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Development and Application of 16S rRNA-Targeted Probes for Detection of Iron- and Manganese-Oxidizing Sheathed Bacteria in Environmental Samples

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Comparative sequence analysis of the 16S rRNA genes from several *Leptothrix* and *Sphaerotilus* strains led to the design of an oligonucleotide probe (PS-1) based on a sequence within the hypervariable region 1 specific for four *Leptothrix* strains and for one of the four *Sphaerotilus natans* strains examined. Another probe (PSP-6) was based on a sequence within the hypervariable region 2. PSP-6 was specific for one of the two evolutionary lineages previously described for *Leptothrix* spp. (P. L. Siering and W. C. Ghiorse, Int. J. Syst. Bacteriol. 46:173–182, 1996). Fluorescein-labeled oligonucleotide probes were synthesized, and their specificity for fluorescence in situ hybridization identification was confirmed by a laser scanning microscopy technique (W. C. Ghiorse, D. N. Miller, R. L. Sandoli, and P. L. Siering, Microsc. Res. Tech. 33:73–86, 1996) to compare whole-cell hybridizations of closely related bacteria. Probe specificity was also tested in dot blots against total RNA isolated from four *Leptothrix* strains, four *Sphaerotilus* strains, and 15 other members of the class *Proteobacteria*. When the probes were tested on samples from the Sapsucker Woods wetland habitat where *Leptothrix* spp. are thought to play a role in manganese and iron oxidation, positive signals were obtained from several sheathed filamentous bacteria including some that were morphologically similar to previously isolated strains of "*Leptothrix discophora*." Other unknown filamentous sheathed bacteria also gave strong positive signals. This work provides a foundation for future studies correlating the presence of members of the *Leptothrix-Sphaerotilus* group of sheathed bacteria with manganese and iron oxidation activity in habitats where biological iron and manganese oxidation are important environmental processes.

The development of culture-independent molecular methods to identify microorganisms and assess their activities in natural environments (3, 4, 13, 24, 34, 39, 41, 54) has led to the successful application of 16S rRNA-targeted fluorescence in situ hybridization (FISH) techniques for the identification of specific microorganisms in genetically diverse natural assemblages (1, 2, 5, 35, 40, 51–53). However, some attempts at employing FISH techniques have yielded unsatisfactory results (6, 27, 36) or been hindered by technical problems such as low signal strength or high background fluorescence (5, 29, 40).

We recently developed a laser scanning microscopy (LSM) procedure for testing FISH protocols that can alleviate some of these technical problems (23). In this study we report the use of the LSM procedure to develop and test a FISH technique for identifying iron- and manganese-oxidizing sheathed bacteria of the *Sphaerotilus-Leptothrix* group in samples from the Sapsucker Woods wetland in Ithaca, N.Y., where these bacteria have been associated with the seasonal cycling of iron and manganese (18, 19, 21) and with the presence of a putative manganese oxidation protein factor gene (11, 47) in environmental samples.

In the development of any 16S rRNA-targeted FISH procedure, the oligonucleotide probe specificity is of primary concern. Therefore, we evaluated the specificity of a previously reported *Leptothrix*-targeted probe (LD1) (51) based on 16S rRNA sequences of "*Leptothrix discophora*" SS-1 (9), which was originally isolated from the Sapsucker Woods wetland (10, 21). LD1 had been shown to hybridize with RNA from "*L. discophora*" SS-1 and "*Aquaspirillum*" *metamorphum* DSM1837^T in dot blots (46); therefore, it is not specific for *Leptothrix* spp. Furthermore, we found that the LD1 target sequence was not included in the 16S rRNA sequences of "*L. discophora*" SP-6 (46), and it did not hybridize with RNA from either "*L. discophora*" SP-6 or *Leptothrix cholodnii* LMG 7171 in dot blots (46). Thus, in this work we designed two new 16S rRNA-targeted probes to distinguish between the available strains of *Leptothrix* spp. and other members of the class *Proteobacteria* (PS-1) and to differentiate between two recently identified lineages of *Leptothrix* spp. (45, 46) (PSP-6).

Finally, we demonstrated by using an optimized FISH procedure that fluorescein-labeled PS-1 and PSP-6 probes were able to identify the *Leptothrix* spp. in laboratory cultures and in actual samples from the Sapsucker Woods wetland.

