

Diversity and Phylogenetic Affiliations of Morphologically Conspicuous Large Filamentous Bacteria Occurring in the Pelagic Zones of a Broad Spectrum of Freshwater Habitats

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Filamentous bacteria with a conspicuous morphology were found in the majority of the bacterioplankton samples from a variety of freshwater habitats that were studied. These heterotrophic filaments typically account for <1 to 11% of the total number of bacteria. The biovolume of this morphotype can exceed 40% of the biovolume for all bacteria. Surprisingly, we found hardly any data on these morphologically conspicuous filaments in the literature. Mixed cultures containing these filamentous bacteria were established by cultivation and isolation experiments with samples from different freshwater lakes. Nearly full-length 16S rRNA gene sequences were obtained from several mixed cultures and environmental samples from habitats in Europe, Africa, China, Australia, and New Zealand. Phylogenetic analysis of the sequences showed that three groups form a single monophyletic cluster, the SOL cluster, in the family *Saprospiraceae*. We developed a set of six nested probes for fluorescence in situ hybridization. Of the six probes, one probe was specific for *Haliscomenobacter hydrossis*, three probes were specific for the three subclusters (each probe was specific for one subcluster), one probe was specific for the entire SOL cluster, and another probe targeted almost the entire *Saprospiraceae* family. Specific hybridization of environmental samples and enrichments showed that the members of the three subclusters exhibited the same filamentous morphology. So far, using the subcluster-specific probes, we have not been able to detect any bacteria with a differing morphology. We conclude that the SOL cluster bacteria are an integral part of bacterioplankton in many freshwater habitats. They potentially account for a large fraction of the total bacterial biomass but have been underrepresented in molecular diversity studies so far.

In the last 9 years, several studies provided insights into the phylogenetic composition of freshwater bacterioplankton communities (5, 6, 7, 8, 11, 13, 21, 23, 30, 31, 35, 37). Most of these studies investigated a single habitat or a few habitats, and only a few studies investigated a larger number of ecosystems (8, 37). Despite the limited number of comprehensive studies, a rough idea surfaced as to which groups of bacteria are typically present in freshwater bacterioplankton (37).

This study focused on a group of planktonic bacteria defined by their morphological characteristics. The conspicuous filamentous morphotype was observed in samples from many freshwater habitats, but we did not find detailed reports on the presence of these bacteria in freshwater in the literature, and not much is known about the phylogenetic affiliation of these striking filamentous bacteria. *Haliscomenobacter hydrossis* (32), a member of the *Saprospiraceae* family (which is found exclusively in wastewater treatment plants [34]), is known to exhibit a similar morphology.

The rationale of this work was to reveal the phylogenetic affiliation and ecological significance of this distinct group of filamentous bacteria. Therefore, molecular tools and culture approaches for the identification and cultivation of these prominent members of the bacterioplankton were developed. Due to the similarity in morphology, the initial steps of the

investigation presented here were directed against *H. hydrossis*. A large number of freshwater habitats were systematically investigated for the presence of the target bacteria. The systems investigated represent a broad range of habitats; habitats differing in size, geographic location, limnological status, etc., were studied. Substantial numbers of the conspicuous filamentous bacteria were detected in the majority of habitats investigated.

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MATERIALS AND METHODS

Study sites and sampling. We investigated samples from the pelagic zones of freshwater habitats in Europe, East Africa, China, Australia, and New Zealand. Samples from freshwater lakes and ponds were taken from a depth of 1 to 2 m with a 10-liter Schindler sampler and kept in 5-liter plastic bottles for less than 2 h until processed in the laboratory. Subsamples for total bacterial counts were fixed with formaldehyde (final concentration, 2%). For fluorescence in situ hybridization (FISH), samples of water (10 to 30 ml) were fixed with paraformaldehyde (final concentration, 2%) for 2 h at room temperature, passed through 0.2- μ m-pore-size polycarbonate filters (45 mm long; Millipore), dried, and frozen until further processing. To collect microbial biomass, samples of water (between 150 and 500 ml) were passed through 0.2- μ m-pore-size polycarbonate filters (45 mm long; Millipore). The filters were frozen at -70°C until the extraction of DNA (biomass filters).

Bacterial abundance and biovolume. Formaldehyde-fixed samples were stained with 4',6'-diamidino-2-phenylindole (DAPI) (final concentration, 6 $\mu\text{g ml}^{-1}$) and enumerated by epifluorescence microscopy (Zeiss Axioplan; Zeiss, Jena, Germany). Between 300 and 700 DAPI-stained bacterial cells were counted at a magnification of $\times 1,250$. High-resolution images (752 by 548 pixels; 8-bit dynamic range) were saved with an analogue monochrome charge-coupled

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TABLE 1. Primers used in this study

Primer	Sequence (5'→3')	Length (nt) ^a	GC content (%)	T _m (°C) ^b	Specificity	Reference
1406F	TGY ACA CAC CGC CCG T	16	65.6	55.6	Bacteria	19
L189R	TAC TGA GAT CYT TMA RTT C	19	34.2	49.1	Bacteria	36
27F	AGA GTT TGA TCM TGG CTC AG	20	47.5	56.3	Bacteria	20
530FC	TCC GTG CCA GCA GCC GCG G	19	78.9	67.5	Bacteria	20 ^c
Hal1R	CTC TAA AAG GAG GTA TTC	18	38.9	49.1	<i>Saprospiraceae</i>	This study
Hal3R	AGC TAT GAG ACT TTG ACT	18	38.9	49.1	<i>Saprospiraceae</i>	This study
26F	AGG ATG AAC GCT AGC GGG	18	61.1	58.2	Bacteria	This study
HAL-1473R	CTT AGC CCC AGT TAC TGG TTT T	22	45.5	58.4	HAL subcluster	This study
LD2-1473R	CTT AGC CCC AGT CAC TAG TTT T	22	45.5	58.4	LD2 subcluster	This study
GKS-1473R	CTT AGC CCC AGT CAT TGG TTT T	22	45.5	58.4	GKS2-217 subcluster	This study
SAP-1490R	CGG CTA CCT TGT TAC GAC TTA G	22	50	60.3	Bacteria	20 ^c

^a nt, nucleotides.

^b T_m, melting temperature [T_m = 69.3 + 41 × [(nG + nC)/s] - (650/s), where nG is the number of G's in the sequence, nC is the number of C's in the sequence, and s is the length of the sequence].

