The ISME Journal (2008) 2, 551–560

www.nature.com/ismej

ORIGINAL ARTICLE

Substrate-induced growth and isolation of *Acidobacteria* from acidic *Sphagnum* peat

Timofei A Pankratov[1], Yulia M Serkebaeva[1,2], Irina S Kulichevskaya[1], Werner Liesack[3] and Svetlana N Dedysh[1]

[1]*Department of Microbial Communities, SN Winogradsky Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia;* [2]*Department of Biology, MV Lomonosov Moscow State University, Moscow, Russia and* [3]*Department of Biogeochemistry, Max-Planck-Institut für terrestrische Mikrobiologie, Marburg, Germany*

Fluorescence *in situ* hybridization (FISH) was applied to estimate the population size of the poorly characterized phylum *Acidobacteria* in acidic peat sampled from nine different *Sphagnum*-dominated wetlands of Northern Russia. The cell numbers of these bacteria in oxic peat layers ranged from 0.4×10^6 to 1.3×10^7 cells per g of wet peat, comprising up to 4% of total bacterial cells. Substrate-induced growth of acidobacteria was observed after amendment of peat samples with glucose, pectin, xylan, starch, ethanol and methanol, while weak or no response was obtained for acetate, pyruvate, mannitol and cellobiose. Using low-nutrient media and FISH-mediated monitoring of the isolation procedure, we succeeded in obtaining nine strains of acidobacteria in pure cultures. These strains belonged to subdivisions 1 and 3 of the *Acidobacteria* and represented strictly aerobic, heterotrophic organisms. Except for methanol, the substrate utilization patterns of these isolates matched the results obtained in our substrate-amendment experiments with native peat. All strains were also capable of utilizing galacturonic acid, a characteristic component of the cell wall in *Sphagnum* spp, which is released during moss decomposition. Most isolates from subdivision 1 were truly acidophilic organisms with the growth optimum at pH 3.5–4.5, while the isolates from subdivision 3 grew optimally at pH 5.5–6.5. Another important phenotypic trait of novel strains was their capability of active growth at low temperatures. Both acidophily and low-temperature growth are consistent with the occurrence of acidobacteria in cold and acidic northern wetlands.
The ISME Journal (2008) **2,** 551–560; doi:10.1038/ismej.2008.7; published online 28 February 2008
Subject Category: microbial ecology and functional diversity of natural habitats
Keywords: *Acidobacteria*; *Sphagnum* peat; uncultured microorganisms

Introduction

The *Acidobacteria* is one of the cosmopolitan but poorly studied phylum of the domain Bacteria (Hugenholtz *et al.*, 1998a). Members of this phylum have been detected by 16S rRNA gene-based molecular surveys in a wide variety of environments, including soils and sediments (Barns *et al.*, 1999; Janssen, 2006), hot springs (Hugenholtz *et al.*, 1998b; Barns *et al.*, 1999; Bryant *et al.*, 2007), peat bogs (Rheims *et al.*, 1996; Dedysh *et al.*, 2006), acidic mining lakes (Kleinsteuber *et al.*, 2008), water distribution systems (Martiny *et al.*, 2005) and caves (Zimmermann *et al.*, 2005; Meisinger *et al.*, 2007). The range of phylogenetic diversity in the *Acidobacteria* is nearly as great as in the *Proteobacteria* (Hugenholtz *et al.*, 1998a). The number of major sequence clusters or subdivisions within the *Acidobacteria* increased from 4–5 in 1997 (Ludwig *et al.*, 1997) to 11 in 2005 (Zimmermann *et al.*, 2005) and 26 in 2007 (Barns *et al.*, 2007). However, in contrast to the rapidly growing pool of 16S rRNA gene sequences, the taxonomically described diversity within this phylogenetic group is still very poor. Currently, it includes only four validly described species: *Acidobacterium capsulatum* (Kishimoto *et al.*, 1991), *Holophaga foetida* (Liesack *et al.*, 1994), *Geothrix fermentans* (Coates *et al.*, 1999) and *Terriglobus roseus* (Eichorst *et al.*, 2007). An acidobacterial isolate of subdivision 3 has been tentatively named '*Solibacter usitatus*' (http://www.jgi.doe.gov), but its detailed description is still missing. Altogether, the physiological information about members of the *Acidobacteria* remains scarce.

Recently, we reported that one-third of all 16S rRNA gene sequences retrieved from acidic *Sphagnum* peat were affiliated with the *Acidobacteria* (Dedysh *et al.*, 2006). These sequences were widely distributed among the subdivisions 1, 3, 4 and 8 of this phylum. However, in contrast to the high proportion of *Acidobacteria*-affiliated sequences in the clone library, the population size of these

Correspondence: SN Dedysh, SN Winogradsky Institute of Microbiology, Russian Academy of Sciences, Moscow 117312, Russia.
E-mail: dedysh@mail.ru
Received 8 October 2007; revised 4 January 2008; accepted 8 January 2008; published online 28 February 2008

bacteria revealed in native peat by fluorescence *in situ* hybridization (FISH) was unexpectedly low, that is, 1.2×10^6 cells per g of wet peat. This study was undertaken to assess the population size of acidobacteria in peat bogs of diverse geographic location and to elucidate how carbon substrate amendments affect the development of acidobacteria in *Sphagnum* peat. The results of the amendment experiment enabled the choice of appropriate substrates for further isolation procedure, which resulted in successful isolation of previously uncultivated members of the *Acidobacteria* and characterization of those phenotypic traits that enable their life in cold and acidic northern wetlands.

Materials and methods

Sampling sites

The peat samples were collected from nine *Sphagnum*-dominated wetlands of West Siberia and European North Russia. Geographic location and some characteristics of these wetlands are shown in Table 1. The sampling was done at the oxic–anoxic interface of the wetland profiles, which corresponded to a depth of 10–20 cm. The samples were transported to the laboratory, homogenized by cutting the peat material into small fragments (about 0.5 cm) with sterile scissors and fixed for FISH or used for substrate-amendment experiment and isolation of acidobacteria.

