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Identification and Targeted Cultivation of Abundant Novel Freshwater Sphingomonads and Analysis of Their Population Substructure▽†

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Little is known with respect to bacterial population structures in freshwater environments. Using complementary culture-based, cloning, and high-throughput Illumina sequencing approaches, we investigated microdiverse clusters of bacteria that comprise members with identical or very similar 16S rRNA gene sequences. Two 16S rRNA phylotypes could be recovered by cultivation in low-nutrient-strength liquid media from two lakes of different trophic status. Both phylotypes were found to be physiologically active *in situ* throughout most of the year, as indicated by the presence of their rRNA sequences in the samples. Analyses of internal transcribed spacer (ITS1) sequences revealed the presence of seven different sequence types among cultured representatives and the cloned *rrn* fragments. Illumina sequencing yielded 8,576 ITS1 sequences that encompassed 15 major and numerous rare sequence types. The major ITS1 types exhibited distinct temporal patterns, suggesting that the corresponding *Sphingomonadaceae* lineages occupy different ecological niches. However, since strains of the same ITS1 type showed highly variable substrate utilization patterns, the potential mechanism of niche separation in *Sphingomonadaceae* cannot be explained by substrate utilization alone and may be related to other traits.

A prokaryotic species is operationally defined as a phenotypically consistent group of strains exhibiting ≥70% similarity of their genomic DNA and ≥97% sequence identity of the 16S rRNA gene (50) (≥98.7% according to a more recent suggestion [57]). However, two-thirds of the diversity present in coastal bacterioplankton resides in clusters of sequences with ≥99% sequence identity (1), so-called microdiverse clusters. Microdiverse bacterial communities also have been found among, e.g., *Synechococcus* populations in an alkaline siliceous hot spring microbial mat (37), *Bacillus simplex* populations in soil (29), and *Firmicutes* and *Bacteroidetes* species in the human distal gut (14). It has been suggested that such microdiverse clusters within the same species arise by periods of selectively neutral diversification that are punctuated by adaptive mutations and followed by selective sweeps (12). Consequently, microdiverse clusters may represent populations of bacterial cells that share ecological niches and adaptations and may therefore be regarded as distinct evolutionary entities, so-called ecotypes (22, 33). Alternatively, such clusters may result from neutral evolution alone (18).

Microdiverse clusters of marine *Vibrio splendidus* occur at different water temperatures and hence may represent individual ecotypes (59). Similarly, different ecotypes of *Synechococcus* seem to be adapted to different temperatures in hot-spring microbial mats (37). Ecotypes of the marine oxygenic phototroph *Prochlorococcus* differ by less than 3% sequence divergence of their 16S rRNA genes and partition themselves according to high or low light intensity in the water column of tropical or subtropical oceans (49). Ecotypes of the soil bacteria *Bacillus subtilis* and *B. licheniformis* that typically diverge by 0.3% of 16S rRNA gene sequences are adapted to different soil temperatures and correspondingly exhibit different compositions of temperature-relevant fatty acids (13). Apart from a study of the marine "*Candidatus* Pelagibacter ubique" (61), the population structure of typical aquatic oligotrophs is largely unexplored. This is particularly true for freshwater environments. Recently, ecological diversification among different lineages of the betaproteobacterium *Polynucleobacter necessarius* subspecies *asymbioticus* has been demonstrated. These lineages inhabit stagnant freshwaters and differ by less than 1% in their 16S rRNA gene sequences, but they seem to occupy distinct niches with respect to pH, conductivity, and dissolved organic carbon and oxygen concentrations (27).

In some oligotrophic freshwater lakes, *Alphaproteobacteria* can account for up to 16 or even 24% of the detectable *Bacteria* (2, 23, 52). Functional differentiation among strains of the alphaproteobacterial genera *Brevundimonas* or among strains of *Sandarakinorhabdus* with identical 16S rRNA sequences has already been demonstrated (21, 26) and may lead to a lasting coexistence of these strains in the natural environment. Sphingomonads represent typical constituents of freshwater bacterioplankton communities (23, 45, 65) that can be recovered by cultivation in defined low-nutrient liquid media (21). Based on the existing information, sphingomonads thus represent a

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suitable novel target group for studies of bacterial population structure and dynamics in freshwater aquatic habitats.

In the present study, we analyzed the population substructure and seasonal dynamics of *Sphingomonadaceae* to gain first insights into the processes that are involved in bacterial speciation and niche formation.

MATERIALS AND METHODS

Sampling sites and environmental parameters. The oligotrophic alpine Walchensee lake is located at 802 m above sea level (a.s.l.) and has a maximum depth of 190 m. Samples were collected by boat at a distance of 30 m from the western shore (47°35′N, 11°20′E). Mesotrophic Starnberger See lake is located 23 km north of Walchensee lake at 584 m a.s.l. and has a maximum water depth of 128 m. Sampling was done from a pier on the eastern shore near the town of Ammerland (47°54′11N, 11°19′54E). Water samples were collected on 20 December 2007 and on 28 April, 14 August, and 23 October 2008 at a water depth of 1 m. The pump system employed consisted of an inlet made of two polyvinyl chloride cones that were spaced 1 cm apart and that were connected to a bilge pump via isoversinic tubing (42). Temperature, pH, and conductivity of the water samples were determined with a WTW Multi 340i multimeter equipped with a SenTix 41-3 and a TetraCon 325 electrode (WTW, Weilheim, Germany) (Table 1).

Bacterial cell counts. Water samples were stained with 4′,6-diamidino-2-phenylindole (DAPI), and bacterial cells were collected onto black polycarbonate filters (pore size, 0.1 μm; Millipore GmbH, Schwalbach, Germany) as described previously (8). Total bacterial cell numbers were determined by epifluorescence microscopy (Zeiss Axiolab microscope equipped with the filter set Zeiss Ex 450–490/FT 510/LP 515).

Cultivation of planktonic *Sphingomonadaceae*. Water samples from both sampling sites obtained in winter 2007 and summer 2008 were used for cultivation. We used basic synthetic freshwater medium buffered with 10 mM HEPES (6) and supplemented with 20 canonical amino acids, glucose, pyruvate, citrate, 2-oxoglutarate, succinate (200 μM each), Tween 80 (0.001%, vol/vol) and a fatty acid mixture containing formate, acetate, and propionate (200 μM each) (25). Trace-element solution SL 10 (final concentration, 1 ml · liter^{-1}) and 10-vitamin solution (final concentration, 10 ml · liter^{-1}) were added (8). For growth stimulation, the inducers cyclic AMP (cAMP), *N*-butyryl homoserine lactone, *N*-oxohexanoyl-DL-homoserine lactone, and ATP were added at a final concentration of 10 μM each (8).