MATERIALS AND METHODS

Cultures and cultivation. The bacterial strains used in this study and their sources are listed in Table 1. *Leptothrix* strains were grown in PTYP medium as previously described (46). For microscopy of *Leptothrix* strains, Mn^{2+} was added to a final concentration of 3 μg of Mn^{2+}/ml from an autoclaved stock solution containing 300 μg of Mn^{2+} per ml as $MnSO_4 \cdot H_2O$. *Sphaerotilus* strains were grown in CGYA medium (16, 46). For microscopic examination, *Sphaerotilus* and *Leptothrix* strains were inoculated by transferring a 100-μl portion of a culture grown for 48 h into 25 ml of medium in a 250-ml flask and incubated at 28°C and 150 rpm; "*L. discophora*" SP-6 was grown without shaking except when cultured for RNA extraction. *Escherichia coli* was grown in Luria broth (43) at 37°C and 200 rpm. *Sphingomonas paucimobilis* RSP1, *Pseudomonas cepacia* 17616, *Pseudomonas* sp. strain CG-21, *Pseudomonas putida* CG-1, *Pseudomonas fluorescens* CG-5, *Pseudomonas glatheii* HG-5, and *Burkholderia gladiolii* HG-10 were all grown in 5% PTYG broth (15) at 30°C and 200 rpm. Unless noted otherwise, all American Type Culture Collection (ATCC)-supplied strains were grown as recommended by ATCC (16). For RNA extractions from all strains, 100-ml cultures were inoculated by transferring a 100-μl portion of a mid-log-phase culture into 100 ml of medium contained in a 500-ml flask; incubations were done as indicated above. Solid media were prepared by adding 1.5% Bacto Agar (Difco Laboratories, Detroit, Mich.).

Probe design and synthesis. Deoxyoligonucleotide probes were designed from alignments of 16S ribosomal DNA (rDNA) sequences which were done as

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TABLE 1. Bacterial strains, their phylogenetic positions in the α, β, and γ groups of the *Proteobacteria*, and their sources

Organism (group)	Strain designation[a]	Source[a]
Sphingomonas capsulata (α)	ATCC 14666^T (T = type)	ATCC
Pseudomonas diminuta (α)	ATCC 11568^T	ATCC
Sphingomonas paucimobilis (α)	RSP1	44
Leptothrix discophora (β)	SS-1 (ATCC 43182)	21
	SP-6 (ATCC 51168)	15
Leptothrix sp. (β)	NC-1[b]	Corstjens[c]
Leptothrix cholodnii (β)	LMG 7171 (= CCM 1827 = Mulder5)	BCCM
Sphaerotilus natans (β)	ATCC 13338^T	ATCC
	ATCC 15291	ATCC
	ATCC 29329	ATCC
	ATCC 29330	ATCC
Alcaligenes eutrophus (β)	ATCC 17697^T	ATCC
Pseudomonas cepacia (β)	ATCC 17616	ATCC
Comamonas testosteroni (β)	ATCC 11996^T	ATCC
Pseudomonas glatheii (β)	HG-5	28
Burkholderia gladiolii (β)	HG-10	28
Pseudomonas maltophilia (γ)	ATCC 13637^T	ATCC
Pseudomonas mendocina (γ)	ATCC 25411^T	ATCC
Aeromonas icthiosmia (γ)	ATCC 49804	ATCC
Pseudomonas putida (γ)	CG-1[d]	28
Pseudomonas fluorescens (γ)	CG-5	28
Escherichia coli (γ)	B/r	Shuler[e]

[a] ATCC, American Type Culture Collection, Rockville, Md.; CCM, Czechoslavak Collection of Microorganisms, Brno, Czech Republic; BCCM and LMG, Belgian Coordinated Collection of Microorganisms and Laboratorium voor Microbiologie, respectively, Universiteit Ghent Bacterial Culture Collection, Ghent, Belgium.

[b] Strain NC-1 was isolated from Bear Trap Creek in Syracuse, N.Y., by Hans and Liesbeth de Vrind and Eleanora Robbins.

[c] Paul L. Corstjens, Department of Chemistry, Leiden University, Leiden, The Netherlands.

[d] *Pseudomonas* sp. strain CG-1 was identified by the BIOLOG identification system as *Sphingobacterium mizutii*, but 16S ribosomal DNA sequence analysis positioned this bacterium within the *Pseudomonas* family in the γ group of the *Proteobacteria* (28).

[e] Michael L. Shuler, Department of Chemical Engineering, Cornell University, Ithaca, N.Y.

described previously (46). The oligonucleotide probes described below were synthesized and labeled at the 5′ end with fluorescein isothiocyanate (FITC) by Oligos etc. (Wilsonville, Oreg.). The base sequences of the probes are given with the corresponding position in the *E. coli* 16S rRNA sequence (8) indicated by an asterisk as follows: PS-1, 5′-fluorescein-GATTGCTCCTCTACCGT-66*; and PSP-6, 5′-fluorescein-GGCTATCCCCCACTACTG-138*. The control probes FZ-1 (universal probe [24]) and FZ-2 (rRNA-like control probe [24]) were also synthesized with 5′-fluorescein labels.