FISH

The cells were serially extracted from peat using a homogenization treatment in a disposable 100-ml BagFilter plastic bag provided with the laboratory stomacher BagMixer (Model 100 'MiniMix;' Interscience, St Nom, France). These bags possess an internal filter unit, which divides them into two compartments. This enables the separation of the peat water enriched with microbial cells from the rough ($\geqslant$2–5 mm) *Sphagnum* debris. Two grams of wet *Sphagnum* peat was placed into one compartment of such a bag, mixed with 10 ml of sterile water and treated in the stomacher at 480 r.p.m. for 1 min. The water enriched with microbial cells was collected from the other bag compartment. The rest of the peat material was mixed with another 10-ml aliquot of sterile water, and the extraction procedure was repeated again. In total, three extraction fractions were obtained from each peat sample and pooled together. As shown previously, three repeated cycles of homogenization and extraction recovered nearly 99% of bacterial cells from a given peat sample (Dedysh *et al.*, 2001). The pooled fraction was centrifuged (10 000 r.p.m.) for 10 min. The supernatant was discarded, and the pellet was resuspended in sterile water to a final volume of 2 ml. An aliquot (0.5 ml) of this suspension was mixed with 1.5 ml of 4% (w/v) freshly prepared paraformaldehyde solution and fixed for 1 h at room temperature. The fixed material was then collected by centrifugation (8000 r.p.m. for 1 min) and washed twice with phosphate-buffered saline ($g\,l^{-1}$: NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 1.44; NaH_2PO_4, 0.2; pH 7.0) to ensure removal of paraformaldehyde. The resulting pellet was resuspended in 0.5 ml of 50% ethanol–phosphate-buffered saline (v/v) and this suspension was stored at $-20\,^{\circ}C$ until use. The Cy3-labeled oligonucleotide probes HoAc1402 and EUB338-mix with reported group specificity for the *Acidobacteria* and members of the domain *Bacteria* (Daims *et al.*, 1999; Juretschko *et al.*, 2002) were used in this study. Hybridization was carried out on gelatin-coated (0.1%, w/v) and dried Teflon-laminated slides (MAGV, Rabenau, Germany) with eight wells for independent positioning of the samples. The fixed samples were applied to these wells, hybridized with the respective probe and stained with the universal DNA stain 4′,6-diamidino-2-phenylindole (DAPI, 1 μM) for 5 min. The cell counts were carried out with a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) equipped with the Zeiss Filters no. 20 and 02 for Cy3-labeled probes and DAPI staining, respectively. Cell counting was performed on 100 randomly chosen fields of view for each test sample. The number of target cells per g of wet peat was determined from the area of the sample spot, the field of view area, the aliquot of the fixed sample used for hybridization and the total volume of the fixed sample.

Amendment of peat with different carbon substrates

Two grams of homogenized peat were placed into 30-ml serum vials and amended with a particular carbon source. The list of substrates tested in this study included glucose, cellobiose, starch, pyruvate, acetate, methanol, ethanol, mannitol, xylan, pectin and gellan gum (Gel-Gro; ICN Biomedicals, Irvine, CA, USA). Each carbon source was dissolved in sterile distilled water and prepared as a stock solution at a concentration designed to deliver 1 mg of respective substrate per ml of water in each peat sample when 0.5 ml was dispensed. The control sample received 0.5 ml of water without dissolved organic carbon. To prevent evaporation of volatile substrates such as methanol and ethanol, the respective vials were sealed with silicon rubber septa. All other vials were sealed with Parafilm 'M' to prevent evaporation of water. One set of vials was incubated at 10 °C, while the other was incubated at 20 °C. After 3 days of incubation, all peat samples were fixed for FISH.

Cultivation approach, screening for the presence of acidobacteria and identification of isolates

Two slightly different basal media (termed MM1 and MM) were used for isolation of peat-inhabiting

Table 1 The number of cells detected by FISH with the *Acidobacteria*-specific probe HoAc1402 in acidic peat sampled from *Sphagnum*-dominated wetlands of different geographic location

Wetland name, location	*Wetland type*	*Plant community composition*	*pH*	*Number of cells per gram of wet peat detected with*	
				$\frac{EUB338\text{-}mix\ (N\times10^8)}{DAPI\ staining\ (N\times10^8)}$	$\frac{HoAc1402 (N\times106)}{\%\ of\ EUB338\text{-}mix}$
Bakchar, West Siberia, Tomsk region, 56°81′N, 82°50′E	Oligo-mesotrophic bog	*Sphagnum fuscum, Oxycoccus palustris, Eriophorum vaginatum, Carex rostrata, Chamaedaphne calyculata*	4.4	$\frac{1.08 \pm 0.13}{3.50 \pm 0.70}$	$\frac{1.53 \pm 0.25}{1.4}$
Blizhnee, West Siberia, Tomsk region, 56°50′N, 83°04′E	Eutrophic fen	*Sphagnum* sp, *Carex* sp, *O. palustris, Andromeda polifolia, C. calyculata, Scheuchzeria palustris, Menyanthes trifoliata*	5.8	$\frac{3.49 \pm 0.58}{7.16 \pm 1.20}$	$\frac{6.25 \pm 0.10}{1.8}$
Gavrilovskoye, West Siberia, Tomsk region, 56°50′N, 83°04′E	Ombrotrophic[a] bog	*Sphagnum* sp, *Carex* sp, *C. calyculata, E. vaginatum, Ledum palustre, O. palustris, S. palustris*	4.5	$\frac{4.02 \pm 0.67}{5.96 \pm 0.99}$	$\frac{2.25 \pm 0.37}{0.6}$
Obskoye, West Siberia, Tomsk region, 56°33′N, 83°06′E	Eutrophic fen	*Sphagnum* sp, *Carex* sp, *Epilobium palustre, Galium uliginosum, Phragmites australis, Polygonum amphibium, Typha latifolia, Scutellaria galericulata*	5.9	$\frac{7.61 \pm 1.26}{10.90 \pm 1.81}$	$\frac{0.69 \pm 0.12}{0.1}$
Obukhovskoye, Yaroslavl region, 58°14′N, 38°12′E	Ombrotrophic bog	*Sphagnum angustifolium, S. fuscum, Carex* spp, *Oxycoccus* sp, *Vaccinium* sp	4.2	$\frac{3.23 \pm 0.72}{11.20 \pm 1.40}$	$\frac{13.30 \pm 0.71}{4.1}$
Darvinskoye, Vologda region, 58°30′N, 37°20′E	Ombrotrophic bog	*Sphagnum* sp, *C. calyculata, A. polifolia, L. palustre, E. vaginatum*	3.8	$\frac{1.00 \pm 0.15}{3.20 \pm 0.46}$	$\frac{0.81 \pm 0.13}{0.8}$
Muksalma, Archangelsk region, 65°01′N, 35°44′E	Aapa-type mesotrophic fen	*Pinus silvestris, Carex* sp, *Calluna vulgaris, S. fuscum, Sph. papillosum, M. trifoliata, Carex livida*	4.8	$\frac{3.21 \pm 0.53}{7.02 \pm 1.17}$	$\frac{0.46 \pm 0.08}{0.1}$
Sekirnoye, Arkhangelsk region, 65°01′N, 35°44′E	Oligo-mesotrophic fen	*S. angustifolium, S. fuscum, L. palustre, E. vaginatum, Rubus chamaemorus, Carex* spp, *Equisetum fluviatile*	4.0	$\frac{2.86 \pm 0.47}{3.79 \pm 0.63}$	$\frac{1.60 \pm 0.27}{0.6}$
Torfjanoye, Archangelsk region, 65°01′N, 35°44′E	Oligo-mesotrophic fen	*S. fuscum, C. vulgaris, Empetrum nigrum, C. calyculata, A. polifolia, L. palustre, R. chamaemorus*	3.8	$\frac{2.03 \pm 0.34}{4.18 \pm 0.69}$	$\frac{0.40 \pm 0.06}{0.2}$