Aliquots of 200 μl growth medium were dispensed into the wells of sterile 96-well round-bottom microtiter plates. Each well was inoculated by employing the multidrop combi apparatus (Thermo Electron Corporation, Vantaa, Finland) with a volume of 50 or 200 cells per well (10). For each microtiter plate, 12 wells served as contamination controls and hence were left inoculated. Plates were incubated for 6 weeks at 15°C. Bacterial cell growth was determined by turbidity measurement, and positive cultures were screened by the *Sphingomonadaceae*-specific PCR with primers Sphingo866f and Alf968r. Cultures that tested positive for *Sphingomonadaceae* were streaked on plates containing different combinations of media and gelling agents. These solid media contained basic synthetic freshwater medium and either 1:10-diluted HD medium (consisting of 0.05% casein peptone, 0.01% glucose, 0.025% yeast extract, wt/vol) or the carbon substrates listed above (amino acids, carbon sources, fatty acids, and inducers). Washed agar and gellan gum were used as gelling agents.

The number of culturable cells per well, x, was calculated from the fraction of positive wells (p) among all inoculated microtiter wells according to $x = -\ln(1 - p)$ (9). The corresponding 95% confidence intervals (CI_{95}) were calculated from p and the total number of inoculated wells (n) according to the following equation: $CI_{95\%} = \pm 1.96 \cdot p/[n \cdot (1 - p)]$.

Nucleic acid extraction and cDNA synthesis. For the extraction of DNA and RNA, cells from 250 to 500 ml of lake water from Walchensee and Starnberger See lakes were collected onto Isopore polycarbonate membrane filters (pore size, 0.1 μm; diameter, 47 mm; Millipore GmbH, Schwalbach, Germany). DNA was extracted using the protocol of Fuhrman et al. (19) as modified by Marschall et al. (35). Concentrations were determined by fluorescent dye binding with PicoGreen (Invitrogen, Karlsruhe, Germany) employing a microtiter plate reader (Tecan Infinite M200; Männedorf, Switzerland).

RNA was isolated using a modification of the method of Eichler et al. (16) as described previously (35). Remaining DNA was degraded with DNase I (Fermentas, St. Leon-Roth, Germany), and RNA was purified using the RNeasy MinElute cleanup kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. RNA concentrations were determined with a nanodrop

TABLE 1. Parameters and total cell counts for the two sampling sites

Water source	Trophic state[a]	Mixing type[a]	Secchi depth (m)	Amt of NO_3-N (mg · liter^{-1})[a]	Amt of P (total; μg · liter^{-1})[a]	Amt of chlorophyll a[a] (μg · liter^{-1})	Amt of DOC[a,b] (mg · liter^{-1})	Sampling date	Water temp (°C)	Conductivity (μS · cm^{-1})	pH	Total cell count ($\times 10^5$ cells · ml^{-1})
Walchensee	Oligotrophic	Dimictic	16	0.58	<5.0	1.5	1.2	20.12.2007	5.3	291	8.14	4.5 ± 0.5
								28.04.2008	7.5	290	8.2	9.2 ± 1.1
								14.08.2008	17.7	266	8.5	8.9 ± 1.3
								23.10.2008	11.9	281	8.32	7.8 ± 0.9
Starnberger See	Mesotrophic	Monomictic	6	0.32	9	2.2–6.2	NA	20.12.2007	4.4	326	7.95	3.8 ± 0.5
								28.04.2008	9.4	324	8.3	16.0 ± 2.8
								14.08.2008	22	304	8.58	9.4 ± 2.1
								23.12.2008	13.4	304	8.4	8.9 ± 1.14

[a] Data are from Gich et al. (21).
[b] DOC, dissolved organic carbon.

TABLE 2. Differences in PCR conditions for each amplification protocol used in this study

Primer[a]	Fragment length (bp)	Total volume (μl)	$MgCl_2$ concn (mM)[b]	Annealing temp (°C) and no. of cycles[c]	Extension time at 72°C (s)	BSA[d] concn (μg · μl^{-1})	DNA polymerase (U)
Alpha19f	2,080–2,130	50	2.25	10×; 67	90	0.8	1.25; *Taq* (Qiagen)
LS48r	2,080–2,130	50	2.25	25×; 62	90	0.8	1.25; *Taq* (Qiagen)
Sphingo866f	103	10	1.5	10×; 63	30		0.25; *Taq* (Qiagen)
Alf968r	103	10	1.5	25×; 61	30		0.25; *Taq* (Qiagen)
Alpha19f	847	50	1.5	10×; 67	60		1.25; *Taq* (Qiagen)
Sphingo866r	847	50	1.5	20×; 60	60		1.25; *Taq* (Qiagen)
GC341f	525	50	2.25	10×; 61	60	0.8	1.25; *Taq* (Qiagen)
Sphingo866r	525	50	2.25	20×; 56	60	0.8	1.25; *Taq* (Qiagen)
G1A-ITS-35f	560	50	1.5	35×; 52	60		1.25; Phusion (Finnzymes)
G1A-ITS-595r	560	50	1.5	35×; 52	60		1.25; Phusion (Finnzymes)

[a] Final concentration of 1 μM.
[b] The PCR buffer concentration was 1.5 mM.
[c] The melting temperature was 94°C for 30 s in each cycle.
[d] BSA, bovine serum albumin.

ND-1000 (Peglab, Erlangen, Germany). Subsequently, RNA was reverse transcribed into cDNA using the ImProm-II reverse transcriptase (Promega, Mannheim, Germany) and random hexamer primers according to the instructions of the manufacturer.

Cloning of *rrn* operon fragments from planktonic *Alphaproteobacteria*. Amplicons of the almost-full-length 16S rRNA gene and the first internal transcribed spacer (ITS1) were generated using the alphaproteobacterial specific primer set Alpha19f (5′-CTG GCT CAG ARC GAA CG-3′ [34]) and LS48r (5′-ACG TCY TTC ATC GCC T-3′ [44]). As previously documented (34, 44), this primer set targets 16S rRNA and 23S rRNA gene sequences, respectively, of *Deltaproteobacteria* and *Verrucomicrobia*. Therefore, we searched for an improved priming site using all 33,637 *Alphaproteobacteria* 16S rRNA gene sequences and 1,180 *Alphaproteobacteria* 23S rRNA gene sequences available in the SILVA database (http://www.arb-silva.de), employing the probe design tool implemented in the ARB software package (32). However, this new analysis did not yield an improved primer.