RNA extraction. Total RNA was isolated from 100-ml cultures of the strains listed in Table 1 by a modified version of the method of Stahl et al. (48). All solutions, glassware, and plasticware used for RNA extraction were treated to decrease RNase activity (43). Cells were harvested, and the pellet was washed in pH 5.1 buffer which contained 50 mM Na acetate and 10 mM EDTA. The pellet was resuspended in 0.25 ml of pH 5.1 buffer and combined with 2 g of 0.1-mm-diameter zirconia-silica beads (prebaked at 200°C for 24 h) (Biospec Products, Bartlesville, Okla.), 50 μl of 20% sodium dodecyl sulfate (SDS), and pH 5.1 buffer-equilibrated phenol to fill a 2-ml screw-cap polypropylene microcentrifuge tube (Lab Product Sales, Rochester, N.Y.). Samples were disrupted by bead beating for 2 min in a Mini Bead Beater (Biospec Products), followed by a 10-min incubation at 60°C, and another 2 min of bead beating. Samples were centrifuged at low speed to pellet the beads, and the aqueous phase was transferred to a clean tube. Beads were rinsed with 200 μl of pH 5.1 buffer and pooled with the aqueous phase. The nucleic acids were further purified by another pH 5.1 phenol extraction, followed by two phenol-chloroform-isoamyl alcohol (125:24:1; pH 4.7) (Sigma Chemical Company, St. Louis, Mo.) and two chloroform-isoamyl alcohol (24:1) extractions. Nucleic acids were precipitated at −20°C by the addition of 0.5 volume of 7.5 M ammonium acetate and 2 volumes of absolute ethanol. Precipitated RNA was collected by centrifugation, washed with 80% ethanol, and electrophoresed on an agarose gel to confirm purity by standard methods (43). In cases where DNA contamination was extensive, the samples were further treated with RNase-free DNase I (Ambion Inc., Austin, Tex.) according to the manufacturer's recommendations. RNA was resuspended in diethylpyrocarbonate (Sigma Chemical Company)-treated double-distilled water (ddH_2O), and concentrations were determined spectrophotometrically (43).

RNA dot blot analysis. Oligonucleotide probes PS-1, PSP-6, and FZ-1 were end labeled with digoxigenin-ddUTP by using the Genius 5 oligonucleotide 3′-end labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to the manufacturer's recommendations. RNA (1 μg) was denatured and transferred onto MagnaGraph nylon transfer membrane (Micron Separations Inc., Westborough, Mass.) as recommended by the manufacturer of the membrane. RNA samples were applied to the membrane with the Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Hercules, Calif.), and membranes were baked at 80°C for 2 h. All solutions and methodology used were in accordance with the Genius System User's Guide for Filter Hybridizations, version 2.0 (Boehringer Mannheim Biochemicals). Prehybridizations, overnight hybridizations with a probe concentration of 5 pmol of probe/ml, and initial washes in 2× wash solution (2× wash solution consists of 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% SDS) were done at 23°C. Subsequent washes were done at either 26°C (PS-1 and FZ-1) or 38°C (PSP-6), at four different stringencies by changing the SSC concentration in the wash solutions (0.4×, 0.2×, 0.04×, and 0.02× SSC corresponding to Na concentrations of 41.67, 16.67, 6.67, and 3.33 mM, respectively). Chemiluminescence detection was done with the Lumi-phos 530 detection system (Boehringer Mannheim Biochemicals) according to the manufacturer's recommendations. Exposures were made on Xar-5 autoradiography film (Eastman Kodak Company, New Haven, Conn.) for time periods ranging from 15 min to 1 h at room temperature.

Environmental samples. Water grab samples containing duckweed (*Lemna* spp.) and detrital material were collected from the surface of the Sapsucker Woods field site in Ithaca, N.Y. (18, 20) in October 1994 (water temperature, 15°C). Samples obtained in sterile 125-ml wide-mouth polypropylene bottles were immediately fixed with formalin as outlined below. After fixation, fluffy brown material was teased away from the *Lemna* roots with the aid of a dissecting microscope by using sterile no. 5 jeweler's tweezers. The separated material was transferred to a test tube with a sterile Pasteur pipette and further processed as described below.

Sample processing. Cultured cells were grown to mid-exponential phase, chilled, and fixed on ice for 15 min (minimum) by the addition of 0.1 volume of formalin, which is a 37% formaldehyde solution containing 10 to 15% methanol (Sigma Chemical Company). Fixation times up to 1 h were used for pure cultures. Field samples were left in fixative for time periods ranging from 1 h to overnight at 4°C. Fixation times greater than 48 h were avoided because they were found to decrease the fluorescence signal intensities of pure cultures and natural samples by using the hybridization protocol described below. Fixed samples were centrifuged, and the pellet was washed twice with an equal volume of phosphate-buffered saline (PBS) which contained 130 mM NaCl and 10 mM sodium phosphate buffer, pH 7.2. Pellets were resuspended in enough PBS to yield a slightly turbid solution. Samples were stored at 4°C for less than 1 month or at −20°C for up to 4 months with no decrease in fluorescence signal intensity after hybridization. Prolonged storage (>2 months) at 4°C did result in a decrease of signal intensity after hybridization, even though there were no morphological changes detectable by phase-contrast microscopy.

In preparation for hybridization, smears were made by spotting 10 μl of sample onto gelatin-coated glass slides treated with chromate (12) and allowing them to air dry. Dried smears were dehydrated, fixed, and washed in sterile ddH_2O as described by Braun-Howland et al. (7). Air-dried smears were stored in desiccators at room temperature for up to 2 months with no loss in fluorescence signal after hybridization.

