's protocol. Cloning and sequencing was performed by an external service provider (Macrogen, Seoul, Korea). For each sample, 96 clone sequences were obtained. Sequences were checked for chimeras using the Decipher online tool (Wright *et al.*, 2012). Remaining sequences were assigned with the Ribosomal Database Project Classifier (RDP Classifier) (Wang *et al.*, 2007) and in comparison analyzed with the nucleotide BLAST comparison tool (Altschul *et al.*, 1990) using the 'Nucleotide collection (nr/nt)' database. Sequences which could not be clearly assigned with the RDP classifier, and BLAST were aligned in ARB (Ludwig *et al.*, 2004) using the Silva database SSU Ref NR 115 from August 2013 (Pruesse *et al.*, 2007). The alignment was checked manually and group-specific trees were constructed with Neighbor Joining (Saitou & Nei, 1987) using termini filter and correction after Jukes and Cantor (Jukes & Cantor, 1969).

Culture media

Lepto-A medium was designed for both isolation and maintenance of iron- depositing bacteria, based on Manganese–Agar (Mulder & Van Veen, 1963). Compared to Manganese–Agar, Lepto-A was modified with respect to the type and concentration of organic substrates, vitamins, and trace elements. The composition was as follows: Lepto-A in g L^{-1}: $MnCO_3$ hydrate, 2; yeast extract (Roth, Karlsruhe, Germany), 0.5; trisodium citrate, 0.15; $Fe(NH_4)_2(SO_4)_2$, 0.2. In addition, 2 mL L^{-1} of both vitamin solution and trace element solution was added, whose compositions were as follows: vitamin solution in mg L^{-1}: biotin, 2; nicotinic acid, 20; thiamin, 10; 4-aminobenzoate, 10; panthotenate, 5; pyridoxamine, 50; cyanocobalamin, 20 (Schlegel & Zaborosch, 1992) and trace element solution in g L^{-1}: nitrilotriacetic acid, 12.8; $FeCl_2.4H_2O$ 1.5; in mg L^{-1}: $ZnCl_2$, 70; $MnCl_2.2H_2O$, 80; H_3BO_3, 6; $CoCl_2.6H_2O$, 190; $CuCl_2.2H_2O$, 2; $NiCl_2.6H_2O$, 24; $NaMoO_4.2H_2O$, 36 (Pfennig & Tschech, 1984). Dur-

ing initial isolation steps, 1 mL L^{-1} of sterile cycloheximide solution (100 mg mL^{-1} in 60% v/v ethanol) was added to suppress growth of eukaryotes, especially fungi (Ha *et al.*, 1995). A variant of Lepto-A medium was also developed by omitting yeast extract and adding 0.6 mL L^{-1} of peat extract (Sera® Blackwater Aquatan, Heinsberg, Germany). Solid Lepto-A additionally contained 20 g L^{-1} of Bacto™ Agar (Becton Dickinson, Franklin Lakes). In all cultivation media, the pH was adjusted to 7.

Isolation procedure, strain maintenance, and cryopreservation

Biofilms were harvested from glass slides under sterile conditions with straight-edged scalpels and transferred into 1.5-mL tubes containing 0.9 mL of PBS buffer. Similarly, small portions of about 0.1 g wet weight of sediments were transferred into tubes. Samples were homogenized using a PTFE pestle homogenizer in the 1.5-mL tubes. Serial decimal dilutions were made in PBS, and 100 μL was plated onto Lepto-A agar plates. Plates were incubated at room temperature and monitored for 1 week or longer for occurrence of dark colonies, assuming that such coloration was due to iron and/or manganese oxide deposition. After growth, colonies were isolated using repeated dilution streaks on the same medium.

The isolates were named following the structure F[sampling site] [number of isolate] (e.g.: FG 67 for isolate from site G, number 67).

Distinctive cell morphological features, such as filamentous growth, were monitored throughout incubation using a Zeiss Axioscope microscope with BF or DIC, or a Leica DM1000 microscope equipped with phase contrast.

Iron or manganese deposition activity of the isolates was verified by checking the presence of visible extracellular deposits in the microscopic appearance to avoid misclassification of pigmented strains as depositing bacteria. This feature was confirmed by dissolving the deposits with dilute oxalic acid which led to decoloration at both macro- and microscopic scales. If both iron and manganese or only one of these metals contributed to formation of deposits was examined by cultivation on Lepto-A variants containing either manganese or iron.

Pure cultures were incubated at room temperature and maintained by transfer on Lepto-A agar every 3–4 weeks. For every strain, several grown plates were stored at 4 °C as backups. As many strains did not respond to a standard cryopreservation protocol using 15% v/v glycerol as cryoprotectant, 1-mL portions of actively growing cultures in liquid Lepto-A were directly frozen without additives in liquid nitrogen using 2-mL screw-cap tubes. Enhancement of regrowth was achieved by incubating the thawed samples at room temperature for 3–6 days before streaking them out on Lepto-A agar.

Molecular characterization of the isolates

Extraction of genomic DNA was performed following a modified protocol (Sambrook *et al.*, 1989) as follows: colony material was harvested, washed twice with 1 mL of sterile deionised water, and collected by centrifugation at 8000 ***g***. Cells were resuspended in 200 μL of solution A (Tris 50 mM, pH 8.0; EDTA 50 mM, pH 8.0; saccharose 25% w/v). RNAse (10 mg mL^{-1}) and lysozyme (20 mg mL^{-1}) were added, and the mixture was incubated at 37 °C for 50 min. After incubation, 400 μL of solution B (Tris 10 mM, pH 8.0; EDTA 5 mM, pH 8.0; SDS 1% w/v) was added and mixed by inversion. Then, 20 μL of proteinase K (40 mg mL^{-1}) was added and incubated for 1 h at 56 °C. Once the mixture reached room temperature, DNA was extracted by the phenol/chloroform procedure as described by Sambrook *et al.* (1989) and pure DNA was dissolved in 50 μL of sterile water. DNA purity was assessed from A_{260}/A_{280} and A_{260}/A_{230} extinction ratios (Johnson, 1994) using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham).

Two pairs of universal bacterial primers labeled as 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′) (Weisburg *et al.*, 1991), and 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1387R (5′-GGGCGGWGTGTACAAGGC-3′) (Marchesi *et al.*, 1998) were used for amplification of nearly complete 16S rRNA genes by PCR. Detection and sizes of PCR products were confirmed by agarose gel electrophoresis (0.8% w/v).

Isolates were first grouped using ARDRA as described by Vaneechoutte *et al.* (1993) with AluI protein (data not shown) which was confirmed by sequencing and resulted in OTUs I to V.