Amplification reactions were performed in a Veriti 96-well thermal cycler (Applied Biosystems, Carlsbad, CA) employing the conditions listed in Table 2 and yielded PCR products with lengths between 2,080 and 2,130 bp. After the purification of PCR products and quantification with PicoGreen (as described above), amplification products generated from the four different DNA samples for each lake were mixed at equal portions and then ligated into the pCR 2.1 TOPO TA cloning vector (Invitrogen, Darmstadt, Germany) according to the instructions of the manufacturer.

Specific detection of *Sphingomonadaceae* 16S rRNA genes. Clones and enrichments containing 16S rRNA genes of *Sphingomonadaceae* were identified by a specific PCR screen developed in the present study. For this purpose, the novel primer Sphingo866f (5′-CGCATTAAGTTATCCGCC-3′) was developed. This primer is specific for the 16S rRNA gene of *Sphingomonadaceae*, except for the deep-branching genus *Sphingosinicella* and *Sphingomonas kaistensis* 16846^T. Primer Sphingo866f was combined with *Alphaproteobacteria* primer Alf968r (5′-GGTAAGGTTCTGCGCGTT-3′ [39]) to yield 103-bp-long 16S rRNA gene fragments employing optimized amplification conditions (Table 2).

To identify particular *Sphingomonadaceae* phylotypes among the primary enrichment cultures for subsequent isolation trials, larger 16S rRNA gene fragments were required. Therefore, an 846-bp-long 16S rRNA gene fragment was generated using primer Alpha19f (as described above), the reverse complementary version of primer Sphingo866 (Sphingo866r; 5′-GGCGGATAACTTAATG CG-3′), and the PCR conditions specified in Table 2 (also see the supplemental material).

Sequencing and phylogenetic analysis. The cloned *rrn* operon fragments were sequenced by the dideoxynucleotide method on an ABI Prism 3730 genetic analyzer (Applied Biosystems, Carlsbad), employing primers Alpha19f, uni1492r (5′-GGTTACCTTGTTACGACTT-3′ [30]), and uni1392f (5′-GYACACACCG CCCGT-3′ [40]) and the BigDye v3.1 chemistry. Amplicons obtained from cultures were sequenced directly. Sequences were edited and assembled with the Vector NTI computer package (Invitrogen).

Sequences were screened for the presence of chimeras by the Greengenes software Bellerophon, and chimeric sequences were removed. The phylogenetic analysis of 16S rRNA gene and ITS1 sequences was conducted with the ARB software package (32). Sequences were automatically aligned with the integrated Fast Aligner tool of the ARB package, and the alignment was corrected manually according to secondary-structure information. Small nucleotide differences of ≤0.35% between 16S rRNA gene or ITS1 sequences are within the error range of the *Taq* polymerase (31, 51). Consequently, such small nucleotide differences were considered in the sequence analysis only if they were verified for different clones and could be confirmed by secondary-structure analysis (i.e., if two single-nucleotide exchanges were found at complementary positions within double helix regions of the 16S rRNA). In addition, small nucleotide differences were confirmed by repeating the PCR and sequencing.

The 16S rRNA gene and ITS1 phylogenetic trees were constructed based on maximum likelihood, neighbor-joining, and maximum parsimony algorithms. Phylogenetic trees were generated with the ARB software package, and bootstrap values were calculated with 1,000 bootstrap resamplings. We identified 16S rRNA gene sequences of the closest relatives by using the NCBI BLAST online tool (3), and classification was verified by the RDP classifier (62). Rarefaction curves, diversity indexes (Shannon and Simpson), and richness analysis (Chao1 and ACE) were calculated with DOTUR (53).

Phylogenetic fingerprinting by DGGE. Seasonal changes in the composition of the active fraction of *Sphingomonadaceae* in Walchensee and Starnberger See lakes were analyzed by comparative phylogenetic fingerprinting employing denaturing gradient gel electrophoresis (DGGE). From the *Sphingomonadaceae* 16S rRNA genes and 16S rRNA-cDNA, 525-bp-long fragments were generated that employed the bacterial primer GC341f (38), the specific primer Sphingo866r (described above), and the appropriate cycling conditions (Table 2). Separate amplification products were generated for the DNA and cDNA of each sampling date and each lake.

PCR products were separated by DGGE in 6% (wt/vol) polyacrylamide gels containing a linear gradient of 35 to 65% denaturant (41) using the Ingeny phorU system (Ingeny International BV, Goes, Netherlands). Electrophoresis was performed at 60°C with 200 V for 15 min, followed by 16 h at 100 V. Polyacrylamide gels were stained with SYBRGold (MoBiTec, Göttingen, Germany) for 30 min. The generated DGGE profiles were analyzed using the Gel ComparII package (Applied Maths, Sint-Martens-Latem, Belgium).

Representative DNA bands were excised from the gel with a sterile scalpel and transferred to 25 μl of 10 mM Tris-HCl buffer (pH 8.0), and DNA was extracted from the gel pieces by overnight incubation at 4°C. One μl of the supernatant was used as the template in subsequent PCR, employing the corresponding primers without a GC clamp. PCR products were sequenced after cleaning. Three DNA fragments yielded two sequence types that were identified after cloning using a TOPO TA cloning kit (Invitrogen, Darmstadt, Germany). Sequence analysis revealed that the presence of two sequences in the same band had to be attributed to cross-contamination by the large amount of *Sandarakinorhabdus limnophila* 16S rRNA genes in the same lane rather than to the formation of heteroduplexes. Sequences were classified using BLAST (3) and the ARB software package.

Population substructure of the G1A and G7A phylotypes. To investigate the population substructure within the G1A and G7A phylotypes, the ITS1 region located between the 16S rRNA gene and the 23S rRNA genes was analyzed for the natural populations as well as the available isolates. A primer set consisting of G1A-ITS-35f (5′-AAGGATTTCGGCGGAA-3′) and G1A-ITS-595r (5′-CT ATTTGATTTGTAACAGCAC-3′) was devised that permitted a specific ampli-

fication of a 560-bp-long ITS1 fragment of bacteria of the G1A phylotype using the PCR conditions listed in Table 2. Separate amplification products were generated for the DNA and cDNA of each sampling date and each lake. The cDNA samples were preamplified for 15 cycles with the described conditions, and 1 μl of the PCR product was used for the second amplification for 35 cycles. Products were purified via the NucleoSpin extract II kit gel extraction protocol (Macherey-Nagel, Düren, Germany).

ITS-PCR products were analyzed by paired-end sequencing (covering 150 bp from each end, including the variable regions) employing the Illumina Genome Analyzer IIx. Libraries of ITS fragments were prepared with NEBNext DNA sample prep master mix set 1 (NEB, Frankfurt am Main, Germany) according to the instructions of the manufacturer. The fluorescent images were processed to sequences using the Genome Analyzer pipeline analysis software, verision 1.9 (Illumina).