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization.
[a]Ombrotrophic bogs are wetlands with no contact to groundwater, so that the only source of mineral nutrients is atmospheric precipitation. This is the most oligotrophic type of northern wetlands.

acidobacteria. Medium MM1 contained the following (per liter of distilled water): $MgSO_4\ 7H_2O$, 40 mg; $CaCl_2\ 2H_2O$, 20 mg; and yeast extract, 50 mg. Variations in the medium composition included the incorporation of some or all of the following (per liter): humic acid dyalizate, 1 ml; galacturonic acid sodium salt, 30 mg; and glucuronic acid, 30 mg. Medium MM contained (per liter): KH_2PO_4, 100 mg; $(NH_4)_2SO_4$, 100 mg; $MgSO_4\ 7H_2O$, 100 mg; and 1 ml of metal salt solution '44' (Staley *et al*., 1992). The solidifying agents used for the media preparations were Gel-Gro (ICN Biomedicals Inc.) or agar (Difco Laboratories, Detroit, MI, USA). After sterilization, one of the following carbon sources was added to these basal media: glucose, pectin, xylan (0.05%, w/v) or methanol (0.05%, v/v). The pH of the prepared media ranged between 4.2 and 5.5. The inoculated plates were incubated in the dark at 10, 15 or 20 °C for 1–2 months. The surveillance of isolation plates for the presence of target bacteria was done by means of plate-wash FISH, a procedure similar to plate-wash PCR described by Stevenson *et al*. (2004) with the only difference that, instead of PCR with *Acidobacteria*-specific primers, we used FISH with *Acidobacteria*-specific probe. When development of the target bacteria had been detected

on one of the plates with a particular carbon substrate, a few other plates were taken for picking representative colonies. The latter procedure involved the use of a dissecting microscope since most of the colonies that appeared on highly dilute and acidic media were very small (<0.5 mm). When subcultures were grown, defined pools of cells were again subjected to FISH-based examination. This procedure was continued until individual colonies of target organisms were ultimately identified and obtained in pure culture. Finally, nearly full-length 16S rRNA gene sequences were determined for the isolates as described by Dedysh *et al*. (2000). Phylogenetic analysis was carried out using the ARB program package (Ludwig *et al.*, 2004). The newly obtained 16S rRNA gene sequences were added to a database, which only contained nearly full-length 16S rRNA sequences and aligned manually. The trees were constructed using distance-based (neighbor-joining) and maximum-likelihood methods. The significance levels of interior branch points obtained in neighbor-joining analysis were determined by bootstrap analysis (1000 data resamplings) using PHYLIP (Felsenstein, 1989).

Phenotypic characterization of isolates
Morphological observations and cell size measurements were made with a Zeiss Axioplan 2 microscope and Axiovision 4.2 software (Zeiss). For preparation of ultrathin sections, cells of exponentially growing cultures were collected by centrifugation and prefixed with 1.5% (w/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 6.5) for 1 h at 4 °C and then fixed in 1% (w/v) OsO_4 in the same buffer for 4 h at 20 °C. After dehydration in an ethanol series, the samples were embedded in a Spurr epoxy resin. Thin sections were cut on an LKB-4800 microtome, stained with 3% (w/v) uranyl acetate in 70% (v/v) ethanol. The specimen samples were examined with a JEM-100C transmission electron microscope. Absorption spectra of methanol extracts of pink-pigmented acidobacteria were determined between 250 and 1000 nm with SPh-56 spectrophotometer.

Physiological tests were performed in liquid media MM1 and MM. Growth of novel strains was monitored by nephelometry in an Eppendorf BioPhotometer at 600 nm for 7–14 days under a variety of conditions, including temperatures of 4–37 °C and pH values of 3.0–8.0. Variations in the acidity level were achieved by mixing 0.1 M solutions of H_3PO_4, KH_2PO_4, K_2HPO_4 and K_3PO_4. Carbon source utilization was determined using mineral medium MM1 supplemented with respective carbon sources (0.05%, w/v). The capacity to utilize methanol was determined at CH_3OH concentrations ranging from 0.01 to 0.5% (v/v). The ability of isolates to degrade different biopolymers was examined by measuring the rate of CO_2 production in tightly closed 120-ml serum bottles containing 20 ml of liquid medium MM1 with 0.05% (w/v) of the corresponding polymer substrate for 1 month at 20 °C. Control incubations were run in parallel under the same conditions but without substrate.

Nucleotide sequence and culture collection accession numbers
The 16S rRNA gene sequences from novel isolates have been deposited in the GenBank, EMBL and DDBJ nucleotide sequence databases under accession numbers AM887754 to AM887762. Three representative acidobacterial isolates from *Sphagnum*-dominated wetlands, that is, the strains KMR, TPO1014 and MPL3, have been deposited in ATCC under accession numbers BAA-1395, BAA-1396 and BAA-1390.

Results and discussion

Quantification of acidobacteria in native peat by FISH
The probe HoAc1402 with reported group specificity for members of the Acidobacteria was applied to native peat sampled from *Sphagnum*-dominated wetlands of different geographic location. According to results of our probe-match check, most of the acidobacterial clone sequences retrieved from acidic peat in our previous study (Dedysh *et al.*, 2006) had no mismatches in the target region of probe HoAc1402. The number of cells detected in different wetlands by this probe ranged from 0.4×10^6 to 1.3×10^7 cells per g of wet peat, comprising 0.1–4.1% of total bacterial cell number (Table 1). The highest population sizes of acidobacteria were revealed in highly acidic (pH 4.2) peat sampled from ombrotrophic bog Obukhovskoye as well as in moderately acidic (pH 5.8) peat sampled from eutrophic fen Blizhnee. Similarly, the lowest numbers of cells targeted with the probe HoAc1402 were detected both in highly acidic (pH 3.8–4.0) peat sampled from oligotrophic bogs Torfjanoye, Sekirnoye and Darvinskoye, and in moderately acidic (pH 5.9) peat from eutrophic fen Obskoye. Thus, we did not observe any correspondence between the pH value of peat water and the cell number of acidobacteria detected with the phylum-specific probe. The sample with the highest population size of acidobacteria, that is, the peat from ombrotrophic bog Obukhovskoye was further used to test the effect of carbon substrate amendments on the development of acidobacteria in *Sphagnum*-derived peat.