Sequences of respective 16S rRNA genes were determined by an external service provider (Macrogen). Primer pair 63F-1387R was used for all isolates. Additionally, 27F-1541R was utilized in one representative strain of each OTU (those sequences are printed in bold type in Fig. 1), as well as for the SLG reference strains mentioned above. Sequence chromatograms were trimmed of low quality portions and assembled using BIONUMERICS software (Applied Maths, Sint-Martens-Latem, Belgium). Resulting consensus sequences were deposited in the NCBI GenBank database under accession numbers JQ945993 to JQ946031.

Sequences were aligned and compared to those published in public databases (GenBank, Ez-taxon) using the BLAST tool. Further sequence analysis of the isolates for phylogenetic tree construction of the SLG was performed with the aid of the ARB program. The alignment was car-

ried out based on the SILVA alignment and revised manually. Type strain sequences of closely related genera were included in the alignment, and 16 type strain sequences of *Burkholderia* were used as out-group. Construction of a similarity matrix (data not shown) and phylogenetic dendrogram was carried out using the neighbor-joining method and Jukes–Cantor distance correction.

Results and discussion

Sampling sites

Iron-depositing communities in freshwater at circumneutral pH have been studied for many decades. However, to our knowledge, this was restricted to the Northern Hemisphere. This work presents the first report of such communities in numerous iron-rich wetland sites that are widespread in the southern part of Tierra del Fuego. A total of 15 sampling sites were identified based on macroscopically visible ochre deposition and named A to R. The SLG isolates presented here originate from five of them namely C, E (Fig. 2a), G, H (Fig. 2b), and J. Positioning and description of the sampling sites are available online in a supplementary section.

Isolation and identification

Colonies of brown or black color were selected from the plated samples and about 70 strains of iron-depositing bacteria were successfully isolated. Thirty of them,

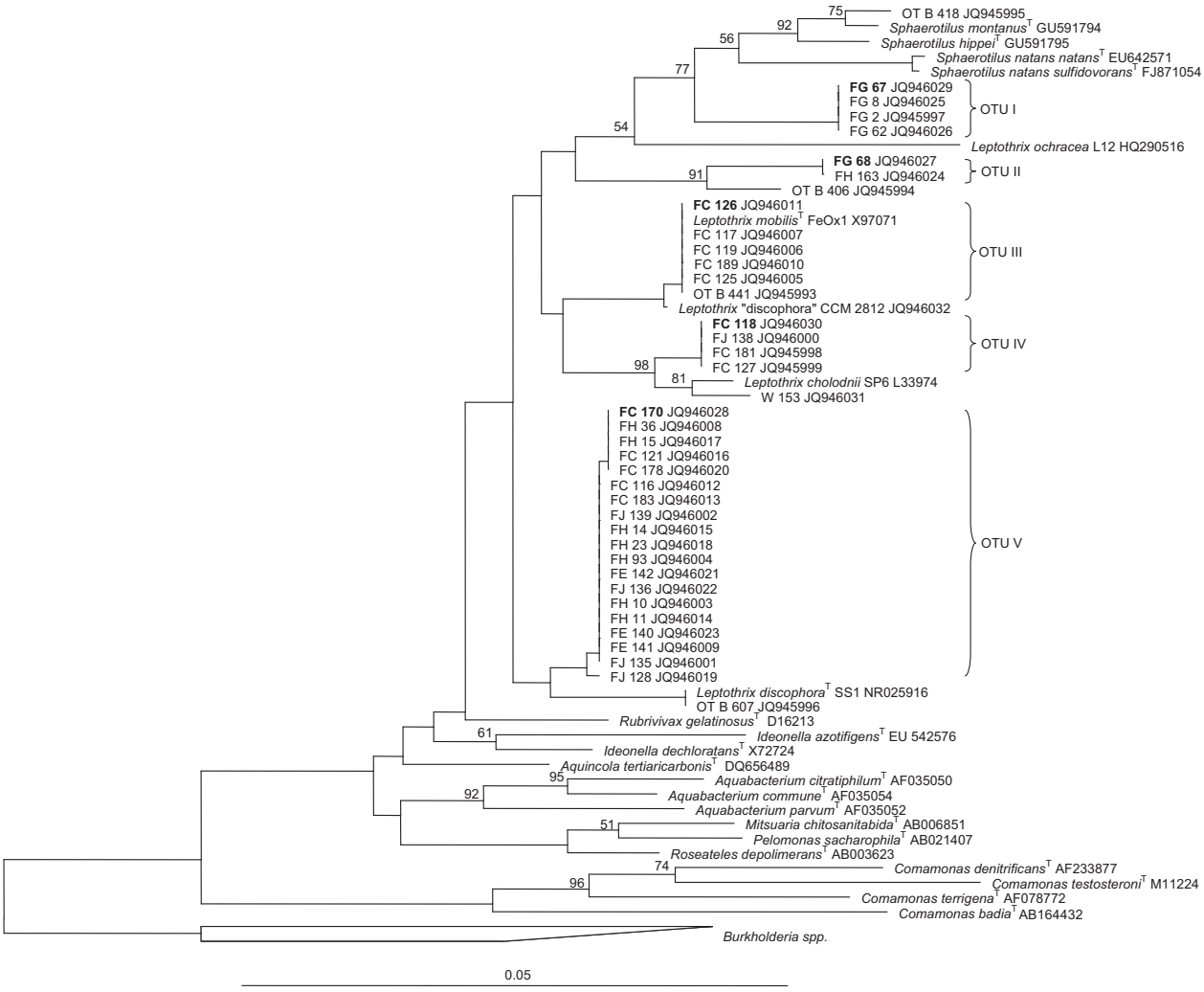

Fig. 1. Phylogenetic tree based on nearly full-length 16S rRNA gene sequences of the isolates, available SLG type strains and closely related species of *Burkholderiales*. The tree was constructed with ARB using neighbor joining, jukes–cantor correction, and termini filter. One sequence per group of identical sequences was used for tree constructions while the remaining was introduced by ARB quick add function. Bootstrap values are based on 1000 repetitions and are displayed if above 50%.

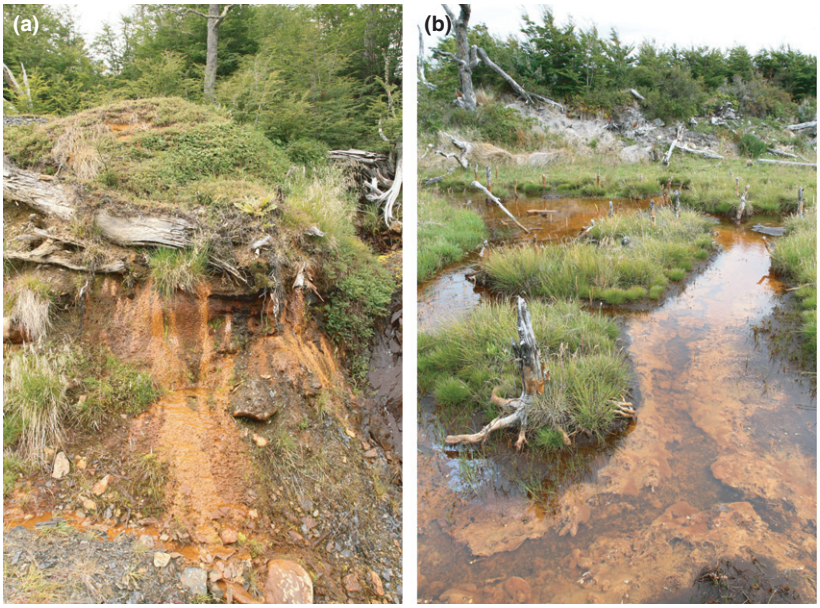

Fig. 2. Two of the sampling sites. (a) ochre well at site E; (b) iron brook at site H.