Sequence reads containing the primers for amplicon sequencing were trimmed to a fixed length of 110 bp because of low sequence quality at the ends of sequence reads, and the forward and reverse reads were concatenated. Because small sequence differences had to be detected, sequences were strongly filtered, allowing no Phred quality score below 20 for each nucleotide. Both tasks were performed by applying custom Java programs. FastQ files were converted to FASTA and Quality files using Biopython (11). Unique sequences were determined by applying the unique.seqs command from Mothur (54) after filtering for remaining ambiguous nucleotides. Based on the sequence database generated for each sampling date, seasonal differences in abundance were determined for unique sequence types that represented a fraction of >5% in at least one of the samples.

Physiological testing of G1A isolates. The Gen III microplate (Biolog, Hayward, CA) tests the utilization of 71 sole carbon sources and the effect of 23 inhibitory substances by measuring cell respiration by the reduction of tetrazolium salts. The cells were streaked on agar plates containing HD diluted 1:10 and incubated for 2 to 7 days to yield sufficient cell mass. Gen III microplates were inoculated with cells resuspended in the inoculation fluid IF-A according to the recommendations of the manufacturer (Biolog). Subsequently, the plates were incubated in the dark at 28°C, and the intensity of the red color resulting from the reduction of the tetrazolium salt was measured after 3 to 6 days using the Omnilog-PM reader in the Single Read ID mode. The maximum intensity value is 400 (Barry Bochner, Biolog, personal communication). Prior to further analysis, the value of the negative-control A1 well was subtracted from all other wells. To determine the variability of individual phenotypic traits for the strains of ITS type 4 ($n = 16$) or the pooled strains of ITS types 2, 3, 5, and 6 ($n = 13$), the variance values were plotted using the R! package ggplot2 (63). Box plots were constructed using the geom_boxplot function in ggplot2.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences obtained in the present study were deposited in the GenBank database under accession numbers JF275006 to JF275059, JF297619 to JF297643, and JN087937 to JN087939.

RESULTS AND DISCUSSION

***Sphingomonadaceae* represent a diverse and dominant group of *Alphaproteobacteria* in Walchensee.** A clone library of alphaproteobacterial *rrn* fragments was established by employing primers Alpha19f and LS48r that target the 5′ ends of the 16S and 23S rRNA genes, respectively. The resulting amplicons cover almost the entire 16S rRNA gene and the complete first internal transcribed spacer (ITS1) region of the *rrn* operon. To cover a larger fraction of the alphaproteobacterial diversity that was present in Walchensee lake, amplification products from bacterioplankton sampled during each of the four seasons were pooled at equal amounts before cloning and sequencing. A total of 725 clones were generated from this mixed sample and subsequently analyzed.

In a first step, a limited number of 125 clones were sequenced to determine the relative abundance of different alphaproteobacterial subgroups in the clone library. Sixty-six of the clones were identified as 16S rRNA gene sequences of *Alphaproteobacteria* (Fig. 1; also see Fig. S1 in the supplemental material). In addition to alphaproteobacterial sequences,

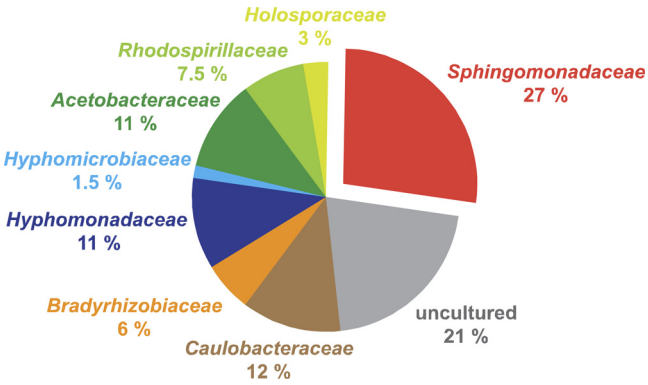

FIG. 1. Fractions of 16S rRNA gene sequences within the clone library of Walchensee bacterioplankton affiliated with the different families of *Alphaproteobacteria*.

clones affiliated with the phyla *Deltaproteobacteria* and *Verrucomicrobia* were present in the library, reflecting the established fact that primer Alpha19f is not fully specific for the 16S rRNA gene sequence, and LS48r is not fully specific for the 23S rRNA gene sequence, of *Alphaproteobacteria* (34, 44). The sequences of *Alphaproteobacteria* were phylogenetically diverse and affiliated with eight families and five subgroups of uncultured members (see Fig. S1). The results for the Walchensee bacterioplankton community therefore are in line with those of previous studies that demonstrated a high diversity of bacteria of this subphylum in other freshwater lakes (17, 23, 65). In the Walchensee clone library, *Sphingomonadaceae* comprised 27% of the cloned sequences and hence constituted the dominant group of planktonic *Alphaproteobacteria* (Fig. 1). *Caulobacteraceae* (12%), *Hyphomonadaceae* (11%), and *Acetobacteraceae* (11%) were less abundant in the clone library.

Sphingomonadaceae represent typical members of freshwater bacterioplankton communities (21, 23, 65) but also are widespread in the marine environment, in pristine and contaminated soils, the rhizosphere, clinical specimens, deep subsurface aquifers, and sewage treatment plants (4, 28, 58). Bacteria of this phylogenetic group are physiologically highly diverse (4). Furthermore, aquatic *Sphingomonadaceae*, including some oligotrophic representatives, have been successfully recovered by cultivation on low-nutrient media (43, 46). Because of their abundance, physiological diversity, and culturability, *Sphingomonadaceae* were chosen as the target group for the subsequent analysis of bacterial population substructure.