Substrate-induced in situ *growth of acidobacteria*
The number of acidobacterial cells in control experiment (with no carbon source) did not change for 3 days of incubation at both 10 and 20 °C. By contrast, addition of most carbon sources resulted in detectable substrate-induced growth of acidobacteria in peat (Figure 1). The most intensive substrate-induced growth was observed with glucose, xylan, methanol, ethanol, starch and pectin. Incubation at

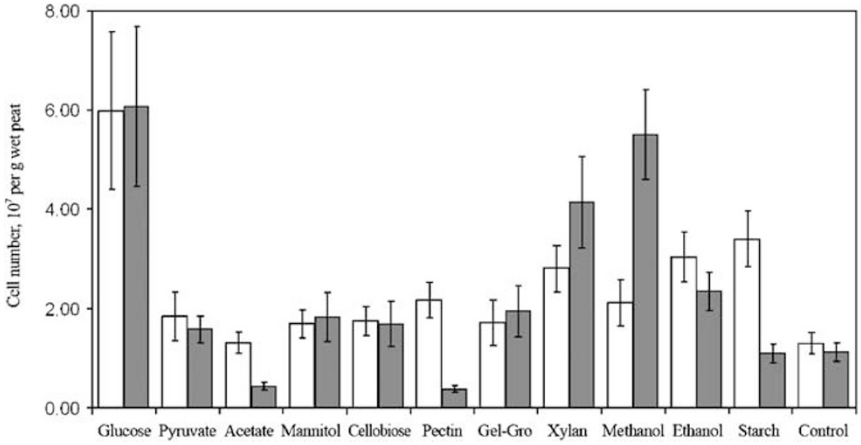

Figure 1 Substrate-induced responses of acidobacteria for 11 carbon sources in an acidic *Sphagnum* peat after incubation at 10 °C (open columns) and 20 °C (shaded columns) as determined by fluorescence *in situ* hybridization with the *Acidobacteria*-specific probe HoAc1402. The error bars indicate ± standard error.

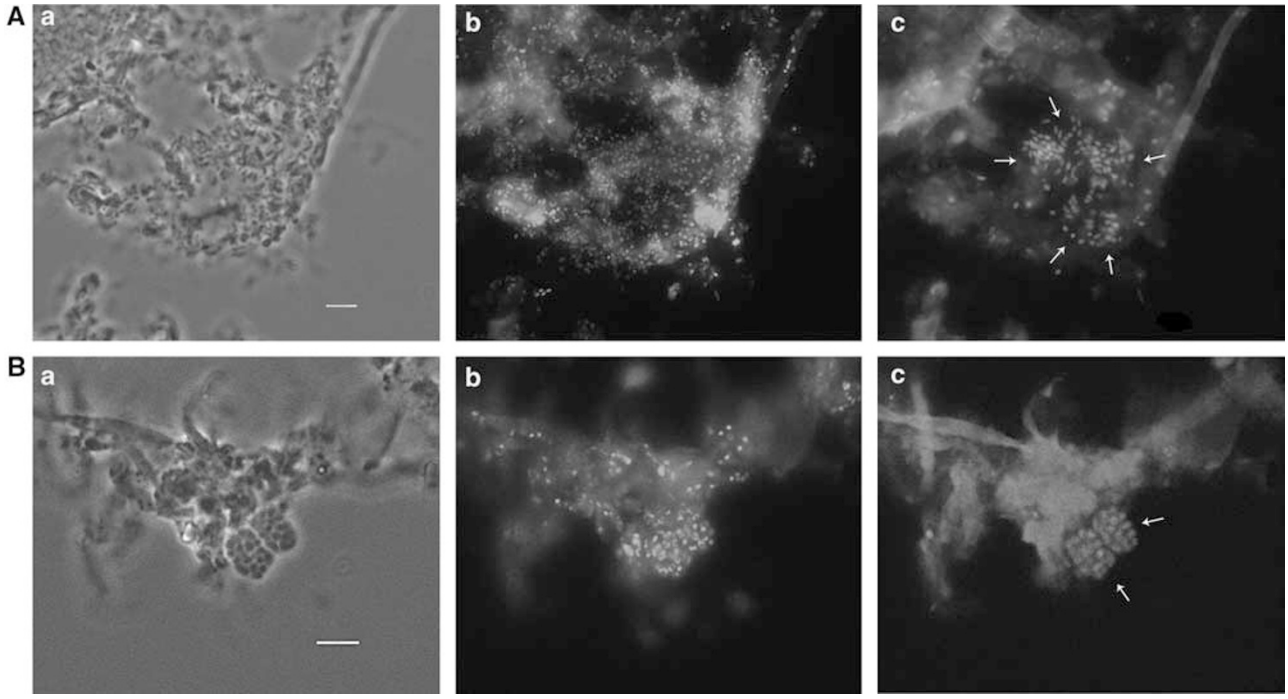

Figure 2 Specific detection of acidobacteria in *Sphagnum* peat amended with pectin (**Aa–c**) and ethanol (**Ba–c**) by fluorescence *in situ* hybridization. Phase-contrast images (**Aa**, **Ba**), 4′,6-diamidino-2-phenylindole (DAPI) staining (**Ab**, **Bb**) and the epifluorescent micrographs of *in situ* hybridizations with Cy3-labeled probe HoAc1402 (**Ac**, **Bc**; arrows indicate cells targeted with this probe) are shown. Bar, 10 μm.

two different temperatures caused no significant difference for growth of acidobacteria on glucose, xylan and ethanol. A clear preference for lower temperatures was observed for acidobacteria that developed on pectin and starch, while the opposite response was characteristic for those developing in methanol-amended peat. The morphology of acidobacterial cells that developed in peat amended with different carbon sources was clearly distinct. For example, *in situ* hybridization with the probe HoAc1402 revealed diffuse growth of rod-shaped acidobacterial cells in peat amended with pectin, while compact, raspberry-like microcolonies of these microorganisms were observed in a sample amended with ethanol (Figure 2). The results of our substrate-amendment experiments thus suggest that the indigenous acidobacterial community was comprised by a number of species differing with respect to their temperature and substrate preferences.

Amendment of peat with different carbon sources resulted in growth stimulation of diverse heterotrophic microorganisms besides that of acidobacteria. For example, addition of glucose and the following incubation at 20 °C led to a fourfold increase in cell numbers detected in FISH with EUB338-mix. Amendments with methanol and xylan caused threefold and twofold increases in bacterial abundance, respectively. Since the respective population responses of acidobacteria on amendments with these three substrates were more pronounced (six-, four- and threefold on glucose, methanol and xylan, respectively), the proportion of acidobacteria in total bacterial community increased up to 5.4, 5.5 and 6.0% in glucose-, methanol- and xylan-amended peat. Thus, these amendments resulted in the enrichment of acidobacteria relative to the rest of the bacterial community.