(Table 1) which additionally featured filamentous morphology, were selected and affiliated to the SLG morphologically which was confirmed by sequencing of the 16S rRNA genes. All SLG strains were isolated on standard Lepto-A agar except for FH 163, which was obtained from Lepto-A agar supplemented with peat extract.

ARDRA and sequencing of the 16S rRNA genes led to grouping into five OTUs affiliated with the SLG within *Burkholderiales* as shown in the phylogenetic analysis presented in Fig. 1.

The strains of OTU III as well as isolate OT B 441 were affiliated to the type strain of *L. mobilis* as they are identical in the examined range of sequence. *Leptothrix* '*discophora*' CCM 2812 also clusters close to *L. mobilis* and not, as was expected to the *L. discophora* type strain SS1 obtained from ATCC indicating that CCM 2812 has been misclassified.

The OTUs I, II, IV, and V represent new taxa within the SLG, while their similarity to validly described SLG type strains is different. OTU I forms a cluster with the *Sphaerotilus* branch with *S. montanus* as its closest relative (97.6%). OTU IV is closely related to *L. cholodnii* (98.8%) and the groundwater isolate W 153. OTU V clusters with *L. discophora* (98%) and includes three slightly different subgroups. The reference isolate OT B 607 shows 100% identity to *L. discophora*.

Deep branching was observed for the OTU II/OT B 406 cluster. The closest relative type strain of OTU II is *L. mobilis* with 96.5% similarity. The position of the OTU II/OT B 406 cluster was not stable using different tree construction methods (data not shown), therefore, it was not clearly assigned either to *Sphaerotilus* or *Leptothrix*. As the *L. ochracea* (Fleming *et al.*, 2011) sequence is also clearly separated, the previous two-genera structure of the SLG no longer seems appropriate.

Low bootstraps of many nodes of the dendrogram suggest that 16S rRNA genes, while showing enough varying positions to differentiate between groups (OTUs), do not contain enough information to unravel the taxonomical structure of the SLG (Spring, 2006). The internal phylogenetic structure of the SLG remains partly unclear, while *Sphaerotilus*, *Leptothrix,* and nearly all of the surrounding genera have to be regarded as *incertae sedis*.

Morphology of the isolates

We regarded iron/manganese deposition in combination with filamentous, sheathed growth as the SLG wild-type morphology which was shown by all of the SLG isolates on Lepto-A. Colonies showed light reddish-brown to black color as well as fringed edges depending on species and incubation time. Microscopic examination showed filaments with partly visible sheaths containing rod-shaped cells. Brown layers of deposits were present on sheaths regarded to be older while younger sheaths were often lacking apparent deposits. Additionally, free cells were frequently observed and motility rarely appeared in liquid Lepto-A medium.

The SLG wild-type morphology was stable in all isolates from Tierra del Fuego even after long-term cultivation over more than 30 transfers on solid media. White

Table 2. Fe/Mn deposition and sheath formation of SLG type strains determined on Lepto-A agar

	Fe/Mn deposition	Sheathed/ filamentous
All isolates included in this publication	+	+
White colored mutants of OT B 418 and OT B 406	–	+
S. natans	–	–
S. hippei	–	–
S. montanus		
Fringed colonies	–	+
Round colonies*	–	–
L. mobilis	+	–
L. discophora	+	(+)†
L. cholodnii	+	+

**S. montanus* type culture grows as a mix of fringed colonies with sheathed cells and round colonies with sheathless cells.
†*L. discophora* shows single cells and short sheaths.

colonies without metal oxidation occurred repeatedly (about every tenth to twentieth transfer) in isolates OT B 418 and OT B 406 from Germany and were conserved separately. Comparison of those strains with the wild-type will possibly open further insight into mechanisms of metal oxidation.

In comparison to the isolates, SLG type strains cultured under the same conditions did not, in many cases, show the wild-type morphology as summarized in Table 2. As it has been repeatedly described that cultures of SLG strains lose morphological characteristics after extended laboratory cultivation (Van Veen *et al.*, 1978; Emerson & Ghiorse, 1992), we ascribed such observations to adaptation or, if irreversible, to degeneration under cultural conditions. The simultaneous occurrence of sheathed and sheathless growth in *S. montanus* type strain culture (Table 2) may be regarded as ongoing degeneration. This was confirmed by separation of both round and fringed type subcultures which retained their morphology.

Comparison of two available *Leptothrix* type strains with new isolates sharing an identical 16S rRNA gene sequence further implies that our recently isolated strains preserved the wild-type morphology and also indicates an influence of culture conditions. As 16S rRNA gene identity does not mean that these pairs of strains were originally identical degeneration in culture is only one possible explanation.

The type strain of *L. discophora* is described to be sheathless (Emerson & Ghiorse, 1992), but showed minor sheath formation in Lepto-A (Fig. 3b) and single cells also occurred. The isolate OT B 607 which has identical 16S rRNA gene sequence as *L. discophora* regularly formed long encrusted sheaths (Fig. 3a).

Leptothrix mobilis, also described as nonsheath-forming and highly motile (Spring *et al.*, 1996) in modified Rouf

Table 3. Clone libraries

	Site G	Site H
Alphaproteobacteria		
Acetobacteraceae		
Roseomonas		1
Betaproteobacteria		
Comamonadaceae		
Rhodoferax/Albidiferax	57	49
Curvibacter/Variovorax	1	4
Hydrogenophaga	1	
Limnohabitans		1
Burkholderiaceae		
Polynucleobacter		3
Burkholderiales		
Rhizobacter/Methylibium		7
Group 1*		
Group 2*	3	5
Group 3*		1
Group 4*		1
Gallionellaceae		
Gallionella	2	3
Sideroxydans	1	2
Rhodocyclaceae		
Georgfuchsia/Sterolibacterium	5	1
Hydrogenophilaceae		
Methylophilus/Methylovorus	1	
Gammaproteobacteria		
Legionellaceae		
Legionella	1	
Methylococcaceae		
Methylobacter		1
Deltaproteobacteria		
Geobacteraceae		
Geobacter	4	1
Cystobacteraceae		
Anaeromyxobacter	1	
Bacteriovoracaceae		
Bacteriovorax	1	1
Epsilonproteobacteria		
Helicobacteraceae		
Sulfuricurvum	1	
Holophagae		
Holophagaceae		
Geothrix	2	1
Sphingobacteria		
Chitinophagaceae		
Ferruginibacter	1	
Cyanobacteria		2
Others		
Chloroplasts	5	3
Sequences not assigned	8	6
Total	Σ 95	Σ 93