To identify all available *Sphingomonadaceae* clones, a specific PCR protocol was developed and used to screen the 725 clones of the alphaproteobacterial 16S rRNA gene library that had been established for Walchensee bacterioplankton. This yielded a total of 80 clones of the bacterial target group. All PCR-positive clones represented 16S rRNA gene sequences of *Sphingomonadaceae*, confirming the high specificity of our new PCR protocol. A detailed phylogenetic analysis placed the 80 sequences in 11 separate phylotypes (G1A, G2B, G2D, G4A, G5B, G6B, G7A, G7B, G7C, G7D, and G8A) (Fig. 2A and B). Six of these phylotypes consisted of more than three clones with 100% sequence identity. The two phylotypes G1A and G7A (Fig. 2A, shaded in gray) by far dominated the clone

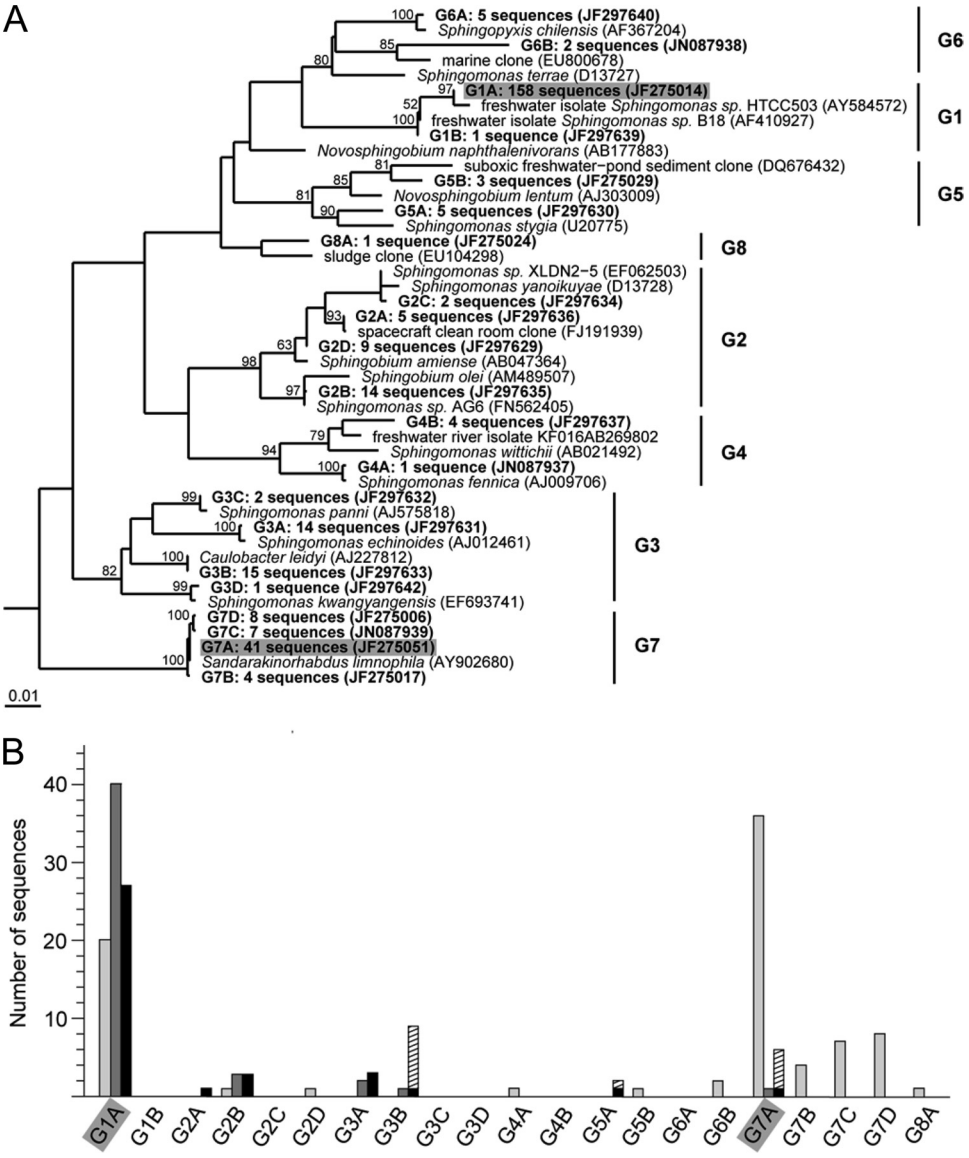

FIG. 2. (A) Maximum likelihood phylogenetic tree of almost-full-length *Sphingomonadaceae* 16S rRNA sequences obtained in the present study (in boldface). Eighty sequences of the environmental clone library, 117 sequences originating from primary liquid enrichment cultures, and 111 sequences of isolated pure cultures of *Sphingomonadaceae* were included in the analysis. The most abundant phylotypes, G1A and G7A, are indicated by gray boxes. Bar represents 0.01 fixed-point mutations per nucleotide. Values at nodes give bootstrap values in percentages (out of 1,000 bootstrap resamplings; only values of ≥50% are given). (B) Frequency of 16S rRNA sequence types from Walchensee lake present in the clone library (light gray columns) and enrichments (dark gray columns) (both from December 2007 samples) and among pure isolates from December 2007 (black columns) and August 2008 (hatched columns).

library and comprised 20 (25%) and 34 clones (42.5% of the clones), respectively (Fig. 2B, black columns). Based on our phylogenetic analysis, phylotype G1A forms a novel genus with ≤95% 16S rRNA gene sequence similarity to established *Sphingomonadaceae*, whereas phylotype G7A is identical to *Sandarakinorhabdus limnophila* DSM 17366^T, which was previously isolated from Walchensee lake.

The value for the richness estimator Chao1 calculated for the *Sphingomonadaceae* 16S rRNA gene clone library amounted to 50 phylotypes. Thus, the 11 phylotypes detected in this study account for ~22% of the existing phylotypes. However, because of the skewed frequency distribution that encompassed two dominant and many unique sequence types, it appears likely that rare phylotypes account for most of the *Sphingomonadaceae* diversity in Walchensee lake that was missed by cloning and sequencing.

The two dominant *Sphingomonadaceae* phylotypes persist and are physiologically active throughout different seasons. In a subsequent step, comparative phylogenetic fingerprinting was conducted to elucidate the seasonal shifts in the composition of planktonic *Sphingomonadaceae*. In parallel, cDNA was generated from total RNA extracts and also was subjected to phylogenetic fingerprinting to identify the physiologically active members of the *Sphingomonadaceae*. Bacterioplankton