^c Primers were modified to amplify preferentially target bacterial sequences.

device camera (Hitachi Denshi, Ltd., Tokyo, Japan) and processed with the image analysis system LUCIA G (Lucia 4.51; Laboratory Imaging, Ltd., Prague, Czech Republic). To determine bacterial cell volumes, the images of 50 filaments and 100 other bacteria per sample were analyzed with LUCIA G. Bacterial cell volumes were calculated by using the formula of Andersson et al. (3). Because of a lack of suitable conversion factors, we did not calculate bacterial biomass.

Cultivation and isolation experiments. In order to isolate and cultivate bacteria of the SOL cluster, a variety of cultivation and isolation experiments were performed. Filaments of the SOL morphotype were generally cultivated and isolated in sterile glassware at 15°C with continuous illumination without agitation. For base media, a complex organic NSY medium (3 g of organic substrate liter⁻¹) (for details, see reference 9), an *H. hydrossis*-specific medium (3.3 g of organic substrate liter⁻¹; DSM 134), and an inorganic IBM medium (10) enriched with different amounts of complex organic substrates (NSY medium) and sterile lake water (15) were tested. Briefly, sterile lake water was prepared by filtering the water through presterilized 0.22-μm-pore-size filtration towers (Steritop; Millipore) directly into sterile 1-liter Schott bottles. These bottles were heated in a microwave to a temperature slightly under the boiling point (heating 1 liter of lake water for 7 min 30 s at 1,000 W) to avoid precipitation of inorganic substances. Microwave treatment was performed twice with subsequent cooling phases to suppress bacterial growth effectively. Media were supplemented with vitamin B₁₂ and thiamine (final concentration, 10 μg liter⁻¹). Growth of the target bacteria was also tested with the following organic substrates added at a concentration of 100 mg liter⁻¹ to an inorganic IBM medium: sodium acetate, deoxycholic acid, pyruvic acid, *n*-caproic acid, caprylic acid, capric acid, D-glucose, D-galactose, potassium citrate, glycolic acid, glutamic acid, and Tween 80. The resistance of *H. hydrossis* strain DSM1100 antibiotics was tested in DSM 134 medium. Antibiotics were added at concentrations of 50 mg liter⁻¹ (for polymyxin thiosulfate, gentamicin sulfate, and neomycin) and 100 mg liter⁻¹ (for nalidixic acid, streptomycin sulfate, and ampicillin). Mixed cultures obtained from environmental samples by the methods mentioned above were diluted or subcultured in new flasks every 4 to 8 weeks. Substrates were added at low concentrations (100 to 200 mg liter⁻¹) in weekly to biweekly intervals. Bacterial growth was monitored by counting samples stained with DAPI with an epifluorescence microscope (Zeiss Axioplan).

DNA extraction. Nucleic acids were extracted either by a standard protocol (including incubation with lysozyme and proteinase K and subsequent extraction with phenol-chloroform-isoamyl alcohol [25:24:1, pH 8] and chloroform-isoamyl alcohol [24:1]) or alternatively with a much faster beadbeater extraction routine (FastDNA kit and FastPrep instrument FP120; QBioGene) following the manufacturer's recommendations. Qualitative comparison of extracts by PCR and denaturing gradient gel electrophoresis (DGGE) did not yield significant differences.

Primer design and evaluation. Reverse primers HAL1R and HAL3R targeting the 16S-23S internal transcribed spacer (ITS) were designed after sequencing the internal transcribed spacers (ITSs) of *H. hydrossis*, *Saprospira grandis*, *Flexibacter elegans*, *Flexibacter sancti*, *Cytophaga hutchinsonii*, *Chitinophaga pinensis*, and *Flectobacillus major* with primers 1406F (19) and L189R (36). The newly developed, more-specific primers 26F, HAL-1473R, LD2-1473R, GKS-1473R, and SAP-1490R (primer sequences and specificities shown in Table 1), were designed on the basis of the first set of SOL cluster bacterial 16S rRNA gene

sequences. Optimization of PCRs for newly designed primers was performed in a Mastercycler gradient (Eppendorf).

PCR and sequencing. Bacterial 16S rRNA genes were amplified in a Primus 96^{plus} thermal cycler (MWG Biotech, Ebersberg, Germany). The reaction mixtures (50 μl) contained 100 μM concentrations of each of the deoxynucleotide triphosphates, 0.3 μM concentrations of each of the primers, 3 mM MgCl₂, 1× PCR buffer, and 1.25 U of *Taq* DNA polymerase (QIAGEN). The universal bacterial primer 27F (20) and the two reverse primers (HAL1R and HAL3R) were used for the amplification of nearly full-length 16S rRNA gene sequences. The PCR program follows: (i) an initial denaturation step (2 min at 94°C); (ii) 30 cycles, with 1 cycle consisting of denaturation (1 min at 94°C), annealing (1 min at 56°C), and extension (2 min at 72°C); and (iii) a final extension step (5 min at 72°C). Specific amplification of nearly full-length 16S rRNA gene sequences of subclusters HAL, GKS2-217, and LD2 in the *Saprospiraceae* family was performed with primers 26F and HAL-1473R, GKS-1473R, and LD2-1473R, respectively. The PCR program follows: (i) an initial denaturation (2 min at 94°C); (ii) 30 cycles, with 1 cycle consisting of denaturation (1 min at 94°C), annealing (1 min at 62°C), and extension (2 min at 72°C); and (iii) a final extension step (5 min at 72°C). For a template, 1-μl samples of the total microbial DNA extracts or small pieces (~5 mm²) of biomass filters were used. Filter PCR was performed by the protocol of Kirchman et al. (17). Comparison of bacterial DNA amplified from PCRs with extracted template DNA and reactions with small pieces of 0.2-μm-pore-size polycarbonate filters from the same sample by DGGE (according to the protocol described in reference 24) did not yield any qualitative differences in the microbial fingerprints obtained. PCR products were sequenced by MWG Biotech. Primer 530FC (modified from the primer in reference 20) was used to sequence the middle section of the 16S rRNA gene. For details on the primers used in this survey, refer to Table 1. Two sequences (MS-Falk1-L and MS-oKlaff1-L) were retrieved from two clone libraries. For the construction of clone libraries, nearly full-length bacterial 16S rRNA gene sequences of a subset of the bacterial community were amplified by PCR using primers 26F and SAP-1490R (modified from the primers in reference 20). Cloning was performed with the QIAGEN PCR Cloning^{plus} kit following the manufacturer's recommendations. Plasmid DNA was extracted using the Nucleospin plasmid kit (Macherey-Nagel, Düren, Germany).