Abundance of phylogenetic groups of clones from sampling sites G and H. Sequences were classified using the RDP classifier compared to BLAST results using the Nucleotide Collection (nr/nt). Sequences with ambiguous assignment were analyzed with ARB. Groups were named after the closest related genus or after two genera when both were similarly related. Known iron-reducing genera are marked in blue and iron-oxidizing bacteria in red.
*Groups of clone sequences which clustered *incertae sedis* within *Burkholderiales* surrounded by the genera *Sphaerotilus*, *Leptothrix*, *Ideonella*, *Rubrivivax*, *Aquabacterium,* and *Aquincola*. The assignment on genus level was not possible based on 16S rRNA gene sequences.

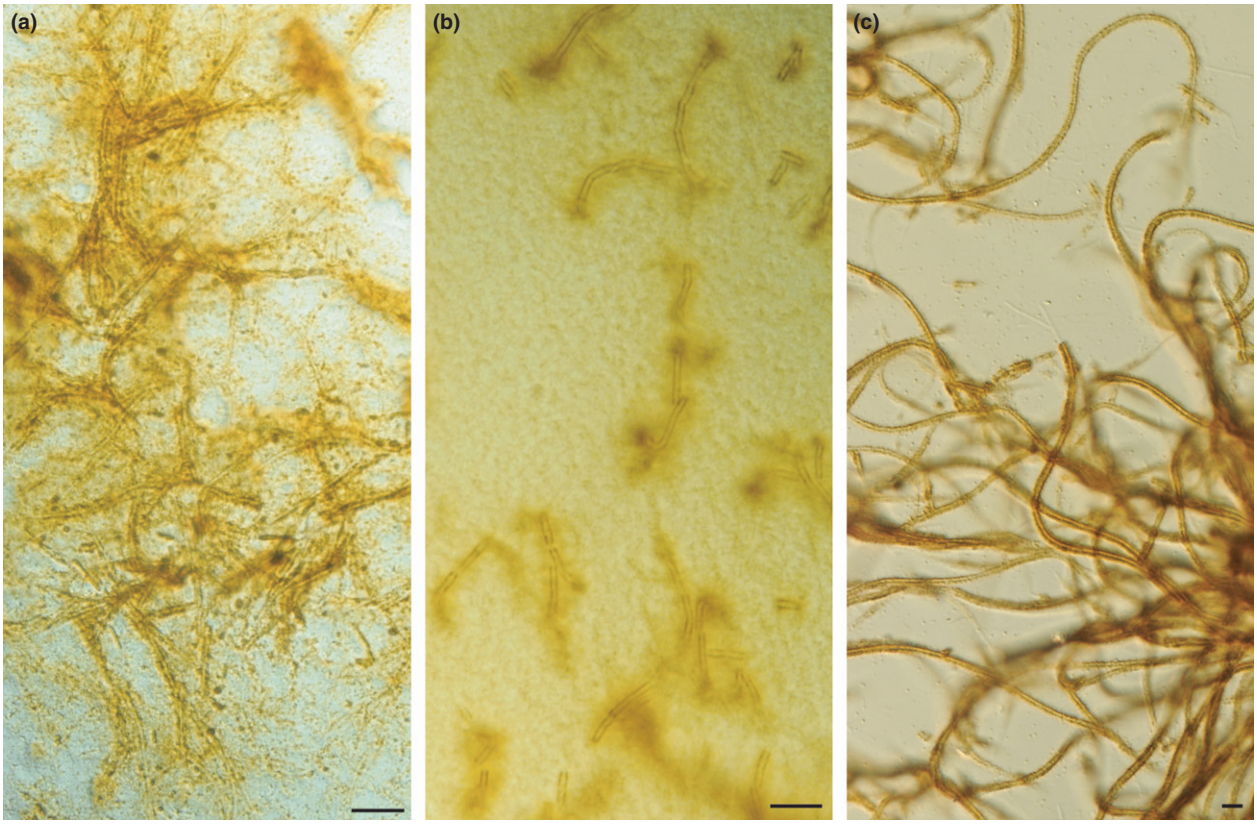

Fig. 3. Formation of encrusted sheaths of (a) OT B 607, (b) *L. discophora* SS1 and (c) FC 126 growing on glass slides in liquid Lepto-A medium, DIC, bars = 10 μm.

and Stokes medium (Rouf & Stokes, 1964; Wagner *et al.*, 1994), shows chains of cells but no visible sheaths and no motility in Lepto-A medium. Under the same culture conditions, the recently isolated FC 126, which again has a 16S rRNA gene sequence identical to *L. mobilis*, formed long, heavily encrusted sheaths (Fig. 3c). As modified Rouf and Stokes medium contained 5 g L^{-1} peptone, in contrast to 0.5 g L^{-1} yeast extract in Lepto-A, lower concentrations of organic substrates seem to preserve the wild-type morphology better.

However, if SLG strains are maintained as growing cultures in liquid media without adequate cryopreservation, the wild type might be overgrown by sheathless or nonmetal depositing cells and can be lost. This demonstrates the value of solid media in enabling control of colony morphology and the importance of cryopreservation (Vollrath *et al.*, 2012).

The ability to oxidize manganese as well as iron is used to distinguish *Leptothrix* from *Sphaerotilus* species (Spring, 2006). All of our isolates were capable of manganese oxidation. Since some of them, for example, FG 67 and OT B 418, are affiliated with *Sphaerotilus*, our results do not support this phenotypic differentiation of *Leptothrix* from *Sphaerotilus*.

Lepto-A turned out to be successful for isolation of new SLG strains as well as for reisolation of known SLG species, as shown for *L. mobilis* and *L. discophora* regarding identification based on 16S rRNA gene sequences. Preservation of wild-type morphology was another benefit of Lepto-A which is characterized by low organic substrate concentration in combination with iron and manganese compounds. Contributing factors may have been extended incubation time (Stevenson *et al.*, 2004) and unusual selective or inhibitory factors, namely high concentration of manganese (Ali & Stokes, 1971; Adams & Ghiorse, 1985). It might be concluded that cultivating 'unculturables' (Rothschild, 2006) does not necessarily require development of elaborate cultivation techniques to imitate complex physicochemical conditions of natural habitats.