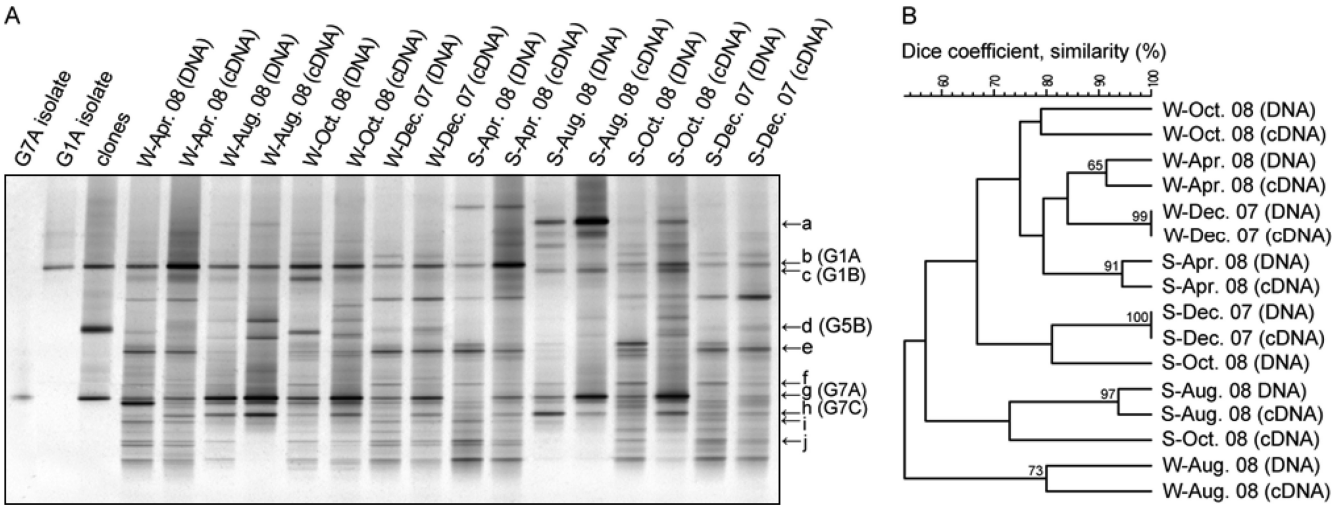

FIG. 3. (A) Seasonal changes in the composition (based on the analysis of 16S rRNA genes; labeled DNA) and in the composition of the active fraction (based on the analysis of rRNA-cDNA; labeled cDNA) of *Sphingomonadaceae* in Walchensee and Starnberger See lakes. A negative image of a SybrGold-stained denaturing gradient gel is shown. Fragments were amplified using a PCR protocol specific for *Sphingomonadaceae* 16S rRNA genes. For comparison, the fingerprints of an isolated representative of phylotype G1A (isolate 505) and phylotype G7A (isolate 407), as well as fingerprints of cloned sequences of phylotypes G1A, G5B, and G7A (compare to Fig. 2A), are shown. (B) Cluster analysis of DGGE fingerprint patterns of seasonal *Sphingomonadaceae* communities using UPGMA. Values at nodes give bootstrap values in percentages (out of 10,000 bootstrap resamplings; only values of ≥50% are given). W, Walchensee lake; S, Starnberger See lake.

communities of oligotrophic Walchensee lake and of the neighboring mesotrophic Starnberger See lake were analyzed during four seasons (Fig. 3).

Among the 16S rRNA gene fragments and 16S rRNA-cDNA of *Sphingomonadaceae* separated by denaturing gradient gel electrophoresis, two different fingerprints showed high signal strengths and were found to be present throughout all four seasons in Walchensee lake (Fig. 3A). The excised bands yielded sequences that were identical to phylotypes G1A and G7A. This corroborates the conclusion that both phylotypes are abundant in Walchensee and also suggests that the corresponding *Sphingomonadaceae* are constantly present. Most notably, the corresponding fragments of G1A and G7A were detected concomitantly in the cDNA, indicating that both phylotypes constitute physiologically active members of the bacterioplankton community throughout the year. Interestingly, both phylotypes also were repeatedly detected and found to be active in the bacterioplankton community of mesotrophic Starnberger See lake, albeit not during all seasons (Fig. 3A). In Starnberger See lake, phylotype G1A was barely detectable during summer, whereas G7A was absent in winter. Out of the 10 melting types analyzed by sequencing, five (G1A, G1B, G5B, G7A, and G7C) were found to correspond to phylotypes detected in the 16S rRNA gene clone library of Walchensee lake (Fig. 3A). In addition, five additional melting types of *Sphingomonadaceae* were recovered by the DGGE approach (see Table S1 in the supplemental material). These melting types were absent from Walchensee samples (melting type *a* in Fig. 3A), showed a low overall abundance (melting types *f*, *i*, and *k*), or were not present during all seasons (melting type *e*), which provides an explanation for the absence of the corresponding sequences from the clone library generated from this lake. The DGGE clearly separated DNA fragments of phylotypes G7A and G7C that differed by only one base pair (Fig. 2A). This provides additional evidence for the sequence difference between both phylotypes (depicted in Fig. 2A) and emphasizes that the DGGE technique is suitable for the separation of fingerprints originating from very closely related phylotypes (38).

Based on a cluster analysis of the DGGE band patterns, DNA and cDNA fingerprints generated for the same water sample were most similar in the majority of cases (Fig. 4B). In fact, DNA and cDNA fingerprint patterns generated for the winter bacterioplankton were virtually identical in Walchensee and Starnberger See lakes, respectively. These results suggest that many of the novel phylotypes of aquatic *Sphingomonadaceae* that were detected in the present study do not represent dormant or dead constituents but rather ribosome-containing and hence physiologically active constituents of the bacterioplankton communities.

Cultivation of the dominant *Sphingomonadaceae* phylotypes. For the targeted isolation of the dominant *Sphingomonadaceae* phylotypes G1A and G7A, a high-throughput cultivation approach in diluted artificial freshwater medium was combined with the PCR-based screening of the generated cultures. To recover isolates of potentially greater phenotypic diversity, cultures were inoculated with samples from both lakes that were obtained in summer as well as winter.

In total, 1,403 primary liquid cultures were obtained and screened for the presence of *Sphingomonadaceae*. The overall cultivation efficiency determined for bacterioplankton in winter 2007 was 0.40% ± 0.05% and 0.56% ± 0.07% for Walchensee and Starnberger See lakes, respectively. In samples obtained from Starnberger See lake in the following summer of 2008, culturability was 0.36% ± 0.05% and thus remained in the same range, whereas the corresponding values for Walchensee lake increased to 2.80% ± 0.035% (Fig. 4). These culturability values are comparable to results of a previous

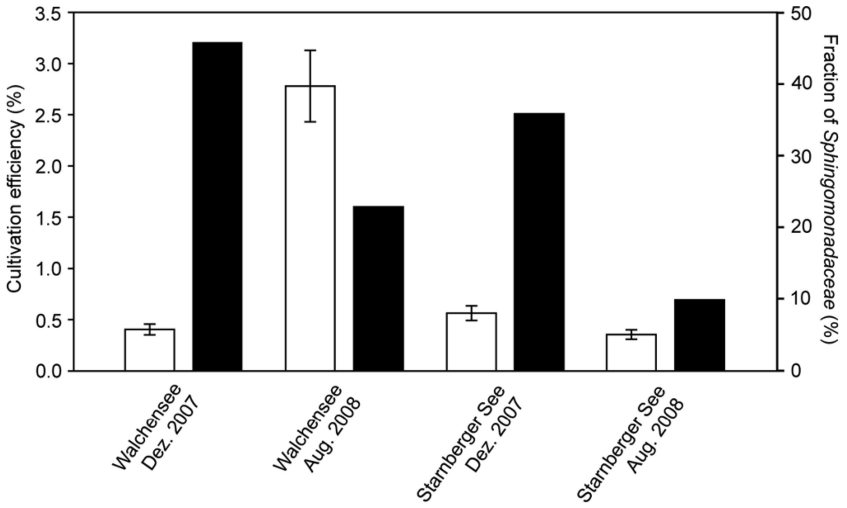

FIG. 4. Cultivation success of planktonic bacteria (white columns, left ordinate) and percentage of *Sphingomonadaceae* cultures among the primary enrichments (black columns, right ordinate) derived from bacterioplankton communities in Walchensee and Starnberger See lakes. Cultivation efficiency is given as the percentage of total bacterial cell counts. Error bars indicate 95% confidence intervals.

study in which the same medium and a comparable inoculation technique were used (9).