Addition of pyruvate, mannitol, cellobiose and Gel-Gro to peat samples caused only a very weak response of acidobacteria (Figure 1). Amendment of peat with acetate resulted in no growth response.

FISH-mediated isolation and identification of acidobacteria

In our previous study, we succeeded in obtaining the first isolate of *Acidobacteria* from *Sphagnum* peat, strain MPL3 (Dedysh *et al*., 2006). On the basis of the results of our amendment experiment, we attempted to isolate additional strains of peat-inhabiting acidobacteria using glucose, xylan, pectin and methanol as growth substrates. FISH-based screening revealed the presence of acidobacteria on most of primary isolation plates with each of the tested substrates. Detailed quantification of acidobacterial cells on primary isolation plates was not possible due to their patchy distribution. However, on some of these plates, *Acidobacteria* constituted approximately 5–15% of all bacteria that were detectable by FISH and thus a clearly higher proportion than in native peat samples. The plates containing highest number of target cells were selected for preparing further subcultures and additional FISH-based examinations. Ultimately, nine strains of bacteria targeted with HoAc1402 were obtained from acidic peat. Strains TPB6028, TPB6011 and TPB6017 were isolated from plates containing basal medium MM1 with pectin. Strains TPO1014, OB1010 and AL were isolated from the same medium with xylan. Strains MPL1011 and MOB76 were obtained using medium MM with glucose. Finally, one strain, KMR, was isolated from medium MM with methanol. However, since its growth on methanol was very poor, strain KMR was further transferred to a medium with glucose.

Sequencing of the 16S rRNA genes from these isolates confirmed that they belong to subdivisions 1 and 3 of the *Acidobacteria* (Figure 3). Strains AL and KMR possessed 98–99% 16S rRNA gene sequence similarity to members of *T. roseus*, a recently described species of soil-inhabiting acidobacteria (Eichorst *et al*., 2007) and 99.5% similarity to termite gut isolates TAA43 (GenBank accession no. AY587228) and TAA48 (AY587229) (Stevenson *et al*., 2004). Strains TPO1014, TPB6028, TPB6011 and OB1010 displayed 94–96% sequence similarity to *T. roseus* and formed a common cluster with soil isolate KBS89 (GenBank accession no. AY587227) (Stevenson *et al*., 2004). Strain TPB6017 belonged to a phylogenetic lineage defined by *A. capsulatum* and exhibited the highest sequence similarity (97%) with the uncultured bacterium UA1 from acidic forest soil (AF200696) (Radajewski *et al*., 2002). Three isolates from subdivision 3, strains MPL1011, MOB76 and MPL3, possessed nearly identical 16S rRNA gene sequences and were only distantly related (90% sequence similarity) to several acidobacterial isolates (Ellin 342, 371, 6071, 6115 and '*S. usitatus*' Ellin 6076) obtained from soils (Sait *et al*., 2002; Joseph *et al*., 2003). Since isolates AL and KMR were phylogenetically very close to the taxonomically characterized *T. roseus* and shared most phenotypic features with members of this species, our future studies were focused on the other newly isolated strains of acidobacteria.

Colony and cell morphologies of acidobacterial isolates

Except for strain TPB6011, all novel acidobacterial isolates formed very small (<0.5 mm in diameter), smooth, circular colonies with an entire margin. The colonies of strain TPB6011 attained the size of 2–3 mm. It took approximately 10–14 days of incubation at 20 °C for strains TPO1014, OB1010, TPB6011 and TPB6028 to form visible colonies, while the colonies of TPB6017, MPL3, MOB76 and MPL1011 became visible by naked eye after 3–4 weeks of incubation. The fast-growing group of strains possessed light pink to pink colonies, while the slow-growing strains had colorless colonies. The main absorption maxima detected in the methanol extracts of pink-pigmented isolates were at 445 and 509 nm, suggesting the presence of α- and β-carotene. Thus, despite the fact that chlorophyll-based phototrophy has recently been demonstrated for some thermophilic representatives of the *Acidobacteria* (Bryant *et al*., 2007), our strains did not possess this capability.

Acidobacterial isolates from *Sphagnum* peat displayed diverse cell morphologies (Figures 4a–c). Subdivision 1 strains were represented by thin (0.6–1 μm) rods of different length that ranged from 1.5 to 3 μm for strains TPO1014, OB1010, TPB6011 and TPB6028, and from 3 to 8 μm for strain TPB6017. The cells occurred singly or in pairs. Cells of subdivision 3 isolates were coccoids or short rods measuring 0.6–0.8 by 0.8–1 μm. They formed shapeless cell aggregates when grown in liquid media. The above reported cell sizes were characteristic for cultures grown on media MM or MM1 with glucose or other sugars. However, these sizes were