Microscopy of environmental samples

Extensive microscopic examination revealed a vast richness of iron-encrusted bacterial morphotypes, which were in most cases similar to those known from comparable habitats of the Northern Hemisphere. Morphotypes of iron bacteria, however, while frequently found in natural sam-

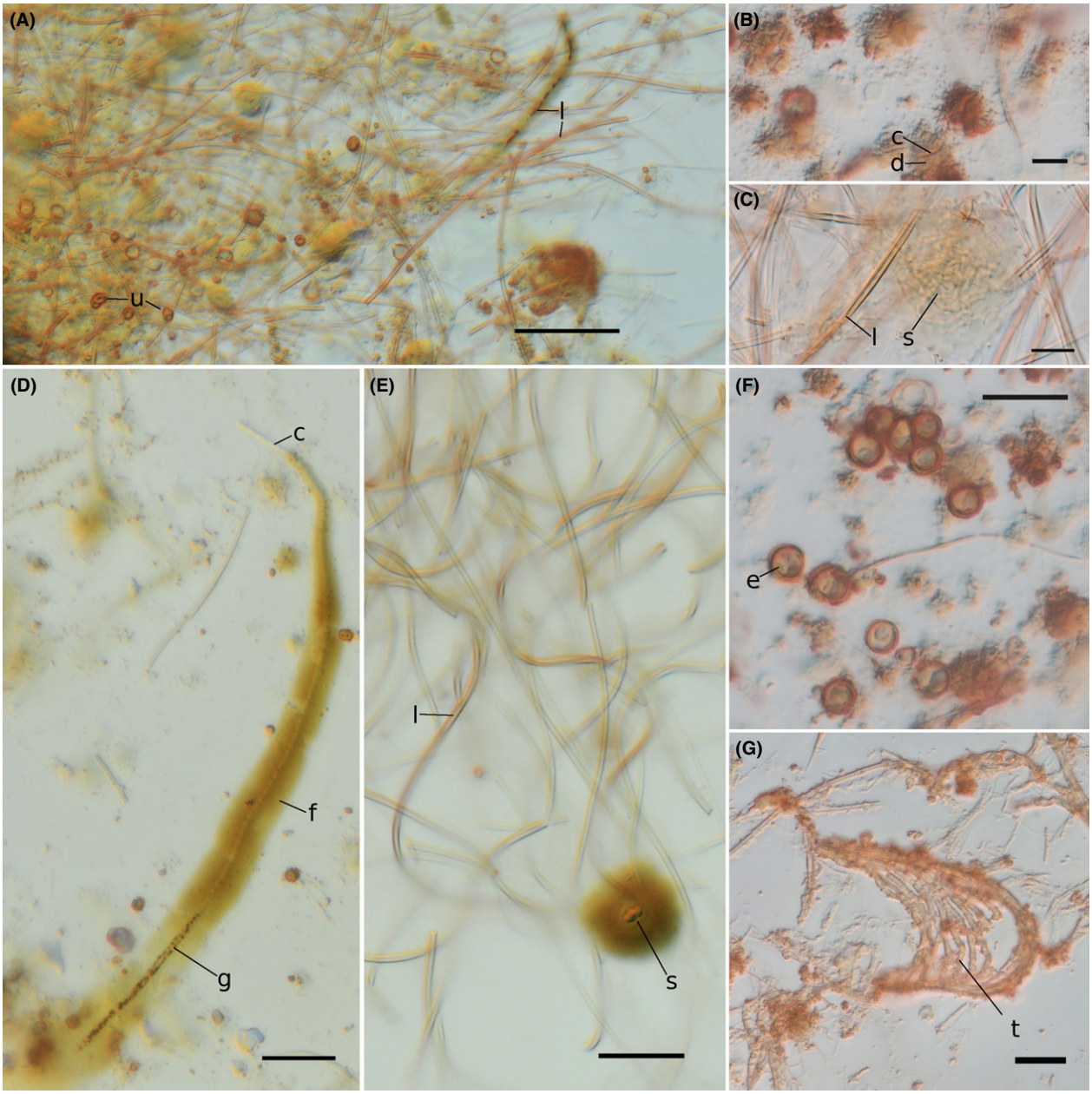

Fig. 4. Iron bacteria morphotypes in natural samples. DIC-microscopy. (A) overview of a diverse iron bacteria community including different *Leptothrix spp*. (l), unicellular morphotypes (u), iron and amorphous iron containing aggregates, bar = 50 μm; (B) 'Mycothrix' growing on a glass surface characterized by round shaped ochreous deposits (d) with a single central channel (c), bar = 10 μm; (C) 'Sideromyces'-structure (s) consisting of tangled thin filaments embedded in an EPS layer surrounded by much larger *L. ochracea* sheaths (l), bar = 10 μm; (D) *Leptothrix sp*., filament of rod-shaped cells (c) coated by a fluffy layer of iron deposits (f) which is tapered to what seems to be the younger part of the filament, the older part features additional dark granules (g) directly on central sheaths surface, bar = 25 μm; (E) 'Siderocapsa' (s) being a cluster of coccoid cells embedded in a mucous layer with ochre precipitates in a microbial mat dominated by *L. ochracea* sheaths (l), bar = 25 μm, (F) iron-encrusted encapsulated eukaryotes (e) growing on a glass surface, bar = 25 μm, (G) "Toxothrix trichogenes"-structure (t), bar = 10 μm.

ples, are in most cases not clearly assigned to valid species because no culture deposits are available.

Using classical morphology-based literature, we identified '*Siderocapsa* spp.' (Molisch, 1909) (Fig. 4E), '*Mycothrix* spp.' (Naumann, 1921) (Fig. 4B), '*Siderocystis* spp.' (Naumann, 1921), 'Toxothrix trichogenes' (Cholodny, 1926) (Fig. 4G), 'Siderococcus' (Dorff, 1934), '*Naumanniella* spp.' (Dorff, 1934), and others. The *Gallionella* mor-

photype, indicated by fragments of encrusted stalks, was present in most samples but not abundant.

In many samples, sheath-forming, iron-depositing morphotypes matching the classic morphologic description for *Leptothrix* formed the predominating group of iron bacteria. The typical *L. ochracea* morphotype with smooth, brown-colored sheaths containing few or no visible cells formed a dominating structural component in microbial mat samples (Fig. 4A, C and E). Occasionally, sheaths resembling *L. ochracea* with a spiral shape occurred. In addition to *L. ochracea,* several different SLG morphotypes were observed, some of them showing rough-granulated encrustations on sheaths, similar to *L. cholodnii* or *L.* 'pseudoochracea' (Mulder & Van Veen, 1963). Other SLG morphotypes were characterized by additional mucous layers of reddish/brown color involving the visible sheath located at the center. Thickness of these layers typically decreased at the ends of filaments. Classical names for morphotypes of this appearance are *L.* 'crassa' (Cholodny, 1926), *L.* 'skujae' (Skuja, 1948), *L.* 'levissima' (Kalbe *et al.*, 1965), or *L.* 'pseudovacuolata' (Skuja, 1948) if internal refractive granules are present in the cells. In some cases, dense dark granules were visible around the sheaths (Fig. 4D), as previously described for *L.* 'levissima' (Häusler, 1982).