FISH protocol. We followed the general strategies for optimizing a FISH protocol as described previously (23). The optimized protocol was as follows: labeled probes were dissolved to a concentration of 5 ng/μl in filter-sterilized (filter pore size, 0.2 μm) hybridization solution of Poulsen et al. (39). Hybridization mix (15 μl) was spotted onto dried smears, and slides were incubated in a humidified chamber at 37°C for 4 to 8 h. Overnight hybridizations routinely resulted in slightly decreased signal intensities. After hybridization, unbound probe was removed by flooding the slide with ice-cold 5× SET (5× SET is 0.750 M NaCl, 0.100 M Tris-HCl [pH 7.8], and 0.005 M EDTA). The slides were then washed three times (for 5 to 10 min each time) in 0.2× to 1× SET at temperatures ranging from 37 to 55°C. After the slides were washed the third time and allowed to air dry, they were mounted under a 22-mm^2 cover glass in 7.5 μl of 0.1% *p*-phenylenediamine (PPD) (Sigma Chemical Company) (pH 8.8) in 90% glycerol–5× SET to decrease fading of fluorescence during microscopy (31). The PPD solution was prepared by dissolving the PPD crystals under a stream of N_2, and aliquots were stored at −20°C for up to 3 months, or until solution darkened. All work with the FISH probes was done under reduced illumination to minimize light exposure. Typically, the slides were viewed immediately after mounting. Occasionally, slides were kept overnight in the dark at 4°C prior to microscopy.

AO staining. Wetland sample smears for direct counts were prepared identically to smears prepared for FISH probing as described above. The staining procedure was a modified version of a previously described method for counting sediment and soil bacteria (20, 26, 50, 55). Smears were stained with 2 drops of a filter-sterilized (filter pore size, 0.2 μm) aqueous solution of 0.01% acridine orange (AO). After the smears were stained for 2 min at room temperature, they were immersed in a coplin jar containing 1 M NaCl and rinsed briefly in sterile

TABLE 2. Aligned regions of the 16S rRNA targeted by *Leptothrix*-specific probes PS-1 and PSP-6

Probe target or strain	Sequence[a]	
	PS-1 probe	PSP-6 probe
Probe target	5′-ACGGUAGAGGAGCAAUC	5′-CAGUAGUGGGGGAUAGCC
Strains		
L. discophora SP-6[b]	ACGGUAGAGG-- ----AGCAAUC	CAGUAGUGGGGGAUAGCC
L. cholodnii CCM1827[b]	ACGGUAGAGG-- ----AGCAAUC	CAGUAGUGGGGGAUAGCC
Leptothrix sp. strain NC-1[b]	ACGGUAGAGG-- ----AGCAAUC	CAGUAGUGGGGGAUAGCC
L. discophora SS-1[b]	ACGGUAGAGG-- ----AGCAAUC	CAGUCGUGGGGGAUAACU
S. natans ATCC 13338[b]	ACGGUAGAGG-- ----GGCAACC	CAGUCGUGGGGGAUAACG
S. natans ATCC 15291[b]	ACGGUAGAGG-- ----GGCAACC	CAGUCGUGGGGGAUAACG
S. natans ATCC 29330[b]	ACGGUAGGGG-- ---GAGCAAUC	CAGUCGUGGGGGAUAACG
S. natans ATCC 29329[b]	ACGGUAGAGG-- ----AGCAAUC	CAGUCGUGGGGGAUAACG
R. gelatinosus ATCC 17011	ACGGUAACAG-- --GCCGCAAGG	CAGUAGUGGGGGAUAGNN
C. testosteroni ATCC 11996	ACGGUAACAG-- --GUCUUCGGA	UAGUAGUGGGGGAUAACU
P. glatheii HG-5[c]	ACGGCAGCAC-- -GGGCUUCGGC	CUGUAGUGGGGGAUAGCC
B. gladiolii HG-10[d]	ACGGCAGCAC-- -GGACUUCG-U	CUGUAGUGGGGGAUAGCC
Bordetella bronchiseptica S-1	ACGGCAGCGCGA GAGAGCUUGCU	CAGUAGCGGGGGAUAACU
Achromobacter xylosoxidans ATCC 15173	ACGGCAGCAC-- -GGGCUUCGGC	CAGUAGCGGGGGAUAACU
Alcaligenes faecalis ATCC 8750	ACGGCAGCAU-C GGGUGCUUNCA	CAGUAGCGGGGGAUAACU
A. eutrophus 335	ACGGUAACGG-- -GNCCUUCGGG	CUGUAGUGGGGGAUAACU
P. cepacia ATCC 25416[d]	ACGGCAGCGG-- GGGUGCUUGCA	CUGUAGUGGGGGAUAGCC
Rhodocyclus purpureus 6770	ACGGUAGCAGGA GAAAGCUUGCU	CUGAAGUGGGGGAUAACG
Azoarcus sp. strain S5b2	ACGGUAACAG-- --GCCUUCGGG	CAGUCGUGGGGGAUAACG
E. coli	ACGGUAACAGGA AGAAGCUUGCU (*E. coli* 66-82)[e]	UGAUGGAGGGGGAUAACU (*E. coli* 138-155)[e]