Phylogenetic analysis. The BLAST program (2) (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to search for related sequences with high similarity values. A comparative analysis of all newly obtained sequences and relatives found in the databases was done with the ARB software package (<http://www.arb-home.de>). Sequences imported into the ARB database were aligned with the Fast Aligner tool of ARB and checked manually for misaligned positions. Phylogenetic trees were constructed for stretches of 1,357 homologous nucleotides. Evolutionary distances were corrected using the algorithm of Jukes and Cantor (14). Partial sequences shorter than 1,357 nucleotides were added to the existing neighbor-joining tree by the ARB parsimony tool without changing the tree topology. Bootstrap values were calculated using the Mega V2.1 program (18) (<http://www.megasoftware.net>). The tree topologies of neighbor-joining trees were validated using maximum-parsimony and maximum-likelihood algorithms as alternative tree construction methods. Sequence similarities were calculated as uncorrected base identities between sequences on the basis of sequence alignments.

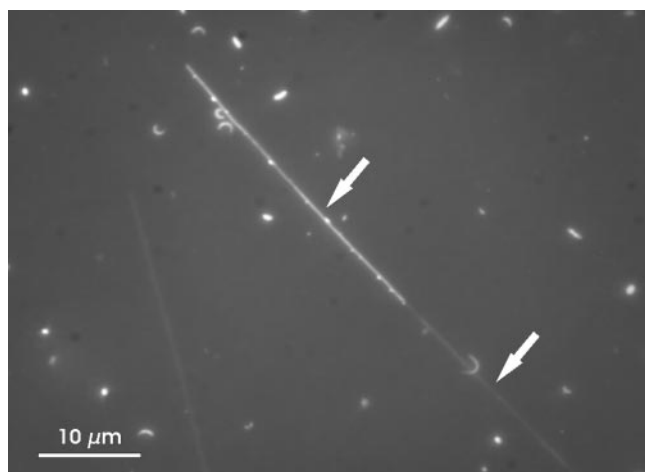


FIG. 1. Microscopic image of a DAPI-stained bacterioplankton sample from Lake Victoria (31 May 2001) presenting a filament of the SOL morphotype. These filaments always possess blunt ends, and flagella were never observed. These characteristics together with the unbowed rigid habit of the filaments allows for a reliable discrimination of members of the SOL morphotype against other filamentous bacteria by standard microscopic techniques. The upper arrow marks the filament with yellowish granule. The lower arrow marks a sheath covering the filament. A similar sheath is also known to cover the wild-type cells of *H. hydrossis*. Magnification, $\times 1,250$.

Probe design and FISH. A nested set of six oligonucleotide probes with different specificities for our target bacteria was designed using the ARB software package. The stringency of the hybridization conditions for the probes was adjusted by a series of solutions of formamide (0 to 60% formamide concentration). The conditions were adjusted with filters from several habitats for all newly designed probes. Signal intensities were evaluated by eye. Probes labeled with the indocarbocyanine dye Cy3 were purchased from Interactiva (Ulm, Germany). FISH was performed on polycarbonate filter sections by the protocol of Alfreider et al. (1). Counting bacteria on FISH filter sections was performed by epifluorescence microscopy (Zeiss Axioplan) at a magnification of $\times 1,250$.

Nucleotide sequence accession numbers. The nearly full-length 16S rRNA gene sequences obtained were deposited in the EMBL nucleotide sequence database under the accession numbers AJ784892 and AJ786318 to AJ7863342.

RESULTS

Water samples from the pelagic zones of 102 different freshwater habitats located on three continents and in three different climatic zones were investigated. In 78 of the 102 habitats (76%), bacteria with a conspicuous morphology were found. These filamentous bacteria exhibit a very distinct morphology and are easily distinguished from the rest of the heterotrophic bacterioplankton. The large cells resemble thin, unbowed sticklike filaments. While the cell diameter is quite stable (between 0.25 and 0.35 μm), the length of the filaments is highly variable (8 to $>100 \mu\text{m}$). A sheath covering the filaments was sometimes visible in DAPI-stained preparations (Fig. 1). In some of the samples, these filaments featured small yellowish particles, which seemed to stick to the surfaces of the cells. Altogether, the described morphotype reminded us remarkably of an Austrian brand of pretzels, so we called this distinct morphology the SOL morphotype.

Bacteria with the conspicuous SOL morphotype were found in plankton samples from a broad range of habitats. Freshwater lakes and rivers inhabited by bacteria of the SOL morphotype were found in the following varied locations: large tropical

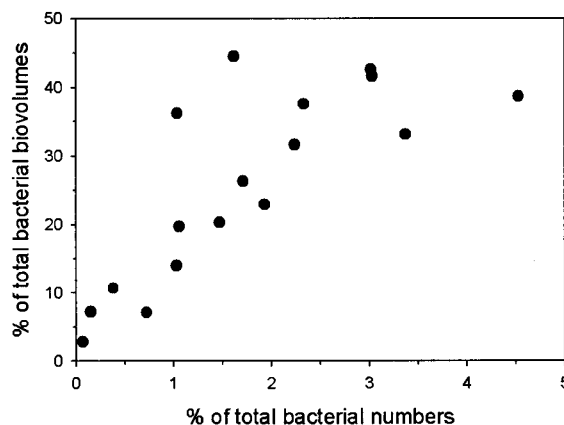


FIG. 2. Contribution of SOL morphotype bacteria to the number and biovolume of all bacteria in 17 samples from 16 different freshwater habitats. Selected samples represent a broad range of ecologically different habitat types.

lakes (e.g., Lake Victoria and Lake Tanganyika in East Africa), subtropical shallow eutrophic lakes and rivers (e.g., Lake Taihu, Changjiang river [Yangtze] in the People's Republic of China), large shallow lowland lakes (e.g., Lake Loosdrecht, The Netherlands), large oligotrophic prealpine lakes (e.g., Lake Attersee, Austria), and small deep ultraoligotrophic high mountain lakes (e.g., Lake Oberer Klaffersee and Lake Kapuzinersee in Austria). SOL morphotype bacteria were not detected in acidic habitats (pH 4.4 to 5.9; seven habitats) or in habitats with short water retention times (4 to 10 days; three habitats) or in nonpermanent freshwater bodies (small, shallow water bodies; two habitats). The other freshwater ecosystems (12 habitats) where it was not possible to detect these filamentous bacteria did not fit in any of those generalized types of habitats. Bacteria of the SOL morphotype were not detected in five acidified Bohemian forest lakes studied, which are known to be frequently dominated by filamentous bacteria (33). The absolute numbers of target bacteria ranged from several hundreds to 200,000 cells ml^{-1} . The relative contribution of the SOL morphotype to the total number of bacteria ranged from 0.1 to 10.8%, with a typical value of around 1% of the total bacterioplankton. Biovolume estimations for the SOL morphotype in several habitats ranged from 2.8 to 44.5% of the total bacterial biovolume (Fig. 2).