By employing the specific PCR screen developed for *Sphingomonadaceae*, between 10 and 46% of the positive primary liquid cultures were identified as *Sphingomonadaceae* (Fig. 4). 16S rRNA gene amplicons were generated from the 117 cultures of *Sphingomonadaceae* obtained from the winter samples, sequenced, and subsequently phylogenetically analyzed. This revealed the presence of 19 different phylotypes (5 phylotypes present in Walchensee are shown in Fig. 2B; six additional rare phylotypes that occurred in neither the clone library nor among pure cultures are listed in Table S2 in the supplemental material). Most notably, 84 (71%) enrichments harbored phylotype G1A, whereas only one culture of phylotype G7A was detected. During subsequent isolation attempts, it was observed that bacteria of the G1A phylotype grew only slowly on solid media, and only after agar concentrations had been decreased to 0.8%. Still, the improved agar media did not permit the isolation of pure cultures from all positive primary enrichments. Our attempts led to 54 pure cultures that were affiliated with the G1A phylotype and an additional single culture of G7A. Although the G1A phylotype was similarly present and active in summer samples (Fig. 3A), this phylotype was almost absent from the corresponding primary enrichments and accordingly could not be obtained from pure culture. In contrast, five additional pure cultures belonging to the G7A phylotype could be recovered from the summer samples (Fig. 2B). The difference in cultivation success observed for equally active *Sphingomonadaceae* in winter and summer samples may be the result of a different physiological status of the bacterial cells or of the presence of physiologically different bacterial lineages in the two different seasons. To elucidate the presence of different subpopulations, the diversity of internal transcribed spacer regions was analyzed in the natural populations and among the isolated strains.

Identification of ITS1 subpopulations within the G1A phylotype and ecological evidence for niche partitioning. The considerable number of cultures of the same 16S rRNA phylotype that could be recovered in the present study was used to analyze the population substructure of the G1A phylotype. The ITS1 segment of the *rrn* operon was chosen for this analysis, since its sequence variability significantly surpasses that of the 16S rRNA genes in most bacteria (5, 36). An initial comparison of ITS1 sequences of all 54 isolates and of the 20 environmental G1A clones (Fig. 2B) revealed the presence of 7 distinct sequence types among the cultured and cloned representatives of G1A (see Table S3 in the supplemental material). Among the cultures and clones, five and four different ITS1 types were detected, respectively. This diversity already surpasses that detected by intergenic spacer analysis among isolates of the marine *Alphaproteobacterium* "*Candidatus* Pelagibacter ubique" that demonstrated the existence of three distinct ITS lineages (48). In fact, two of our observations, namely, that congruence between the cultured and cloned G1A representatives was limited to two sequence types and that many of the sequence types were detected at low frequency, is indicative of a larger diversity in the natural G1A population.

To cover the ITS1 diversity in Walchensee and Starnberger See lakes more adequately, we used an Illumina sequencing approach and employed primers that amplified the most variable central portion of the ITS1 segment selectively for G1A. DNA and cDNA samples obtained in the four seasons were analyzed separately by multiplexing to follow the seasonal dynamics of individual ITS types and their activity in both lakes.

Due to the rigid quality control used (see Materials and Methods), the remaining data set comprised a total of 8,576 ITS1 sequences. This high-throughput sequencing uncovered the presence of significantly greater ITS1 diversity than observed that among the cultures and the clone library (see Table S3 in the supplemental material). The subsequent analysis of temporal shifts was limited to the 15 ITS1 sequence types that surpassed a value of relative abundance of 5% on at least one of the four sampling dates. This revealed strong seasonal shifts in the abundance of the ITS1 types, which followed an unexpectedly large number of temporal abundance patterns (Fig.

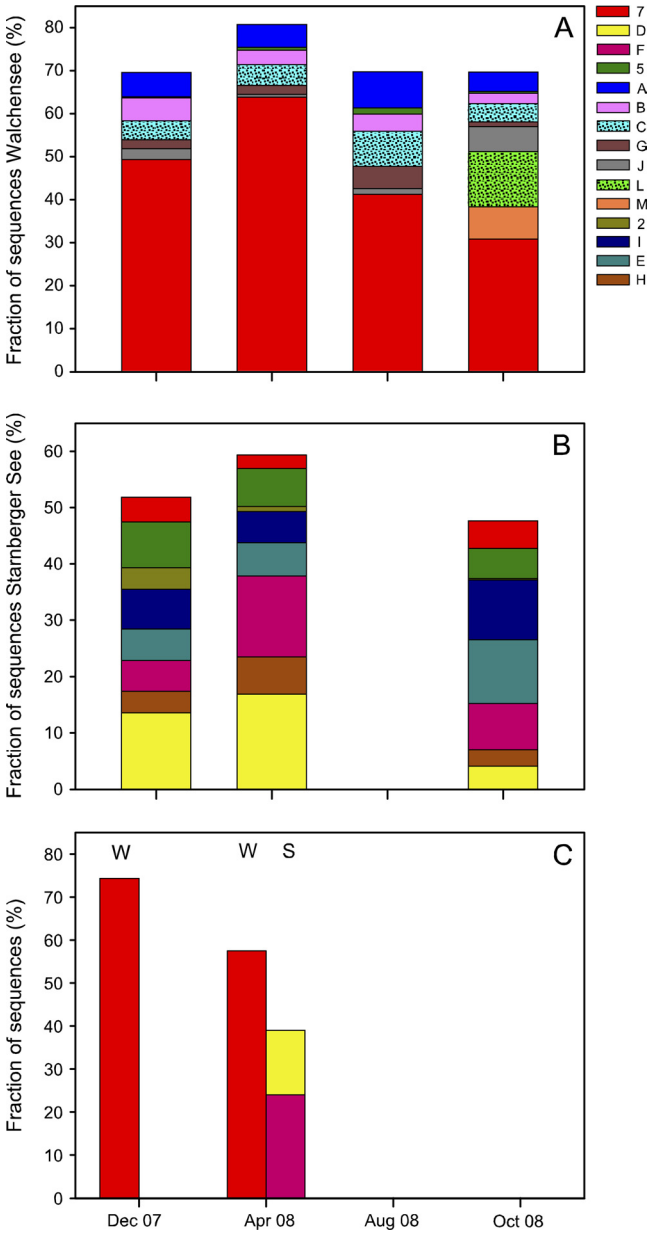

FIG. 5. Annual fluctuations of G1A ITS types in Walchensee (A) and Starnberger See (B) lakes. (C) Active fraction of G1A ITS1 types detected in cDNA samples. The remaining ITS types were present at a relative abundance of <5% and are not shown.