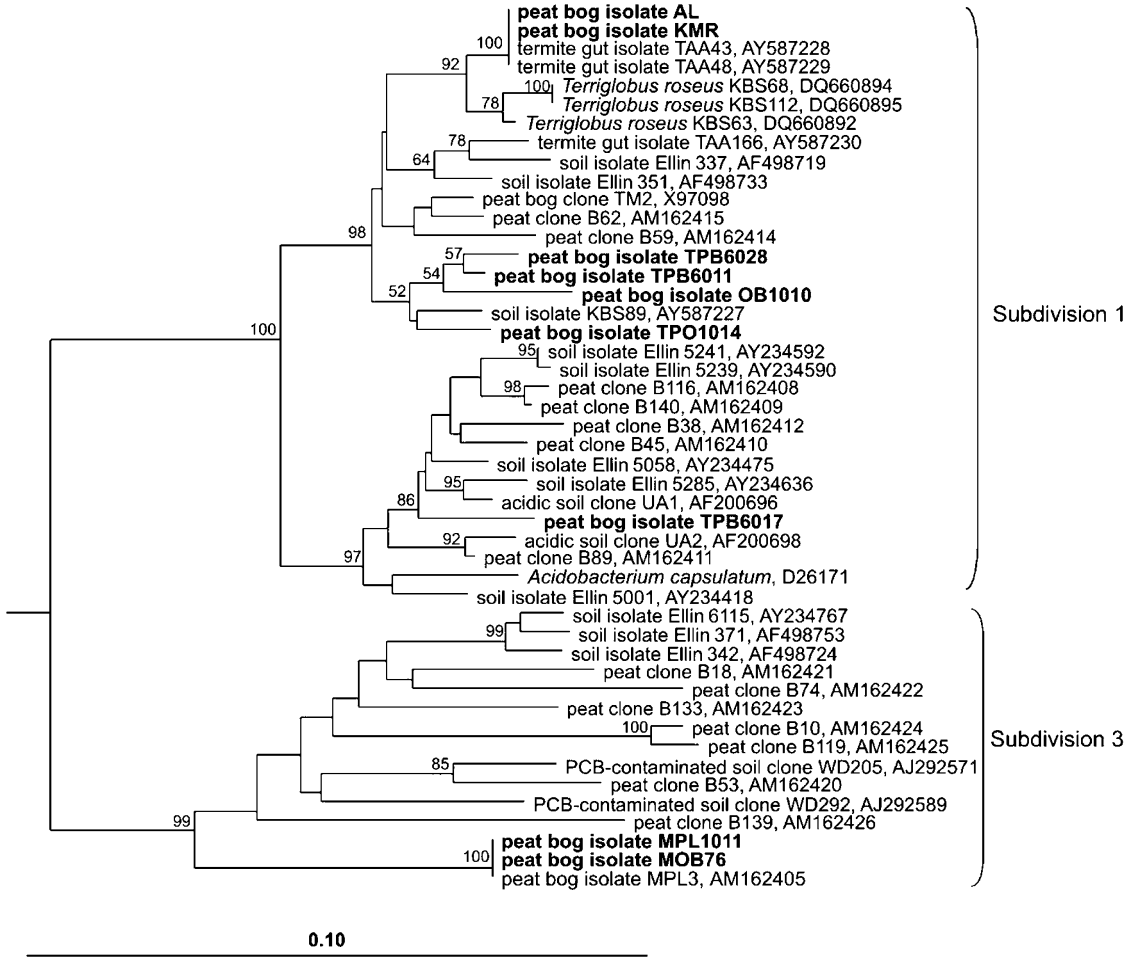

Figure 3 16S rRNA gene-based neighbor-joining tree (Jukes–Cantor correction) showing the phylogenetic relationship of novel isolates from peatlands to representative members of subdivisions 1 and 3 of the phylum *Acidobacteria*. Bootstrap values (1000 data resamplings) of >50% are shown. 16S rRNA sequence of *Geothrix fermentans* (U41563) was used as an outgroup. The scale bar represents 0.1 substitution per nucleotide position. Similar tree topology was obtained using maximum-likelihood method. Sequences obtained in this study are shown in bold.

not stable and varied depending on growth substrate and growth conditions. For example, the cells of strains TPO1014 and OB1010 were twice longer their 'regular' size when grown on pectin. The same was true for cells of subdivision 3 strains grown on ethanol.

Ultrathin sections of representative acidobacterial isolates revealed the cell wall structure typical of Gram-negative bacteria (Figures 4d and e). No intracytoplasmic membranes or other specialized structures were observed in cells of acidobacterial strains. As revealed by contrasting the acid mucopolysaccharides with ruthenium red, the cells of subdivision 1 members produced amorphous extracellular polysaccharide substance, while subdivision 3 isolates possessed very thin capsules covering the cells.

Growth substrates

All strains obtained in this study represented strictly aerobic, heterotrophic organisms. Most sugars and *N*-acetyl-D-glucosamine were the preferred growth substrates of subdivision 1 isolates. They were also capable of hydrolyzing xylan, pectin, laminarine and starch. Subdivision 3 isolates grew best on media with sugars and ethanol, and were capable of hydrolyzing xylan and starch. Most organic acids were either poorly or not utilized. However, all isolates from peat were capable of utilizing galacturonic acid, a characteristic component of the cell wall in *Sphagnum* spp (Clymo, 1963, 1964; Kremer *et al*., 2004), which is released during moss debris decomposition. None of our isolates including strain KMR, which was isolated on agar medium with methanol, were capable of methylotrophic growth. Thus, with the only exception of methanol, the substrate utilization patterns of our isolates matched the results obtained in our substrate-amendment experiments with native peat.

pH and temperature responses

Capability of growth at low pH and low temperatures is of special importance for microbial inhabi-

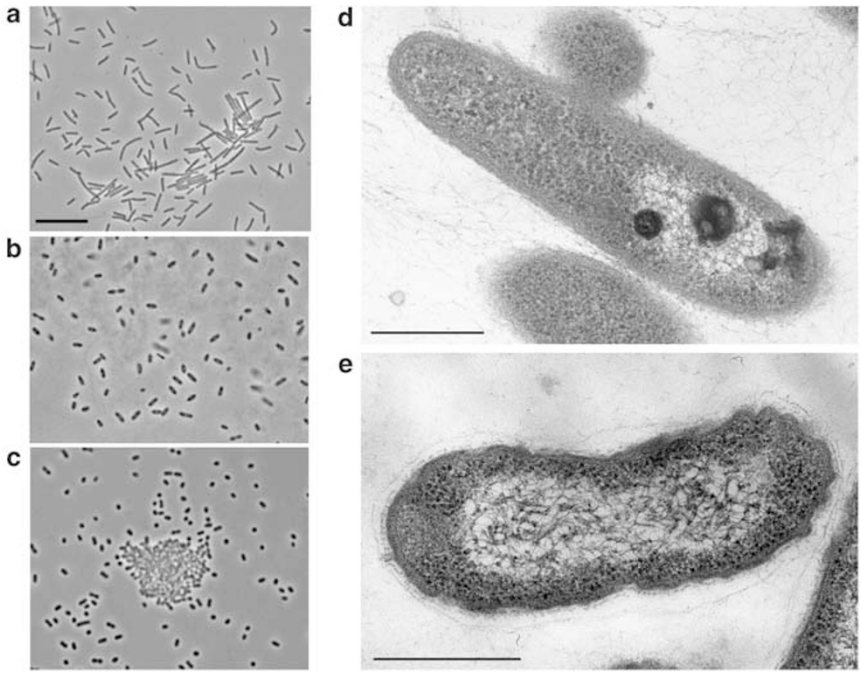

Figure 4 Phase-contrast micrographs of cells of strains TPB6017 (**a**), TPO1014 (**b**) and MPL3 (**c**). Electron micrographs of ultrathin sections of cells of subdivision 1 acidobacterium strain TPO1014 (**d**) and subdivision 3 acidobacterium strain MPL3 (**e**). Bars, 10 μm (**a–c**) and 0.5 μm (**d**, **e**).

tants of northern acidic peatlands. Our experiments showed that peat-inhabiting acidobacteria are adapted to both extremes. Subdivision 1 isolates TPO1014, TPB6011, TPB6028 and OB1010 were truly acidophilic bacteria. They were capable of growth at pH values between 3.1 and 7.8, with an optimum at pH 3.5–4.5 (Figure 5). The highest growth rate ($\mu = 0.1\,h^{-1}$) was observed at pH 3.8–4.0, which is exactly the pH value typical for ombrotrophic *Sphagnum* bogs. Two other isolates from subdivision 1, strains KMR and AL that were phylogenetically related to *T. roseus*, and all isolates from subdivision 3 were less acidophilic and grew between pH 4.5 and 7.2 with an optimum at pH 5.5–6.5. This difference in pH responses of acidobacteria from subdivisions 1 and 3 is consistent with the absence of clear correspondence between the pH value of peat water and the total cell number of acidobacteria detected in our study. As has been shown before, the proportion of subdivision 1 acidobacteria in clone libraries of 16S rRNAs and 16S rRNA genes from different soils increases with decreasing soil pH (Sait *et al.*, 2006; Eichorst *et al.*, 2007). The situation with pH responses of acidobacteria from other subdivisions remains unclear. In our study, we counted acidobacteria with the phylum-specific probe HoAc1402 and were unable to determine the proportion of members from different subdivisions. As a consequence, we failed to observe any kind of relationship between the total cell number of these bacteria and the peat pH.