[a] Underlined bases indicate positions of mismatch with probe target. Dashes are gaps used to optimize alignment.
[b] Sequences were reported previously (46).
[c] Sequence from Herrick (28).
[d] No sequence information is available for *B. gladiolii* HG-10 (28), and for *P. cepacia* ATCC 17616 used in Fig. 1. The sequence information indicated is for *B. gladiolii* ATCC 10248 and EY3258 which are identical in the regions presented above and for *P. cepacia* ATCC 25416, respectively.
[e] Sequence and numbering Brosius et al. (8).

ddH_2O. Smears were allowed to dry in the dark and mounted under a 22-mm^2 cover glass with 10 μl of a filter-sterilized (filter pore size, 0.2 μm) aqueous solution of 32.7 mg of 1,4-diazabicyclo [2.2.2]octane (DABCO) per ml. Smears were immediately viewed under the microscope as described below. Individual cells were counted on two smears, eight fields per smear, and the numbers were compared to counts obtained from samples hybridized with the fluorescein-labeled universal probe FZ-1 (24), following the protocol described above with washes at 37°C in 1× SET.

Microscopy. All samples were examined on a Zeiss LSM-210 laser scanning microscope outfitted with an external argon laser providing 488-nm-wavelength excitation and conventional light sources for transmitted phase-contrast epifluorescence imaging as described previously (23). Fields containing representative cells were viewed under oil immersion with a phase-contrast 63× Plan-apochromat objective lens (numerical aperture, 1.4) and a matching oiled condenser lens. To allow for comparison of relative fluorescence intensities between samples, image intensities were normalized by the constant contrast and brightness technique described previously (23). Electronic background noise was filtered from laser-scanned phase-contrast and fluorescent images by line averaging with LSM-210 software. The laser-scanned images and their corresponding parameters were stored on a 32-megabyte disk drive in the host computer or displayed on an RGB video monitor (Sony Trinitron, model CPD-1402E Character Display monitor). Photomicrographs of stored images were recorded on 35 mm T-MAX 100 black-and-white film through a color video image recorder (MATRIX Instruments Inc., Orangeburg, N.Y.). Conventional epifluorescence imaging for fluorescein and AO was done at an excitation wavelength of 450 to 490 nm with a filter combination consisting of a dichroic FT510 mirror and a LP520 barrier filter.

RESULTS AND DISCUSSION

Comparative sequence analysis and probe design. Complete sequencing of the 16S rRNA genes of "*L. discophora*" SP-6 and SS-1 (46) revealed sequences within the V1 and V2 hypervariable regions which had the potential to distinguish *Leptothrix* spp. from other members of the beta group of the class *Proteobacteria* (β-proteobacteria). In order to confirm that these regions would be useful for in situ identification of *Leptothrix* cells in environmental samples, we generated partial sequences (corresponding to the first 315 bp) of one strain of *Leptothrix cholodnii* (LMG 7171), a *Leptothrix* strain not identified to the species level (NC-1), and three strains of *Sphaerotilus natans* (ATCC 13338^T, ATCC 15291, and ATCC 29330) (46). We aligned these sequences to the most similar sequences retrieved from the GenBank/EMBL and Ribosomal DataBase Project (RDP) databases (14, 33) as previously described (46). All sequences were also aligned and compared to sequences from gram-negative aerobic heterotrophic bacteria isolated in our laboratory (28) (Table 2).

We designed the PS-1 probe (Table 2) to be specific for *Leptothrix* spp. We later determined a partial 16S ribosomal DNA sequence for *S. natans* ATCC 29329 (46), which was found to have sequence identical to that of all of the *Leptothrix* strains within the PS-1 target region (Table 2). Although the normal habitat of *S. natans* is eutrophic freshwater contaminated with organic matter (i.e., sewage or dairy wastewater), it has been known to occur in unpolluted waters where the growth substrates are unidentifiable (38). While *S. natans* has never been isolated from the wetland field site (17), there is a possibility that *Sphaerotilus* spp. occurs in association with *Lemna* root material which harbors a diverse microbial community (21, 22).

Our previous phylogenetic analysis of the *Sphaerotilus-Leptothrix* group (46) showed that two distinct lineages of *Leptothrix* species exist. One goal of this work was to be able to distinguish between the two *Leptothrix* lineages and between the two genera to enable the tracking of distinct *Leptothrix* populations during active periods of iron and manganese oxidation (21). Probe PSP-6 (Table 2), was designed to achieve this goal. The *Rubrivivax gelatinosus* sequence contains two unidentified bases in the last two positions of the PSP-6 target