Cultivation and isolation experiments. In order to cultivate and isolate bacteria of the SOL morphotype, we attempted several different strategies. (i) Dilution cultures were established in the wells of cell culture plates and in sterile glass flasks. Sterile lake water, NSY medium (3 g of organic substrate liter $^{-1}$), and *Haliscomenobacter* medium (DSM 134; 3.3 g of organic substrate liter $^{-1}$) were used as the base media. The plates and flasks were incubated for several days to weeks and screened by epifluorescence microscopy. No pure isolates could be obtained from these dilution experiments. (ii) Live filaments were sorted with a MoFlo cell sorter (Cytomation, Fort Collins, Colo.) with the assistance of Stefan Andreatta. For details on the cytometer settings, see reference 4. SOL morphotype filaments were sorted with a low-salt sheath fluid in cell culture plates containing sterile filtered lake water or inorganic IBM medium enriched with small amounts of or-

TABLE 2. Environmental samples used for cultivation and enrichment experiments and details on the sequences retrieved

Geographic origin of sample	Total no. of bacteria (10 ⁶ ml ⁻¹) ^a	No. of SOL morphotype bacteria (10 ⁴ ml ⁻¹) ^a	Sequence	Affiliation (subcluster)	Accession no.	Length (nt) ^b
Lake Egelsee	1.59	0.19	MS-Egel1-L	LD2	AJ786318	940
Lake Egelsee	1.59	0.19	MS-Egel1-H	HAL	AJ786324	765
Jaegerteich pond	5.94	7.13	MS-Jaeger1-L	LD2	AJ786319	1,135
Lake Wolfgangsee	1.30	1.11	MS-Wolf1-H	HAL	AJ786322	1,414
Lake Wolfgangsee	ND ^c	2.00	MS-Wolf2-H	HAL	AJ786323	1,478

^a On the sampling day.

^b nt, nucleotides.

^c ND, not determined.

ganic substrates (25 or 50 μ l of NSY medium to 2 ml of IBM medium). One, 10, 100, and 1,000 cells were sorted into the different media in replicate wells. No growth of filaments was observed for any of the different treatments after incubation of the plates for 2 weeks at 15°C. (iii) In another experiment, a series of environmental samples and enrichment cultures were plated on agar plates containing NSY medium (3 g liter⁻¹), and *Haliscomenobacter* medium (DSM 134; 3.3 g liter⁻¹). After 1 week of incubation at 15°C, colonies were screened by epifluorescence microscopy. No colony formation of filamentous bacteria was detected.

While the attempt to establish pure cultures failed, it was possible to enrich and cultivate (long-term) the mixed cultures. The enrichment of SOL morphotype bacteria was accomplished partly by reducing the diversity and total number of bacteria in samples and partly by increasing the number of target bacteria through modified growing conditions. The most successful treatments were the addition of glutamic acid (concentration of 100 mg liter⁻¹) as an organic substrate to filtered (0.2- μ m-pore-size filters) and microwave-treated lake water and inhibition of other bacteria by treatment with nalidixic acid (final concentration, 100 mg liter⁻¹). Cultivation of bacteria of the SOL morphotype normally resulted in a substantial increase of the target bacteria (up to 42% of the total number of bacteria). With this type of treatment, we were able to obtain several mixed cultures of members of the HAL and LD2 subclusters (details on phylogenetic analysis given below) of the SOL cluster bacteria. Mixed cultures were subcultured every 4 to 8 weeks. Cultures were maintained for several weeks to more than 1 year. For more details on the samples used for the enrichment experiments, see Table 2.

Primer design and specific direct sequencing. The first specific primers developed, primers HAL1R and HAL3R (Table 1), were derived from 16S-23S ITS sequences of *H. hydrossis* and several related bacterial strains available in culture collections. The primer binding positions are at the very beginning of the ITS region and the first tRNA gene located in the ITS. These primers were designed with a broader phylogenetic specificity to recover sequences of *H. hydrossis* and close relatives in the family *Saprospiraceae*. These reverse primers were combined with the universal bacterial primer, primer 27F. First, nearly full-length sequences of the target bacteria allowed the development of more-specific primers 26F, SAP-1490R, HAL-1473R, LD2-1473R, and GKS-1473R. Primer 26F was designed to match all of the available sequences from members of the *Saprospiraceae* family but is not strictly specific for this group. Primer SAP-1490R was derived from universal

primer 1490R (20) to preferentially fit known *Saprospiraceae* sequences but also matches a broad spectrum of other bacterial 16S rRNA gene sequences. The combination of the 26F primer with subcluster-specific primers HAL-, LD2-, and GKS-1473R allowed a subcluster-specific amplification (details on phylogenetic analysis given below) of 16S rRNA gene sequences from a variety of environmental samples (Table 3) and mixed cultures. The sequences consisted of 765 to 1,478 nucleotides and contained 0 to 7 ambiguous positions (average, 1.17). Resequencing *H. hydrossis* strain DSM1100 with primers 27F and HAL1R yielded a slightly longer 16S rRNA gene sequence (1,509 nucleotides) with fewer ambiguous positions than a previously published sequence of the same strain (accession no. M58790).

Comparative analysis of 16S rRNA gene sequences. The alignment and phylogenetic analysis of the 25 16S rRNA gene sequences obtained and previously published sequences yielded three phylogenetic groups. Only the first group contains a described species, namely, *H. hydrossis*; therefore, it was named the HAL subcluster. The second group of sequences joined the LD2 cluster introduced by Zwart et al. (37). The rest of the retrieved sequences formed a phylogenetic group with a sequence obtained from Lake Gossenköllesee. We named this new group GKS2-217 according to the clone name introduced by Glöckner et al. (8). These three groups form a monophyletic cluster (the SOL cluster) affiliated with the family *Saprospiraceae* (*Bacteroidetes* phylum). Aside from the sequence of the only described species (*H. hydrossis*), all other previously published sequences that were found to be affiliated with the SOL cluster were retrieved only by culture-independent methods from freshwater habitats. Some shorter sequences that were also affiliated with the SOL cluster (Table 4) were omitted in the calculation of the phylogenetic tree (presented in Fig. 3), due to their limited length.