Predominance of empty sheaths was only observed for *L. ochracea* while all other SLG morphotypes featured sheaths which were at least partly filled with cells. The latter morphotypes were probably the origin of the isolates, although the morphology in culture might be divergent. However, a fraction of empty sheaths was also observed in older cultures of the isolates.

Clone libraries

As displayed in Table 3, both clone libraries of sites G and H show dominance of *Betaproteobacteria*. In addition, other classes of *Proteobacteria* as well as *Holophagae* and *Sphingobacteria* were detected at low frequencies.

Clone distribution indicated that bacteria connected with the iron cycle were present at the examined sites. Sequences of iron-reducing bacteria dominated the clone libraries due to the abundance of *Rhodoferax*- and *Albidiferax*-related sequences. These genera were also found in other iron-oxidizing communities (Haaijer *et al.*, 2008; Fleming *et al.*, 2011; Johnson *et al.*, 2012; Roden *et al.*, 2012; Lu *et al.*, 2013). Also, members of the genera *Anaeromyxobacter*, *Geothrix*, *Geobacter,* and *Georgfuchsia*, which were found in the clone libraries, are known to be capable of iron reduction (Coates *et al.*, 1999; Treude *et al.*, 2003; Nevin *et al.*, 2005; Weelink *et al.*, 2009).

Clones related to the known iron-oxidizing genera *Gallionella* and *Sideroxydans* (Hanert, 1968; Weiss *et al.*, 2007) were found.

Group 1 to group 4 (Table 3) contain clone sequences which were assigned to *Burkholderiales* within the branch defined by, for example, *Sphaerotilus*, *Leptothrix*, *Ideonella*, *Rubrivivax*, *Aquabacterium,* and *Aquincola*. As mentioned before for the SLG isolates, the structure of that cluster of species is not stable based only on 16S rRNA gene sequence. Besides *Sphaerotilus* and *Leptothrix* other iron bacteria that are closely related to those clones have been isolated by our group (data not shown). Also, an *Aquabacterium* strain has been discussed to be able of iron oxidation coupled to nitrate reduction (Straub *et al.*, 2004). However, the group is metabolic diverse including, for example, phototrophic *Rubrivivax gelatinosus* (Willems *et al.*, 1991).

Although SLG morphotypes were most abundant in microscopical findings, no sequences in the clone libraries were clearly assigned to the known SLG. This might be caused by a bias in our methods that influenced the distribution of clones (Philippot *et al.*, 2001; Acinas *et al.*, 2005; Feinstein *et al.*, 2009). A specific bias concerning iron bacteria might result if encrustations cause resistance to DNA extraction techniques and resultant underrepresentation in clone libraries.

Alternately, the distribution of clones might reflect the real ratio of 16S rRNA genes in the samples. Striking sheath structures may lead to an overestimation of the number of SLG cell during the microscopical observation.

Finally, not all sheath-forming iron-encrusted *Leptothrix* morphotypes are necessarily phylogenetic members of the SLG. Sheath formation is widespread and, in combination with iron deposition, found in the *GammaproteobacteriaClonothrix fusca,* and *Crenothrix polyspora* (Völker *et al.*, 1977; Vigliotta *et al.*, 2007). It is not possible to unambiguously assign morphotypes observed in nature to cultured or genetically known species (species candidates resp.), without combining microscopy and molecular tools such as FISH.

This problem has been shown for the iron-oxidizing *Betaproteobacteria*, *Gallionella ferruginea* and *Leptothrix ochracea*, which feature a characteristic morphology and therefore apparently easy to identify microscopically. Recently they were joined by 'doppelgangers' sharing their obvious morphological features. Both a *Gallionella*-like isolate, *Mariprofundus ferrooxydans,* and a sheathed bacterium assigned by FISH, belonged to the *Zetaproteobacteria* (Singer *et al.*, 2011; Fleming *et al.*, 2013). These latter isolates originated from marine environments, and it has been suggested that physicochemical factors, in this case salinity, can be useful to differentiate between these pairs of very similar morphotypes (McBeth *et al.*, 2013). Even in freshwater systems, the *G. ferruginea* morphotype is expressed by isolate R-1 which shares only 93,55% 16S rRNA gene sequence similarity with *G. ferruginea* (Krepski *et al.*, 2012).

Clone library data indicate that bacteria of both parts of the iron cycle are present and diverse at the sampling sites. Due to changes in flow conditions, activity might switch between oxidizing or reducing processes, depending mainly of the availability of oxygen as suggested for other iron-rich freshwater habitats (Sobolev & Roden, 2002; Blöthe & Roden, 2009; Roden *et al.*, 2012).

Conclusions

A high diversity of iron bacteria morphotypes, similar to those described from Northern Hemisphere was detected in Tierra del Fuego wetlands.

Our successful isolation method extended the diversity of cultivated *Sphaerotilus* and *Leptothrix* significantly, including new strains from Tierra del Fuego, Argentina; the Bogengraben, and a groundwater system in Germany.

Cultivation of the isolates on Lepto-A preserves the sheathed, iron, and manganese-depositing morphology.

The phenotypic differentiation based on manganese oxidation which was ascribed only to *Leptothrix* was not validated.

Phylogenetic analysis based on 16S rRNA genes indicates that the structure of the *Sphaerotilus-Leptothrix* group should be revised.

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References

Acinas SG, Sarma-Rupavtarm R, Klepac-Ceraj V & Polz MF (2005) PCR-Induced sequence artifacts and bias : insights from comparison of two 16S rRNA clone libraries constructed from the same sample. *Appl Environ Microbiol* **71**: 8966–8969.

Adams LF & Ghiorse WC (1985) Influence of manganese on growth of a sheathless strain of *Leptothrix discophora*. *Appl Environ Microbiol* **49**: 556–562.

Ali SH & Stokes JL (1971) Stimulation of heterotrophic and autotrophic growth of *Sphaerotilus discophorus* by manganous ions. *Antonie Van Leeuwenhoek* **37**: 519–528.

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic Local Alignment Search Tool. *J Mol Biol* **215**: 403–410.

Blöthe M & Roden EE (2009) Microbial iron redox cycling in a circumneutral-pH groundwater seep. *Appl Environ Microbiol* **75**: 468–473.

Cholodny N (1926) *Die Eisenbakterien*, Vol. 4. Gustav Fischer Verlag. Pflanzenforschung, Jena.