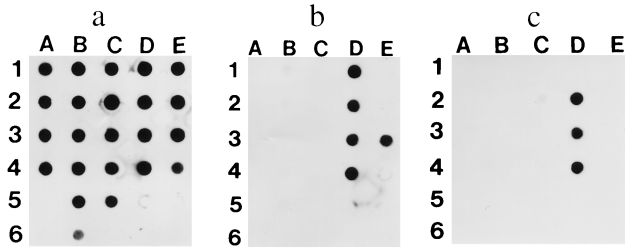

FIG. 1. Dot blot hybridization matrices of total RNA extracted from the 4 *Leptothrix* strains, 4 *Sphaerotilus* strains, and 15 other proteobacterial strains (Table 1). RNA extracted from each strain was probed with three digoxigenin-ddUTP-labeled 16S rRNA-targeted oligonucleotide probes, FZ-1 universal probe (a), PS-1 probe (b), and PSP-6 probe (c), as described in Materials and Methods. Position identification in each matrix: A1 to A4, *Sphingomonas capsulata* ATCC 14666^T, *Pseudomonas diminuta* ATCC 11568^T, *Sphingomonas paucimobilus* RSP-1, and *Pseudomonas* sp. strain CG-21, respectively; B1 to B6, *Pseudomonas maltophilia* ATCC 13637^T, *Pseudomonas mendocina* ATCC 25411^T, *Aeromonas icthiosmia* ATCC 49804, *Pseudomonas putida* CG-1, *Pseudomonas fluorescens* CG-5, and *Escherichia coli* B/r, respectively; C1 to C5, *Alcaligenes eutrophus* ATCC 17697, *Pseudomonas cepacia* ATCC 17616, *Comomonas testosteroni* ATCC 11996^T, *Pseudomonas glatheii* HG-5, and *Burkholderia gladiolii* HG-10, respectively; D1 to D4, "*Leptothrix discophora*" SS-1 ATCC 43182, "*L. discophora*" SP-6 ATCC 51168, *Leptothrix* sp. strain NC-1, and *Leptothrix cholodnii* CCM 1827, respectively; E1 to E4, *Sphaerotilus natans* ATCC 13338^T, ATCC 15291, ATCC 29329, and ATCC 29330, respectively.

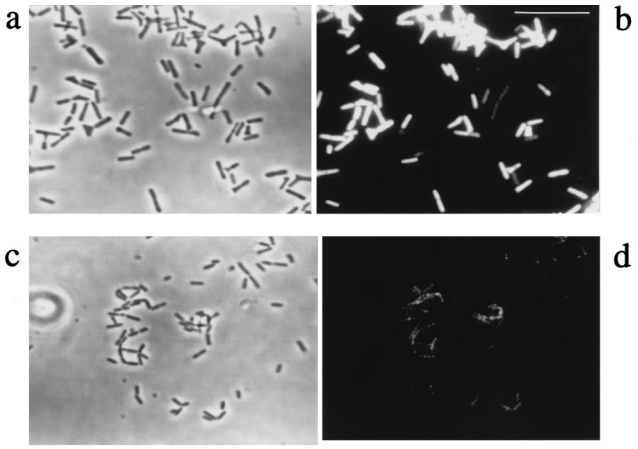

FIG. 2. Matched laser scanning phase-contrast (left) and epifluorescence images (right) showing whole-cell hybridizations of *S. natans* (ATCC 13338) (a and b) and "*L. discophora*" SS-1 (ATCC 43182) (c and d) cultures with the fluorescein-labeled universal probe FZ-1 (24). Hybridizations were done as described in the text, and washes consisted of three 10-min washes in 1× SET at 45°C. Note the less-intense signal of *L. discophora* SS-1 relative to that of *S. natans*, indicating growth rate and therefore ribosome content differences between the two strains (see text for discussion). These intensity differences were normalized by imaging under constant brightness and contrast conditions with the LSM (contrast/brightness ratio of 1.65) as described previously (23). Bar = 15 μm.

sequence (Table 2). Although the PSP-6 probe target sequence may be present in the *R. gelatinosus* rRNA, potential cross-reactivity can be discerned on the basis of morphological and physiological differences between the two genera.

Determination of probe specificity by RNA dot blot hybridization and FISH analysis. Total RNA was isolated from 15 different representative α-, β-, and γ-proteobacteria, 4 strains of *Leptothrix*, and 4 strains of *Sphaerotilus* (Table 1). Six of the reference strains of *Proteobacteria* were selected to represent either the type strain or a species (49), and the remaining strains were random environmental isolates from surface water, sediment, or soil. The probes PS-1, PSP-6, FZ-1 (universal positive-control probe [24]), and FZ-2 (rRNA-like negative-control probe [24]) were 3′ end labeled with digoxigenin-ddUTP and hybridized with isolated RNA in identical dot blots (Fig. 1). Temperature and NaCl concentrations of wash buffers were determined empirically as described in Materials and Methods. For all probes tested, the optimal specificity was achieved by using the wash temperatures indicated in Materials and Methods in a wash solution containing 0.04× SSC corresponding to 6.67 mM Na^+. The FZ-1 universal probe bound all isolated RNAs (Fig. 1a); probe PS-1 bound to only the RNA from four *Leptothrix* strains and *S. natans* ATCC 29329 (Fig. 1b, D1 to D4 and E3); probe PSP-6 bound only to RNA from "*L. discophora*" SP-6, *L. cholodnii* LMG 7171, and *Leptothrix* sp. strain NC-1 (Fig. 1c, D2 to D4). As expected, the FZ-2 probe did not bind to any of the isolated RNAs (data not shown).