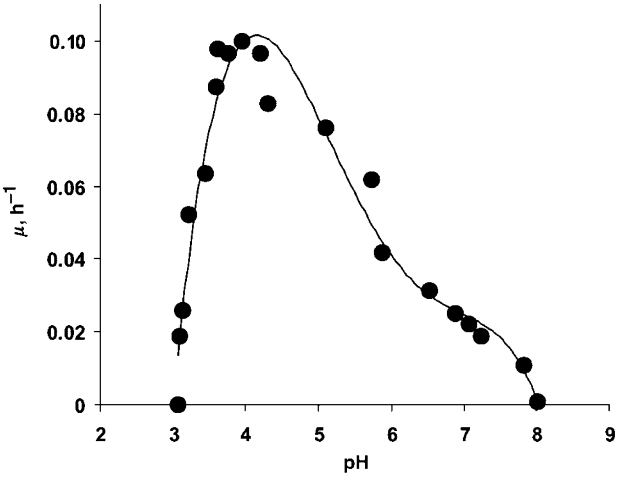

Figure 5 Effect of medium pH on the growth of subdivision 1 acidobacterium strain TPB6011.

The temperature range for growth was similar for all our *Acidobacteria* strains. They grew between 2 and 32 °C, with an optimum at 15–22 °C. Their tolerance of cold conditions was clearly demonstrated by their ability to grow exponentially ($\mu = 0.014\,h^{-1}$), without a lag phase, at 4 °C.

In summary, we showed that members of the *Acidobacteria* are a characteristic component of the microbial community in *Sphagnum*-dominated acidic wetlands. The total number of acidobacterial cells detected in peat by FISH comprised 0.1–4.1%

of total bacterial cells or 0.05–1.2% of all DAPI-stained cells. For comparison, in a highly acidic (pH 2.6) mining lake, acidobacteria comprised 1.4–7.9% of all DAPI-stained cells (Kleinsteuber *et al.*, 2008), although absolute cell numbers of acidobacteria in lake water ($0.4–3.7 \times 10^4$ cells ml^{-1}) were significantly lower than those detected in acidic peat samples. In acidic soils, RNA of acidobacteria accounted for 1–6% of total bacterial rRNA (Buckley Schmidt, 2003).

Substrate availability is one of the major factors that determine the population size of acidobacteria in peat. The preferred growth substrates of aerobic peat-inhabiting members of this phylum are sugars. They gave rise to the most pronounced growth stimulation of acidobacteria, but they are also among the most deficient compounds in peat water. Thus, it is most likely that the survival of acidobacteria in peat is based on the utilization of various heteropolysaccharides and galacturonic acid, which are released during decomposition of *Sphagnum* moss and vascular plant debris. At present, we cannot offer any experimental data to explain the response of acidobacteria to amendment of peat with methanol, since none of our isolates were able to grow on this substrate. We are also not aware about any direct proofs for the occurrence of methylotrophic metabolism in members of the *Acidobacteria*. The situation remains unclear due to limited diversity of cultured acidobacteria, while the results of cultivation-independent studies are not always unequivocal. For example, a few 16S rRNA gene sequences affiliated with the *Acidobacteria* were previously retrieved from a ^{13}C-labelled DNA fraction obtained after amendment of acidic soil with ^{13}C-methanol (Radajewski *et al.*, 2002). One of these sequences, clone UA1, is most similar to the 16S rRNA gene sequence of one of our isolates, strain TPB6017. However, our repeated attempts to cultivate strain TPB6017 in liquid media with various concentrations of methanol were unsuccessful. One possible explanation may be a crossfeeding of acidobacteria on slime-producing methylotrophs, which are highly abundant in acidic peat. This hypothesis coincides well with our observation that on agar media acidobacteria often develop in a coculture with other bacteria that produce slimy, mucous colonies.

Important phenotypic traits that enable development of acidobacteria in *Sphagnum*-dominated northern wetlands are acidophily and cold tolerance. Acidophilic nature of members of subdivision 1 has already been reported for *A. capsulatum* (Kishimoto *et al.*, 1991), *T. roseus* (Eichorst *et al.*, 2007) and some taxonomically noncharacterized isolates obtained from soil (Sait *et al.*, 2006). Most of our isolates from subdivision 1 were truly acidophilic organisms with the growth optimum at pH 3.5–4.5. Subdivision 3 isolates were clearly less acidophilic, although they were also capable of growth at low pH values typical for *Sphagnum*-dominated wetlands. All *Acidobacteria* strains isolated from peat were able to develop at 4 °C and grew well at 10–15 °C, which is the actual temperature of subsurface peat layers in spring and summer seasons.

Thus, our results demonstrate that the metabolic and phenotypic diversity of peat-inhabiting acidobacteria is consistent with their ubiquitous distribution in northern acidic wetlands. The set of novel isolates from acidic peat greatly extends the cultured diversity of acidobacteria and calls for further taxonomic studies. However, since our amendment experiments and cultivation attempts were focused only on aerobic members of the *Acidobacteria*, the above reported data may not be representative for all peat-inhabiting members of this phylum. Our previous FISH-based assessment of depth distribution of *Acidobacteria* in a *Sphagnum* peat bog Bakchar revealed numerically significant population size of these bacteria in an anoxic part of the bog profile (Dedysh *et al.*, 2006). Thus, further research with a special emphasis on anaerobic acidobacteria is needed to obtain a more detailed picture of the metabolic diversity and functional role of members of the *Acidobacteria* in northern wetlands.

Acknowledgements

This work was supported by the Program 'Molecular and Cell Biology' of Russian Academy of Sciences, the Russian Fund of Basic Research (Grant no. 07-04-91561), the Russian Science Support Foundation and the Deutsche Forschungsgemeinschaft (436 RUS 113/543/0-3).