Trees constructed by alternative methods (maximum-parsimony and maximum-likelihood algorithms) validated the topology of the neighbor-joining tree shown in Fig. 3. The high bootstrap values for the SOL cluster and the HAL, LD2, and GKS2-217 subcluster levels support the topology shown in Fig. 3. The short sequence of the clone LiUU-11-221 was added to the tree by way of the ARB parsimony method. The exact position of this sequence inside the SOL cluster could not be determined. To validate the position of this clone in the SOL cluster, we truncated the sequences of our data set in order to include some shorter sequences in the tree-building process. This additional tree building (data not shown) supported the position of this clone in the SOL cluster with high bootstrap

TABLE 3. Details on habitats from which sequences were retrieved by direct sequencing without enriching SOL cluster bacteria

Sampling site	Trophic status	Country	Climatic zone	Total no. of bacteria (10^6 ml^{-1})	No. of SOL morphotype bacteria (10^4 ml^{-1})	SOL morphotype bacteria (% of total)	% of bacterial biovolume	Sequence	Affiliation (subcluster)	Accession no.
Lake Irrsee	Oligomesotrophic	Austria	Temperate	1.05	1.09	1.04	36.2	MS-Irr1-H	HAL	AJ786320
Lake Irrsee	Oligomesotrophic	Austria	Temperate	1.05	1.09	1.04	36.2	MS-Irr2-H	HAL	AJ786329
Lake Mondsee	Oligomesotrophic	Austria	Temperate	2.66	0.39	0.15	7.2	MS-Mond1-H	HAL	AJ786321
Lake Ahomsee	Oligotrophic	Austria	Temperate	0.62	1.39	2.24	31.6	MS-Ahom1-G	GKS2-217	AJ786325
Lake Kapuzinersee	Oligotrophic	Austria	Temperate	0.3	0.91	3.03	41.6	MS-Kapu1-G	GKS2-217	AJ786326
Lake Oberer Klaffersee	Oligotrophic	Austria	Temperate	0.55	1.66	3.02	42.6	MS-oKlaff1-G	GKS2-217	AJ786327
Lake unterer Sonntagskarsee	Oligotrophic	Austria	Temperate	0.18	0.59	3.28	33.1	MS-uSonn1-G	GKS2-217	AJ786328
Lake Wolfgangsee	Oligotrophic	Austria	Temperate	1.12	2.61	2.33	37.6	MS-Wolf1-L	LD2	AJ786330
Lake Toplitzsee	Oligotrophic	Austria	Temperate	1.39	1.43	1.03	14.0	MS-Topli1-L	LD2	AJ786332
Lake Schwarzensee	Oligotrophic	Austria	Temperate	1.05	0.4	0.38	10.7	MS-Schwa1-L	LD2	AJ786334
Lake Mondsee	Oligomesotrophic	Austria	Temperate	2.19	1.57	0.72	7.1	MS-Mond1-L	LD2	AJ786337
Lake Irrsee	Oligomesotrophic	Austria	Temperate	1.87	1.98	1.06	19.7	MS-Irr1-L	LD2	AJ786338
Lake Attersee	Oligotrophic	Austria	Temperate	1.12	5.07	4.53	38.7	MS-Atter1-L	LD2	AJ786340
Lake Falkertsee	Oligotrophic	Austria	Temperate	0.96	1.85	1.93	22.9	MS-Falk1-L	LD2	AJ786341
Lake Krottensee	Oligotrophic	Austria	Temperate	1.13	1.66	1.47	20.3	MS-Krott1-L	LD2	AJ786342
Lake Loosdrecht	Eutrophic	The Netherlands	Temperate	3.13	5.07	1.62	44.5	MS-Loos1-L	LD2	AJ786339
Pond in Dunedin	ND ^a	New Zealand	Temperate	ND	ND	ND	ND	MS-NZ1-L	LD2	AJ786335
Pond in Sydney	ND	Australia	Temperate	ND	ND	ND	ND	MS-Aus1-L	LD2	AJ786336
Lake Taihu	Hypertrophic	China ^b	Subtropical	ND	ND	ND	ND	MS-Taihu1-L	LD2	AJ786333
Lake Tanganjika	Oligotrophic	Zambia	Tropical	1.75	0.12	0.07	2.8	MS-Tang1-L	LD2	AJ786331

^a ND, not determined.^b People's Republic of China.

values for the SOL cluster in any case. The minimal sequence similarity values inside the subclusters are 98.7% for the HAL subcluster, 97.6% for the LD2 subcluster, and 99.7% for the GKS2-217 subcluster. The maximum similarities between subclusters are 92.7% for the HAL and LD2 subclusters, 95.5% for the HAL and GKS2-217 subclusters, and 92.1% for the LD2 and GKS2-217 subclusters. The overall minimum similarity value inside the SOL cluster is 88% (Table 5).

FISH. We used nested FISH probes to detect phylogenetic groups of various levels in lake water samples (Table 6). The new probe HHY-441 is specific for the *H. hydrossis* species. All other sequences inside the broader HAL subcluster contain one to two mismatches at the probe binding position. We used this probe in order to determine whether we could find filaments of *H. hydrossis* in lake water samples. Hybridization of samples from natural freshwater habitats did not yield any

positive signals with this very specific probe. This indicates the absence of *H. hydrossis* as found in wastewater treatment plants (34) in all of the natural freshwater ecosystems tested. Nevertheless, we were able to detect bacteria of the HAL subcluster in a broad range of habitats with the subcluster-specific probe HAL-844.