Coates JD, Ellis DJ, Gaw CV & Lovley DR (1999) *Geothrix fermentans* gen. nov., sp. nov., a novel Fe(lIl)-reducing bacterium from a hydrocarbon-contaminated aquifer. *Int J Syst Bacteriol* **49**: 1615–1622.

Crerar DA, Knox GW & Means JL (1979) Biogeochemistry of bog iron in the New Jersey pine barrens. *Chem Geol* **24**: 111–135.

Dorff P (1934) *Die Eisenorganismen - Systematik und Morphologogie*, Vol. 16. Gustav Fischer Verlag. Pflanzenforschung, Jena.

Emerson D & Ghiorse WC (1992) Isolation, cultural maintenance and taxonomy of a sheath-forming strain of *Leptothrix discophora* and characterization of manganese-oxidizing activity associated with the sheath. *Appl Environ Microbiol* **58**: 4001–4010.

Emerson D, Weiss J & Megonigal J (1999) Iron-oxidizing bacteria are associated with ferric hydroxide precipitates (Fe-plaque) on the roots of wetland plants. *Appl Environ Microbiol* **65**: 2758–2761.

Feinstein LM, Sul WJ & Blackwood CB (2009) Assessment of bias associated with incomplete extraction of microbial DNA from soil. *Appl Environ Microbiol* **75**: 5428–5433.

Fleming EJ, Langdon AE, Martinez-Garcia M, Stepanauskas R, Poulton NJ, Masland EDP & Emerson D (2011) What's new is old: resolving the identity of *Leptothrix ochracea* using single cell genomics, pyrosequencing and FISH. *PLoS ONE* **6**: e17769.

Fleming EJ, Davis RE, McAllister SM, Chan CS, Moyer CL, Tebo BM & Emerson D (2013) Hidden in plain sight: discovery of sheath-forming, iron-oxidizing *Zetaproteobacteria* at Loihi Seamount, Hawaii, USA. *FEMS Microbiol Ecol* **85**: 116–127.

Gridneva E, Chernousova E, Dubinina G, Akimov V, Kuever J, Detkova E & Grabovich M (2011) 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. *Int J Syst Evol Microbiol* **61**: 916–925.

Ha SD, Pillai SD, Maciorowski KG & Ricke SC (1995) Cycloheximide as a media amendment for enumerating bacterial populations in animal feeds. *J Rapid Methods Autom Microbiol* **4**: 95–105.

Haaijer SCM, Harhangi HR, Meijerink BB, Strous M, Pol A, Smolders AJP, Verwegen K, Jetten MSM & Op den Camp HJM (2008) Bacteria associated with iron seeps in a sulfur-rich, neutral pH, freshwater ecosystem. *ISME J* **2**: 1231–1242.

Hanert H (1968) Untersuchungen zur Isolierung, Stoffwechselphysiologie und Morphologie von *Gallionella ferruginea* Ehrenberg. *Arch Microbiol* **60**: 348–376.

Häusler J (1982) *Schizomycetes Bakterien*, Vol. 20. Gustav Fischer Verlag. Süßwasserflora von Mitteleuropa, Stuttgart.

Johnson JL (1994) Similarity analysis of DNAs. *Methods for General and Molecular Bacteriology* (Gerhardt PE, Murray RG, Wood WA & Krieg NR, eds), pp. 655–681. ASM Press, Washington, DC.

Johnson KW, Carmichael MJ, McDonald W, Rose N, Pitchford J, Windelspecht M, Karatan E & Bräuer SL (2012) Increased abundance of *Gallionella* spp., *Leptothrix* spp. and total bacteria in response to enhanced Mn and Fe concentrations in a disturbed southern appalachian high elevation wetland. *Geomicrobiol J* **29**: 124–138.

Jukes TH & Cantor CR (1969) Evolution of protein molecules. *Mammalian Protein Metabolism*, Vol. 3 (Munro HN, ed), pp. 21–132. Ac.Press, New York.

Kalbe L, Keil R & Thiele M (1965) Licht- und elektronenmikroskopische Studien an Arten von *Leptothrix*, *Siderocapsa* und *Planctomyces*. *Arch f Protistenk* **108**: 29–40.

Krepski ST, Hanson TE & Chan CS (2012) Isolation and characterization of a novel biomineral stalk-forming iron-oxidizing bacterium from a circumneutral groundwater seep. *Environ Microbiol* **14**: 1671–1680.

Kützing FT (1843) *Phycologia Generalis oder Anatomie; Physiologie und Systemkunde der Tange*. Brockhaus Verlag, Leipzig.

Lu S, Chourey K, Reiche M, Nietzsche S, Shah MB, Neu TR, Hettich RL & Küsel K (2013) Insights into the structure and metabolic function of microbes that shape pelagic iron-rich aggregates ("iron snow"). *Appl Environ Microbiol* **79**: 4272–4281.

Ludwig W, Strunk O, Westram R, *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Dymock D & Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* **64**: 795–799.

McBeth JM, Fleming EJ & Emerson D (2013) The transition from freshwater to marine iron-oxidizing bacterial lineages along a salinity gradient on the Sheepscot River, Maine, USA. *Environ Microbiol Rep* **5**: 453–463.

Molisch H (1909) Siderocapsa treubii eine neue, weit verbreitete Eisenbakterie. *Ann Jard Bot Buit* **3**: 29–34.

Mulder EG & Van Veen WL (1963) Investigations on the *Sphaerotilus-Leptothrix* Group. *Antonie Van Leeuwenhoek* **29**: 121–153.

Naumann E (1921) Untersuchungen über die Eisenorganismen Schwedens. Almqvist & Wiksells Booktryckeri, Stockholm.

Neubauer SC, Toledo-Durán GE, Emerson D & Megonigal JP (2007) Returning to their roots: iron-oxidizing bacteria enhance short-term plaque formation in the wetland-plant rhizosphere. *Geomicrobiol J* **24**: 65–73.

Nevin KP, Holmes DE, Woodard TL, Hinlein ES, Ostendorf DW & Lovley DR (2005) *Geobacter bemidjiensis* sp. nov. and *Geobacter psychrophilus* sp. nov., two novel Fe(III)-reducing subsurface isolates. *Int J Syst Evol Microbiol* **55**: 1667–1674.

Pfennig A & Tschech N (1984) Growth yield increase linked to caffeate reduction in *Acetobacterium woodii*. *Arch Microbiol* **137**: 163–167.

Philippot L, Hallet S, Chaussod R, Soulas G & Catroux G (2001) DNA extraction from soils: old bias for new microbial diversity analysis methods. *Appl Environ Microbiol* **67**: 2354–2359.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J & Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**: 7188–7196.

Roden EE, McBeth JM, Blöthe M, Percak-Dennett EM, Fleming EJ, Holyoke RR, Luther GW, Emerson D & Schieber J (2012) The microbial ferrous wheel in a neutral pH groundwater seep. *Front Microbiol* **3**: 1–18.