References

Barns SM, Cain EC, Sommerville L, Kuske CR. (2007). *Acidobacteria* phylum sequences in uranium-contaminated subsurface sediments greatly expand the known diversity within the phylum. *Appl Environ Microbiol* **73**: 3113–3116.

Barns SM, Takala SL, Kuske CR. (1999). Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. *Appl Environ Microbiol* **65**: 1731–1737.

Bryant DA, Costas AMG, Maresca JA, Chew AG, Klatt CG, Bateson MM *et al.* (2007). *Candidatus* Chloracidobacterium thermophilum:an aerobic phototrophic acidobacterium. *Science* **317**: 523–526.

Buckley DH, Schmidt TM. (2003). Diversity and dynamics of microbial communities in soils from agro-ecosystems. *Environ Microbiol* **5**: 441–452.

Clymo RS. (1963). Ion exchange in *Sphagnum* and its relation to bog ecology. *Ann Bot (Lond)* **27**: 309–324.

Clymo RS. (1964). The origin of acidity in *Sphagnum* bogs. *Bryologist* **67**: 427–431.

Coates JD, Ellis DJ, Gaw CV, Lovley DR. (1999). *Geothrix fermentans* gen.nov., sp.nov., a novel Fe(III)-reducing

bacterium from a hydrocarbon-contaminated aquifer. *Int J Syst Bacteriol* **49**: 1615–1622.

Daims H, Brühl A, Amann R, Schleifer K-H, Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**: 434–444.

Dedysh SN, Derakshani M, Liesack W. (2001). Detection and enumeration of methanotrophs in acidic *Sphagnum* peat by 16S rRNA fluorescence *in situ* hybridization, including the use of newly developed oligonucleotide probes for *Methylocella palustris*. *Appl Environ Microbiol* **67**: 4850–4857.

Dedysh SN, Liesack W, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD *et al.* (2000). *Methylocella palustris* gen.nov., sp.nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs. *Int J Syst Evol Microbiol* **50**: 955–969.

Dedysh SN, Pankratov TA, Belova SE, Kulichevskaya IS, Liesack W. (2006). Phylogenetic analysis and *in situ* identification of *Bacteria* community composition in an acidic *Sphagnum* peat bog. *Appl Environ Microbiol* **72**: 2110–2117.

Eichorst SA, Breznak JA, Schmidt TM. (2007). Isolation and characterization of soil bacteria that define *Terriglobus* gen. nov., in the phylum *Acidobacteria*. *Appl Environ Microbiol* **73**: 2708–2717.

Felsenstein J. (1989). PHYLIP—phylogeny inference package (version 3.2). *Cladistics* **5**: 164–166.

Hugenholtz P, Goebel BM, Pace NR. (1998a). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol* **180**: 4765–4774.

Hugenholtz P, Pitulle C, Hershberger KL, Pace NR. (1998b). Novel division level bacterial diversity in a Yellowstone hot spring. *J Bacteriol* **180**: 366–376.

Janssen PH. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl Environ Microbiol* **72**: 1719–1728.

Joseph SJ, Hugenholtz P, Sangwan P, Osborne CA, Janssen PH. (2003). Laboratory cultivation of widespread and previously uncultured soil bacteria. *Appl Environ Microbiol* **69**: 7210–7215.

Juretschko S, Loy A, Lehner A, Wagner M. (2002). The microbial community composition of a nitrifying-denitrifying activated sludge from industrial sewage treatment plant analyzed by the full-cycle rRNA approach. *Syst Appl Microbiol* **25**: 84–99.

Kishimoto N, Kosako Y, Tano T. (1991). *Acidobacterium capsulatum* gen.nov., sp.nov.: an acidophilic chemoorganotrophic bacterium containing menaquinone from acidic mineral environment. *Curr Microbiol* **22**: 1–7.

Kleinsteuber S, Müller F-D, Chatzinotas A, Wendt-Potthoff K, Harms H. (2008). Diversity and *in situ* quantification of *Acidobacteria* subdivision 1 in an acidic mining lake. *FEMS Microbiol Ecol* **63**: 107–117.

Kremer C, Pettolino F, Bacic A, Drinnan A. (2004). Distribution of cell wall components in *Sphagnum* hyaline cells and in liverwort and hornwort elaters. *Planta* **219**: 1023–1035.

Liesack W, Bak F, Kreft JU, Stackebrandt E. (1994). *Holophaga foetida* gen.nov., sp.nov., a new homoacetogenic bacterium degrading methoxylated aromatic compounds. *Arch Microbiol* **162**: 85–90.

Ludwig W, Bauer SH, Bauer M, Held I, Kirchhof G, Schulze R *et al.* (1997). Detection and *in situ* identification of representatives of a widely distributed new bacterial phylum. *FEMS Microbiol Lett* **153**: 181–190.

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

Martiny AC, Albrechtsen H-J, Arvin E, Molin S. (2005). Identification of bacteria in biofilm and bulk water samples from a nonchlorinated model drinking water distribution system: detection of a large nitrite-oxidizing population associated with *Nitrospira* spp. *Appl Environ Microbiol* **71**: 8611–8617.

Meisinger DB, Zimmermann J, Ludwig W, Schleifer K-H, Wanner G, Schmid M *et al.* (2007). *In situ* detection of novel *Acidobacteria* in microbial mats from a chemolithoautotrophically based cave ecosystem (Lower Kane Cave, WY, USA). *Environ Microbiol* **9**: 1523–1534.

Radajewski S, Webster G, Reay DS, Morris SA, Ineson P, Nedwell DB *et al.* (2002). Identification of active methylotroph populations in an acidic forest soil by stable-isotope probing. *Microbiology* **148**: 2331–2342.

Rheims H, Rainey FA, Stackebrandt E. (1996). A molecular approach to search for diversity among bacteria in the environment. *J Ind Microbiol* **17**: 159–169.

Sait M, Davis KER, Janssen PH. (2006). Effect of pH on isolation and distribution of members of subdivision 1 of the phylum *Acidobacteria* occurring in soil. *Appl Environ Microbiol* **72**: 1852–1857.

Sait M, Hugenholtz P, Janssen PH. (2002). Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. *Environ Microbiol* **4**: 654–666.

Staley JT, Fuerst JA, Giovannoni S, Schlesner H. (1992). The order *Planctomycetales* and the genera *Planctomyces*, *Pirellula*, *Gemmata* and *Isosphaera*. In: Balows A, Truper H, Dworkin M, Harder W, Schleifer K-H (eds). *The Prokaryotes: a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, 2nd edn. Springer-Verlag: New York, pp 3710–3731.

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.

Zimmermann J, Gonzalez JM, Saiz-Jimenez C, Ludwig W. (2005). Detection and phylogenetic relationships of highly diverse uncultured acidobacterial communities in Altamira Cave using 23S rRNA sequence analysis. *Geomicrobiol J* **22**: 379–388.