Probe CF-319a (22), which was designed for the broad *Cytophaga-Flavobacteria-Bacteroides* group shows one mismatch with all available SOL cluster 16S rRNA sequences. This results in weak to undetectable signals of SOL cluster filaments under stringent hybridization conditions. We designed a probe for the family *Saprospiraceae* (probe SAP-309), which targets all of the members of the SOL cluster as well as more than 80% of all other available sequences from members of the *Saprospiraceae* family. This probe detected all filaments of the SOL morphotype in each of our samples. The SOL-852 probe

TABLE 4. Sequences affiliated with the SOL cluster but not presented in the tree shown in Fig. 3 due to insufficient sequence length^a

Sequence name	Accession no.	Length (nt) ^b	Affiliation (subcluster)	Sample type	Reference
Clone BCM3S-7B	AY102912	719	HAL	Subtropical freshwater marsh	Unpublished
Clone csbio160175	AY187353	942	HAL	Drinking-water biofilm	28
Clone Urk0-01	AJ416169	709	LD2	The Netherlands, Lake IJssel	37
Clone Med0-02	AJ416157	699	LD2	The Netherlands, Lake IJssel	37
Clone DC5-0-5	AY145578	506	LD2	Germany, Weser estuary	Unpublished
Clone CR98-5-53	AF428783	600	LD2	China, Changjiang River (Yangtze)	29
Clone CR98-35-61	AF428943	600	LD2	China, Changjiang River (Yangtze)	29
Clone CR98-5-24	AF428754	600	LD2	China, Changjiang River (Yangtze)	29
Clone CRP99-04	AF428658	600	LD2	China, Lake Poyang	29
Clone LiUU-5-268	AY509298	763	LD2	Sweden, freshwater bacterioplankton	Unpublished
Clone LiUU-9-278	AY509321	800	LD2	Sweden, freshwater bacterioplankton	Unpublished
Clone LiUU-9-266	AY509369	798	LD2	Sweden, freshwater bacterioplankton	Unpublished
Clone LiUU-3-375	AY509275	800	LD2	Sweden, freshwater bacterioplankton	Unpublished

^a The affiliation of the sequences with the SOL cluster was proven by detailed phylogenetic analysis.^b nt, nucleotides.

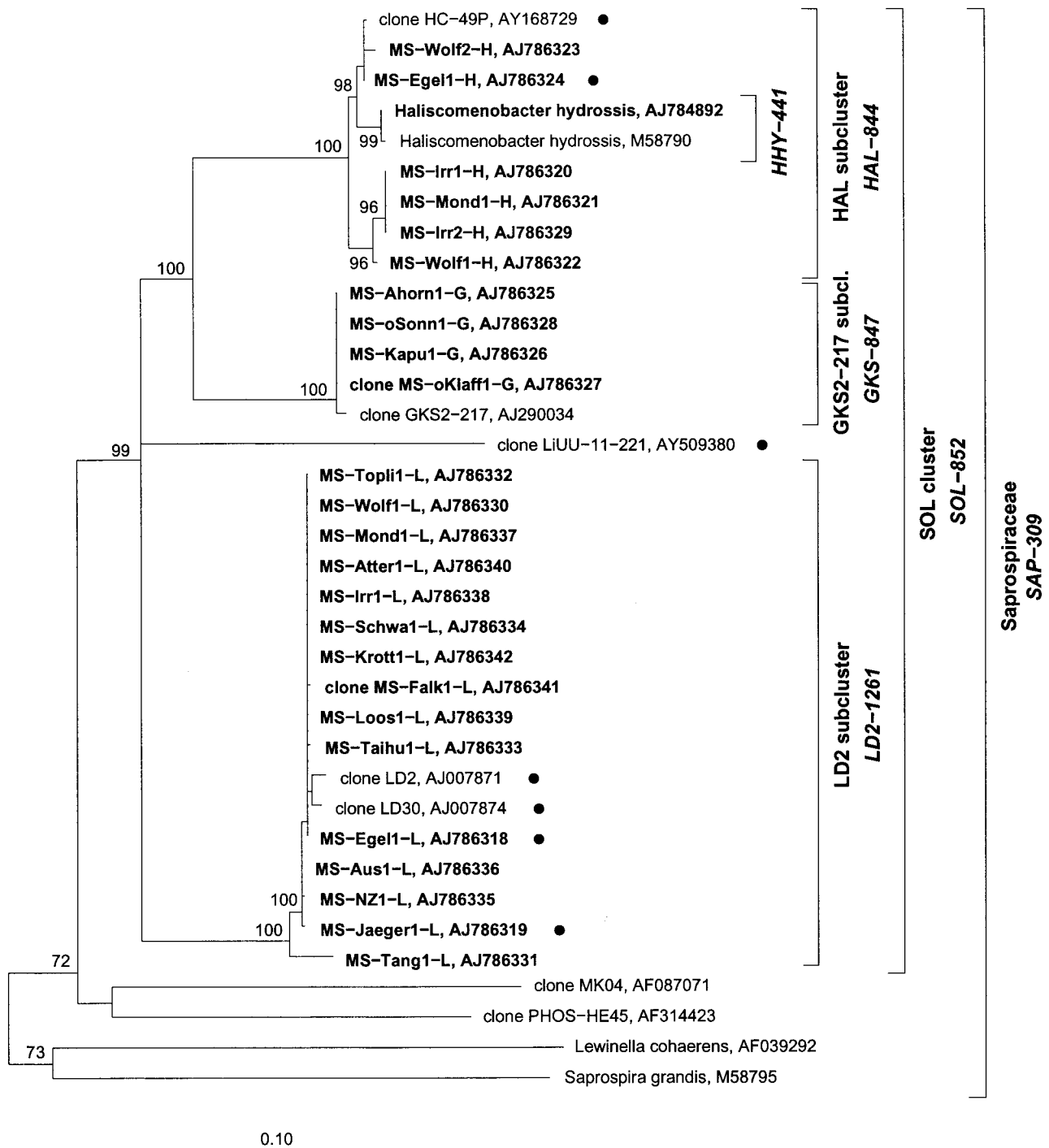


FIG. 3. Phylogenetic tree showing the affiliation of new bacteria of the *Saprospiraceae* family (in bold type) from mixed cultures and environmental samples. The neighbor-joining tree was calculated with almost full-length 16S rRNA gene sequences. Seven shorter sequences (765 to 1,165 nucleotides) (indicated by a small solid circle) were added by the ARB parsimony method without changing the tree topology. *H. hydrossis* sequence AJ784892 is the resequenced sequence. Bootstrap values (1,000 iterations) of >50% are given. Brackets indicate the specificity of FISH probes (probe names in italic type). Probe SAP-309 targets all SOL cluster bacteria and >80% of the remaining sequences within the *Saprospiraceae* family.

was designed to cover all SOL cluster sequences in our database, yet it also perfectly matched the sequence of the clone PHOS-HE19 (accession no. AF314428), which does not belong to the SOL cluster. Additionally, some members of the *Bacte-*

roidetes show only weak mismatches with this probe. This reduced specificity of the probe results in signals from bacteria with different morphologies (rods and cocci) in some environmental samples. However, we did not observe FISH signals of

TABLE 5. Similarity values within and between subclusters HAL, LD2, and GKS2-217

Subcluster	16S rRNA gene sequence similarity value ^a					
	HAL		LD2		GKS2-217	
	Min	Max	Min	Max	Min	Max
HAL	98.7	100				
LD2	90.1	92.7	97.6	100		
GKS2-217	93.8	95.5	89.9	92.1	99.7	100
LiUU-11-221 ^b	88	91.5	88.7	91.5	89.5	89.7

^a Minimum (Min) and maximum (Max) similarity values within and between subclusters.