5). Notably, the G1A population in mesotrophic Starnberger See lake comprised eight ITS types that fluctuated in their relative abundance (Fig. 5B), while the G1A population in Walchensee lake was strongly dominated by ITS1 type 7 (constituting 64% of the ITS1 sequences), which is consistent with the results of our cloning approach (see Table S3 in the supplemental material). Furthermore, some lake-specific ITS types were detected (for Walchensee, types L, M, and C; for Starnberger See, types H and 2). The much higher diversity of temporal patterns in Starnberger See lake may be caused by a larger number of ecological niches available to G1A *Sphingomonadaceae* in this different environment (Table 1) and/or a more dynamic competition between the different ITS1 types of G1A. Niche partitioning has been shown to occur on different levels of diversity in marine *Prochlorococcus*, where 16S rRNA phylotypes that differ by 3% sequence divergence occupy either high- or low-light-intensity niches in the water column of tropical or subtropical oceans (49), and the more closely related (high-light-adapted) strains partition themselves according to ambient temperature (36).

Members of G1A were detected in an active state (i.e., in the cDNA sample) only in the December and April samples from Walchensee lake and in the April sample from Starnberger See lake (Fig. 5C). As for the DNA sample, ITS1 type 7 dominated the cDNA from Walchensee; the failure to detect G1A among the active fraction in summer and autumn is consistent with the concomitant decline of this population during these month (Fig. 5A). Similarly, the ITS1 types D and F that dominated in Starnberger See lake in spring also constituted the dominant fraction in the cDNA sample at this time (Fig. 5C).

A high physiological diversity resides within individual ITS1 subpopulations of the G1A phylotype. In some bacteria, ITS sequences have too little resolution to define all ecotypes in a natural population (37). In a last approach, we therefore analyzed physiological differences within and between the different ITS1 types of G1A cultivated. Sixteen randomly chosen isolates belonging to the ITS1 type 4 and 13 representatives of the four other types (types 2, 3, 5, and 6) were selected for testing to obtain comparable sample sizes. The substrate utilization pattern, as revealed by the Biolog assay, clearly showed that the variance of substrate utilization within the 16 isolates with identical ITS sequences (type 4) was even higher than that among all other ITS types combined (Fig. 6). No distinct patterns of substrate utilization could be detected for the different ITS1 types, and no differences with respect to the origin of the isolates from the two lakes were observed. Twenty-four different substances, of which 80% could be identified as carbon sources and 20% as inhibitory substances (antibiotics and low-pH substances), exhibited the highest variance within type 4 (denoted as triangles in Fig. 6; also see Table S4 in the supplemental material). The four other ITS1 types showed a high variance only for six conditions (four carbon sources, two inhibitors; see Table S4 in the supplemental material). The substrate group that exhibited the highest variability in utilization was sugars plus sugar alcohols (42% of the substrates). In addition, very high variance values were detected for resistance against the antibiotics vancomycin and rifamycin, where rifamycin showed the highest variance value of all tested substrates and inhibitors (see Table S5 in the supplemental material).

Bacterial strains of ITS1 type 4 differed considerably with respect to many central metabolic properties. Similarly to the situation in *Bacillus simplex* (56), standard physiological parameters that typically are employed as one of the elements of species delineation do not provide information about possible mechanisms of niche separation that could underlie the differences in abundance patterns of *Sphingomonadaceae* observed in the natural environment. Instead, a mechanism of niche adaptation in *Sphingomonadaceae* might include, among others, the degradation of refractory high-molecular-weight organic compounds (4), resistance to UV radiation (24), a planktonic or sessile lifestyle (4, 7, 55), differences in adaptation to

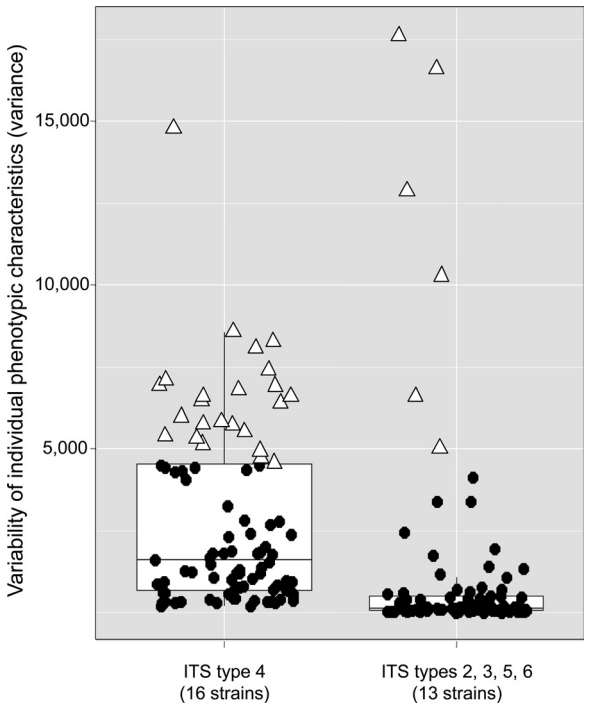

FIG. 6. Variances of individual phenotypic traits. The box indicates the first and third quartile, the horizontal line in the box the median value, and the whiskers extend to a maximum of 1.5-fold of the box size. The values above the threshold value of the third quartile of ITS type 3 strains are depicted as open triangles for all data. The highly variable substances coded by the open triangles are listed in Table S4 in the supplemental material.

different growth-limiting substrates (15, 47, 60), aerobic anoxygenic phototrophy (20, 28), or a different susceptibility to bacteriophage attack (64). Future research along these lines is needed to elucidate the mechanisms that maintain the large variety of different ITS1 subpopulations of *Sphingomonadaceae* detected in the present study.

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