Roth AW (1797) *Catalecta botanica quibus plantae novae et minus cognitae describuntur atque illistrantur*. Lipsiae in Bibliopolio I.G. Mülleriano, Leipzig.

Rothschild LJ (2006) Short statement: a microbiologist explodes the myth of unculturables. *Nature* **443**: 249.

Rouf M & Stokes JL (1964) Morphology, nutrition and physiology of *Sphaerotilus discophorus*. *Arch Microbiol* **49**: 132–149.

Saitou N & Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schlegel HG & Zaborosch C (1992) *Allgemeine Mikrobiologie*. Thieme Verlag, Stuttgart, New York.

Singer E, Emerson D, Webb EA *et al.* (2011) *Mariprofundus ferrooxydans* PV-1 the first genome of a marine Fe(II) oxidizing Zetaproteobacterium. *PLoS ONE* **6**: e25386.

Skuja H (1948) Taxonomie des Phytoplanktons einiger Seen in Uppland, Schweden. *Symb Bot Upsal* **9**: 1–399.

Sobolev D & Roden EE (2002) Evidence for rapid microscale bacterial redox cycling of iron in circumneutral environments. *Antonie Van Leeuwenhoek* **81**: 587–597.

Spring S (2006) The genera *Leptothrix* and *Sphaerotilus*. *The Prokaryotes*, Vol. 5 (Dworking M, Falkow S, Rosenberg E, Schleifer K-H & Stackebrandt E, eds), pp. 758–777. Springer, New York.

Spring S, Kämpfer P, Ludwig W & Schleifer K-H (1996) Polyphasic characterization of the genus *Leptothrix*: new descriptions of *Leptothrix mobilis* sp. nov. and *Leptothrix discophora* sp. nov. nom, rev. and emended description of *Leptothrix cholodnii* emend. *Syst Appl Microbiol* **19**: 634–643.

Stevenson BS, Eichorst SA, Wertz JT, Schmidt TM & Breznak JA (2004) New strategies for cultivation and detection of previously uncultured microbes. *Appl Environ Microbiol* **70**: 4748–4755.

Straub KL, Schönhuber WA, Buchholz-Cleven BEE & Schink B (2004) Diversity of ferrous iron-oxidizing, nitrate-reducing bacteria and their involvement in oxygen-independent iron cycling. *Geomicrobiol J* **21**: 371–378.

Szewzyk U, Szewzyk R, Schmidt B & Braun B (2011) Neutrophilic iron-depositing microorganisms. *Biofilm Highlights*, Vol. 5 (Flemming HC, Wingender J & Szewzyk U eds), pp. 63–79. Springer Verlag, Berlin, Heidelberg.

Thronicker O & Szewzyk U (2011) *WellMa-DNA end Report – Documentation of Data Acquisition and Conclusions*. TU Berlin, published online by Kompetenz Zentrum Wasser Berlin. http://www.kompetenz-wasser.de/WELLMA-2.494.0.html

Tindall BJ, Rosselló-Móra R, Busse H-J, Ludwig W & Kämpfer P (2010) Notes on the characterization of prokaryote strains for taxonomic purposes. *Int J Syst Evol Microbiol* **60**: 249–266.

Treude N, Rosencrantz D, Liesack W & Schnell S (2003) Strain FAc12, a dissimilatory iron-reducing member of the *Anaeromyxobacter* subgroup of *Myxococcales*. *FEMS Microbiol Ecol* **44**: 261–269.

Van Veen WL, Mulder EG & Deinema MH (1978) The *Sphaerotilus-Leptothrix* group of bacteria. *Microbiol Rev* **42**: 329–356.

Vaneechoutte M, De Beenhouwer H, Claeys G, Verschraegen G, De Rouck A, Paepe N, Elaichouni A & Portaels F (1993) Identification of *Mycobacterium* species by using amplified ribosomal DNA restriction analysis. *J Clin Microbiol* **31**: 2061–2065.

Vigliotta G, Nutricati E, Carata E, Tredici SM, De Stefano M, Pontieri P, Massardo DR, Prati MV, De Bellis L & Alifano P (2007) *Clonothrix fusca* Roze 1896, a filamentous, sheathed, methanotrophic gamma-proteobacterium. *Appl Environ Microbiol* **73**: 3556–3565.

Völker H, Schweisfurth R & Hirsch P (1977) Morphology and ultrastructure of *Crenothrix polyspora* Cohn. *J Bacteriol* **131**: 306–313.

Vollrath S, Behrends T & Van Cappellen P (2012) Oxygen dependency of neutrophilic Fe(II) oxidation by *Leptothrix* differs from abiotic reaction. *Geomicrobiol J* **2**: 550–560.

Wagner M, Amann R, Kämpfer P, Assmus B, Hartmann A, Hutzler P, Springer N & Schleifer K-H (1994) Identification and *in situ* detection of Gram-negative filamentous bacteria in activated sludge. *Syst Appl Microbiol* **17**: 405–417.

Wang Q, Garrity GM, Tiedje JM & Cole JR (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**: 5261–5267.

Wang J, Muyzer G, Bodelier PL & Laanbroek HJ (2009) Diversity of iron oxidizers in wetland soils revealed by novel 16S rRNA primers targeting *Gallionella*-related bacteria. *ISME J* **3**: 715–725.

Weelink SA, van Doesburg W, Saia FT, Rijpstra WI, Röling WF, Smidt H & Stams AJ (2009) A strictly anaerobic betaproteobacterium *Georgfuchsia toluolica* gen. nov., sp. nov. degrades aromatic compounds with Fe(III), Mn(IV) or nitrate as an electron acceptor. *FEMS Microbiol Ecol* **70**: 575–585.

Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**: 697–703.

Weiss JV, Rentz JA, Plaia T, Neubauer SC, Merrill-Floyd M, Lilburn T, Bradburne C, Megonigal JP & Emerson D (2007) Characterization of neutrophilic Fe(II)-oxidizing bacteria isolated from the rhizosphere of wetland plants and description of *Ferritrophicum radicicola* gen. nov. sp. nov., and *Sideroxydans paludicola* sp. nov. *Geomicrobiol J* **24**: 559–570.

Willems A, Gillis M & De Ley J (1991) Transfer of *Rhodocyclus gelatinosus* to *Rubrivivax gelatinosus*. *Int J Syst Evol Microbiol* **41**: 65–73.

Winogradsky S (1888) Über Eisenbakterien. *Bot Ztg* **46**: 262–270.

Wright ES, Yilmaz LS & Noguera DR (2012) DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. *Appl Environ Microbiol* **78**: 717–725.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Positions of the sites showing iron depositing communities.
Fig. S2. Sampling site C.
Fig. S3. Sampling site E.
Fig. S4. Sampling site G.
Fig. S5. Sampling site H.
Fig. S6. Sampling site J.
Table S1 Position data of the sites.