^b Representative of a potential fourth subcluster inside the SOL cluster.

these rods and cocci with the SAP-309 probe (which covers all SOL cluster sequences), so we can exclude the possibility that members of these morphotypes belong to the SOL cluster. Probe SOL-852 detected all SOL filaments in any of the environmental samples investigated so far. The subcluster-specific probes HAL-844, LD2-1261, and GKS-847 (Fig. 4) did not yield any signals from cells other than the SOL morphotype.

DISCUSSION

Between several hundred to more than 100,000 individuals of the remarkable SOL morphotype per ml were found in most of the bacterioplankton samples that were analyzed. This morphotype typically accounted for around 1% of the total bacteria. The use of standard procedures for direct counts of microbial abundance (16) should lead to the detection of some of these striking organisms in almost every sample from a freshwater habitat inhabited by SOL bacteria. Given the observed abundance of SOL cluster bacteria and the fact that these filamentous bacteria produce quite bright and conspicuous signals when they were stained by standard procedures (26), one would think that these conspicuous filamentous bacteria would be mentioned in a number of articles in journals with a microbial and limnological focus. Surprisingly, the reverse is true. There are few mentions of these filamentous forms, and if they are mentioned or depicted, it looks rather accidental. Schmaljohann et al. (27) detected long straight filaments with diameters of 0.4 μm and lengths of up to 30 μm in Lake Kinneret in Israel. Alfreider and coworkers (1) pictured some of these filamentous members of bacterioplankton hybridized with eubacterial probe EUB338 on a figure presenting FISH data on Lake Gossenköllesee, a mountain lake high in the Austrian

Alps. Hofer et al. (12) worked on the same lake and demonstrated that filamentous viruses infect bacteria of the SOL morphotype. Recently, Pernthaler et al. (25) also demonstrated that members of the LD2 subcluster derived from Lake Schöhsee in Germany possess the conspicuous SOL morphology.

We demonstrate here that this distinct morphotype consists of a genetically quite diverse (Table 5) yet monophyletic group. In view of the high frequency of occurrence of members of the SOL cluster in a broad range of different freshwater habitats, as well as their remarkable contribution to the total bacterial biomass, this group of bacteria was clearly underrepresented in bacterial diversity studies performed by culture-independent methods. Apart from the sequence of the only described species (*H. hydrossis*), there was only a limited number of 16S rRNA gene sequences affiliated with the SOL cluster available up to now. The sequence of clone HC-49P (accession no. AY168729) was obtained from an arsenite-oxidizing biofilm (unpublished data). The short sequences of clones BCM3S-7B (accession no. AY102912) and csbio160175 (accession no. AY187353) were retrieved from a subtropical freshwater marsh (unpublished data) and a drinking water biofilm (28), respectively. The GKS2-217 clone comes from a clone library of the high mountain Lake Gossenköllesee (8). Zwart and coworkers (38) retrieved some sequences from Lake Loosdrecht (clone LD2 [accession no. AJ007871] and clone LD30 [accession no. AJ007874]) and Lake IJssel (clone Urk0-01 [accession no. AJ416169] and clone Med0-02 [accession no. AJ416157]) and designated these four sequences as the typical freshwater bacterial cluster LD2 (37). Sequencing of a DGGE band retrieved from a dilution culture of the Weser estuary in Germany yielded the short sequence of clone DC5-0-5 (unpublished data) (accession no. AY145578). Sekiguchi et al. (29) obtained short sequences from the Changjiang (Yangtze) river and Lake Poyang in the People's Republic of China (accession no. AF428658, AF428754, AF428783, and AF428943). Five sequences 763 to 1,038 nucleotides long were obtained from different lakes in Sweden by Eiler et al. (unpublished data) (accession no. AY509298, AY509321, AY509369, AY509275, and AY509380). The monophyletic SOL cluster was found by phylogenetic analysis of the 25 16S rRNA sequences retrieved from a variety of different habitats in this study together with some of the sequences already known. The three subclusters HAL, LD2, and GKS2-217 form this monophyletic SOL cluster. The hybridization of a broad spectrum of bacterioplankton samples with subcluster-specific probes

TABLE 6. Oligonucleotide probes used in this study

Probe	Sequence (5'→3')	GC content (%)	T _m (°C) ^a	Length (nt) ^b	Specificity	Formamide concn (%)	No. of unspecific matches ^c
SAP-309	TCT CAG TAC CCG TGT GGG	61.1	48.3	18	<i>Saprospiraceae</i>	25	8
SOL-852	ACG CTT TCG CTT GGA CAC	55.6	50.9	18	Soletticluster	30	20
HAL-844	CGC TTG GAC ACT CAC TCC	61.1	48.1	18	HAL subcluster	25	1
GKS-847	TTC GCT TGG ACA CAC AAT C	47.4	48.9	19	GKS2-217 subcluster	25	1
LD2-1261	GGC TCC GCT TCA CAG CTT	61.1	52.7	18	LD2 subcluster	30	3
HHY-441	CCA GAT TTC TTC CCA AGC	50	46.8	18	<i>Haliscomenobacter hydrossis</i>	20	17

^a T_m, melting temperature (according to the manufacturer).

^b nt, nucleotides.

^c Number of known sequences matching (<1 weighted mismatch) the probes but not affiliated to the respective target groups.