Probe specificity was also tested by FISH with whole cells grown in axenic culture. Hybridization conditions were kept constant, and stringency was optimized by changing the temperature and NaCl concentration of the wash buffer as described previously (23). The probe specificity results from FISHs of cultured cells (e.g., see Fig. 3 and 4) agreed with the dot blot hybridization results in all cases. However, determining the washing stringencies of whole-cell hybridizations was significantly more difficult because of the problems of differences in fluorescence signal intensity presumably due to variations in rRNA content as a function of growth rate and cell size. An extreme example of this variation was observed when comparing *S. natans* cells hybridized with the universal probe FZ-1 with *Leptothrix* cells hybridized with the same probe under the same washing conditions (compare Fig. 2b and d). Note that the signal intensity for *S. natans* (Fig. 2b) was far brighter than for *L. discophora* (Fig. 2d). Indeed, cells of the *S. natans* strains used in this work were larger, grew more rapidly, and thus presumably contained more ribosomal target sequences than *Leptothrix* cells (46). Even though *S. natans* ATCC 13338^T and 15291 differed in 2 bases of the target sequence of PS-1 (Table 2), eliminating the PS-1 hybridization signals from these *S. natans* strains required very high stringency washes (one 10-min wash and two 5-min washes with 1× SET and at 50 to 55°C). These wash conditions resulted in detectable fluorescence differences between the target and non-target cells, including a significant reduction of the fluorescence intensity of target *Leptothrix* cells (Fig. 3). This problem made direct comparisons of different hybridization experiments difficult to interpret unless the contrast and brightness settings of the LSM were normalized to the brightest image in a given set of hybridizations as described previously (23) and as was done in Fig. 3. Similar difficulties were encountered when attempting to balance the specificity and signal intensity of PSP-6 hybridization to cells of *B. cepacia*, *B. gladioli*, and *P. glatheii*, each of which contained only one base mismatch from the PSP-6 target sequence (Table 2). For all strains tested, it was possible to achieve a detectable, although often suboptimal, signal in the PSP-6 target organisms ("*L. discophora*" SP-6, *L. cholodnii* LMG 7171, and *Leptothrix* sp. strain NC-1), while eliminating signal from non-target organisms by washing under the high-stringency conditions described above (data not shown). In each case, normalization of the LSM contrast and brightness parameters allowed the critical comparisons to be made.

The PSP-6 probe was designed specifically to differentiate between the two lineages of *Leptothrix* spp. (46). To test this specificity, we applied the PSP-6 probe to a mixed sample containing "*L. discophora*" SS-1 and SP-6 cells used as a pos-

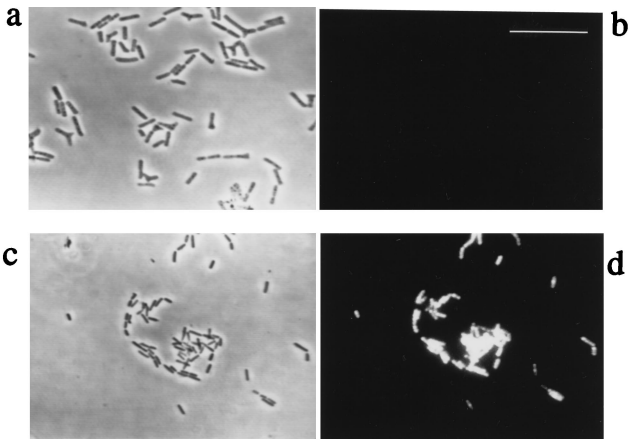

FIG. 3. Matching laser scanning phase-contrast (left) and epifluorescence (right) images showing whole-cell hybridizations of *S. natans* (ATCC 13338^T) (a and b), and *L. discophora* SS-1 (c and d) with probe PS-1. Hybridizations were done as described in the text, and washes consisted of three 10-min washes in 1× SET at 50°C. There are two positions of mismatch between the PS-1 target sequence and *S. natans* 13338^T. The PS-1 probe does not hybridize with *S. natans* 13338^T under the conditions employed. Note the brighter apparent image of *L. discophora* SS-1 (d) compared to that in Fig. 2d due to the use of an increased contrast/brightness ratio of 2.0 for the epifluorescence images in the figure (see the legend to Fig. 2). Bar = 15 μm.

itive control under the standardized hybridization, washing, and microscopic observation conditions described above (Fig. 4). When grown statically, cultured cells of strain SP-6 occur mostly as long sheathed filaments (15, 46), while those of strain SS-1 occur mostly as individual cells and some short filaments devoid of a sheath (46). Hybridizations of the mixed sample with the universal probe (data not shown) or with probe PS-1 (Fig. 4a and b) showed that all cells were labeled. Hybridization with the PSP-6 probe specifically labeled SP-6 cells (Fig. 4c and d). This confirmed the specificity of PSP-6 for the sheathed filamentous cells of "*L. discophora*" SP-6. Similar