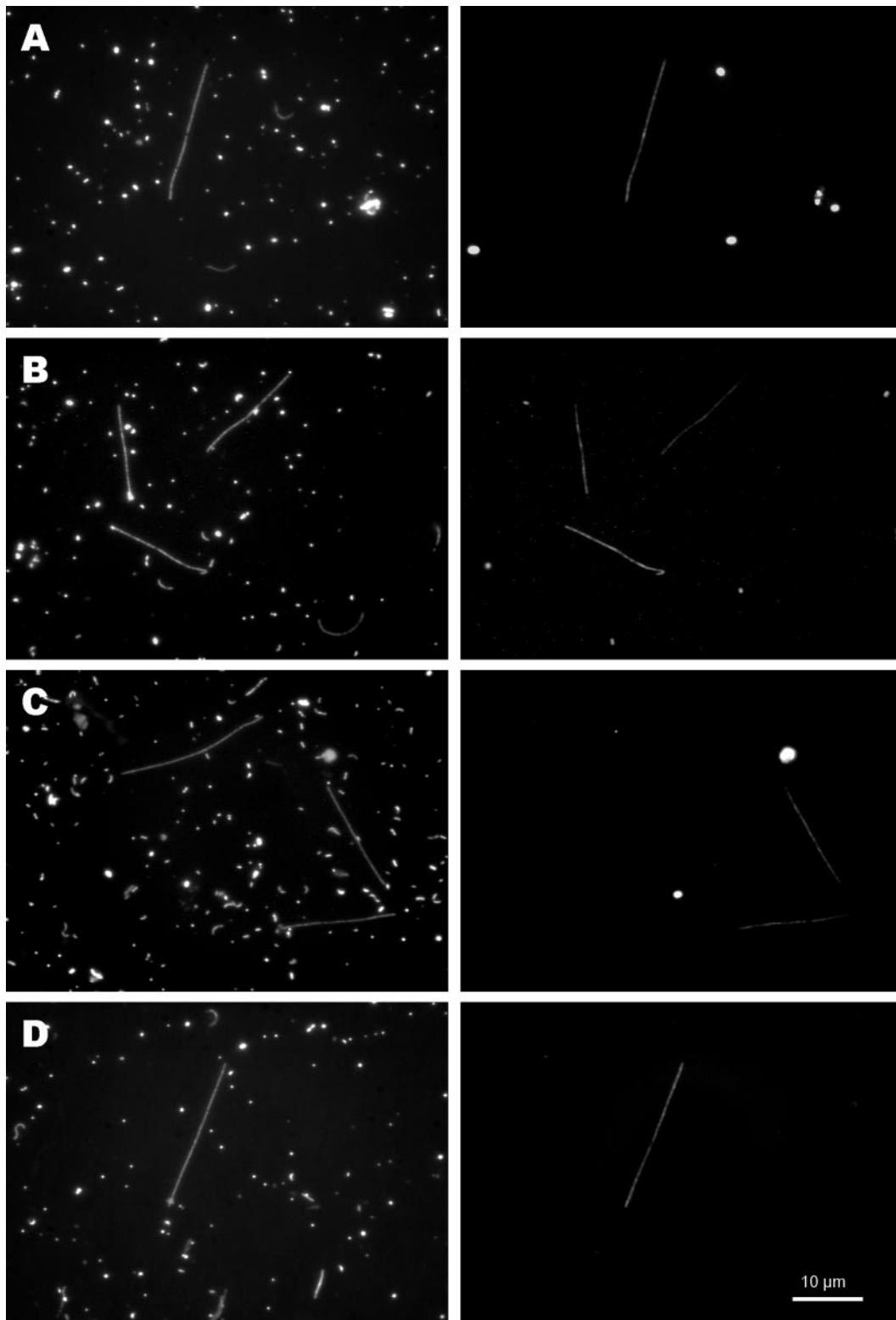


FIG. 4. Pairs of microscopic images of the same microscopic field with UV excitation (DAPI stained; left column) and green excitation (hybridized with Cy3-labeled oligonucleotide probes; right column). Probes SAP-309 (A), SOL-852 (B), HAL-844 (C), and LD2-1261 (D) were used. All coccoid signals on the right-hand images arise from phototrophic organisms (autofluorescent signals). All photomicrographs were taken at a magnification of $\times 1,250$. The scale bar is valid for all microscopic images.

proved that members of all three subclusters have the same conspicuous morphology.

However, in some environmental samples, a small fraction of typical SOL morphotype bacteria did not hybridize with any of the three subcluster-specific probes. This finding together with the phylogenetic position of the LiUU-11-221 clone in the neighbor-joining tree shown in Fig. 3 gives rise to the question of whether there are more than three subclusters belonging to the SOL cluster. Taking into account the high genetic diversity of this group and the scarce data on the bacterioplankton community of freshwater habitats in major parts of the world, one would not be surprised by the appearance of additional phylogenetic subclusters of bacteria also possessing the SOL morphology. Nevertheless, with probes SAP-309 and SOL-852, we were able to securely identify all SOL morphotype bacteria in all environmental samples investigated so far.

Members of the SOL cluster were found in a wide spectrum of different freshwater habitats. Samples were taken on three continents over temperate, subtropical, and tropical zones. The limnological characterization of the habitats ranged from ultraoligotrophic high mountain lakes (e.g., Lake Zwerfenbergsee, Austria) to hypertrophic parts of large shallow lakes (e.g., Mailing bay, Lake Taihu, People's Republic of China). Bodies of water where we were able to find SOL cluster bacteria ranged from sea level (e.g., Lake Loosdrecht, The Netherlands) to up to 2,309 m above sea level (e.g., Lake Oberer Klaffersee, Austria). The surface area of the habitats ranged from around 1 ha (e.g., Lake Egelsee, Austria) to 68,870 km² (Lake Victoria, East Africa). SOL cluster filaments were also found in small artificial ponds used for aquaculture (e.g., Jäger-teich pond, Austria). Considering the large number of different habitats where it was possible to identify these conspicuous filaments, we conclude that these SOL cluster bacteria form an integral part of the bacterioplankton in diverse freshwater habitats. This group potentially makes up a significant portion of the total number of bacteria. These facts, together with the filamentous morphology, lead to a significant share of the total bacterial biomass of this group in many freshwater habitats. However, we still do not know details about the ecology of this intriguing group of bacterioplankton. Nevertheless, this study established a phylogenetic basis for these monophyletic SOL cluster bacteria, with at least three subclusters with the same conspicuous morphology. On this basis, we will try to elucidate more details of the ecology of this interesting group of bacteria.

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