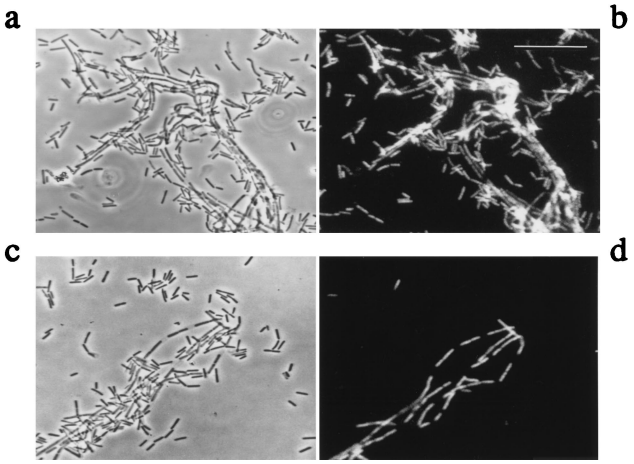

FIG. 4. Matching laser scanning phase-contrast (left) and epifluorescence (right) images showing whole-cell hybridizations of a mixed culture of *L. discophora* SP-6 and SS-1 with probe PS-1 (a and b), and probe PSP-6 (c and d). Hybridizations were done as described in the text, and washes consisted of one 10-min wash and two 5-min washes in 1× SET at 45°C. There are three positions of mismatch between the PSP-6 target sequence and *L. discophora* SS-1. Note that probe PSP-6 is specific for the sheathed, filamentous cells of *L. discophora* SP-6 (c and d), while probe PS-1 targets both *L. discophora* SS-1 and SP-6 cells (a and b). Bar = 15 μm.

results were obtained with probe PSP-6 positive-control cells of *Leptothrix* sp. strain NC-1 and *L. cholodnii* LMG 7171 (data not shown). During this work we found that the growth rate (and hence the probe-mediated ribosomal fluorescence intensity) was greater when SP-6 was grown under high aeration (i.e., shaking at 200 rpm in PTYP medium) (data not shown); however, these growth conditions resulted in fewer sheathed cells in the culture (15). Therefore, in planning experiments with SP-6 cultures as positive controls for environmental samples, static growth conditions were used to better mimic the natural situation in which *Leptothrix* would be found.

Additional work revealed no differences in fluorescence intensity between hybridizations of the same strain when the PS-1, PSP-6, and FZ-1 probes were applied under standardized hybridization and fluorescence microscopy conditions. This implied that the target sequences for each probe were equally accessible and that the probes were each labeled with comparable efficiencies. Also, when the SDS concentration of the hybridization buffer was increased to concentrations of up to 1%, fluorescence intensity did not increase, suggesting that cell envelope permeability did not limit the FISH intensity (data not shown). These results contribute evidence that ribosome abundance, rather than cell envelope permeability, is a major controlling factor affecting FISH results of *Proteobacteria* (23). Two factors which repeatedly led to decreased fluorescence intensity were prolonged fixation times in formalin and prolonged storage of formalin-fixed and washed cells stored in PBS at 4°C.

A *Leptothrix*-specific problem observed during this work was the reductive dissolution of Mn oxides during the hybridization procedure. Mn oxidation is a diagnostic property of the genus *Leptothrix* (19). When *Leptothrix* strains are grown in a low-carbon-source medium such as PTYP containing small amounts of Mn^{2+}, Mn oxides accumulate extracellularly either on the sheath of "*L. discophora*" SP-6 (15, 46) or in extracellular clumps associated with the cells of "*L. discophora*" SS-1, *L. cholodnii* LMG 7171, or *Leptothrix* sp. strain NC-1 (46). Similarly, in the wetland or other environmental samples containing Fe and Mn, many sheathed bacteria which are morphologically similar to *Leptothrix* characteristically display metal oxide-encrusted sheaths (19, 23). However, smears of the wetland samples displayed significantly lower numbers of the characteristic metal-encrusted sheaths after they were subjected to the standardized hybridization, washing, and mounting protocol (see left panels in Fig. 5 and 6). Subsequent experiments showed that the PPD mountant acted as a reducing agent to dissolve the Mn oxides and probably the Fe oxides as well (data not shown). No loss of metal oxides was seen in smears incubated in the individual solutions used in the hybridization protocol including 100 or 30% formamide (in 0.9 M NaCl), oligonucleotide-containing hybridization mixture, 0.1% SDS in 0.9 M NaCl, and 1× SET. However, oxides were rapidly dissolved in smears mounted in PPD (for 15 min at room temperature) regardless of whether they were treated and washed for hybridization (data not shown). The dissolution of metal oxides in environmental samples has important consequences in our work, because in natural samples, iron- and manganese-oxidizing bacteria traditionally are identified on the basis of their metal-encrusted extracellular structures (19, 22, 37, 38). Maintaining the oxides in the sheath by omitting the PPD antifading reagent or changing to another one, such as DABCO or sodium azide, may allow for better identification of filamentous sheathed organisms during FISH analysis of natural communities; however, more rapid fading rates and less control of normalized fluorescence imaging may result (25, 26).

FISHs of bacteria in wetland samples. To assess whether the bacteria in the wetland samples would be detectable with FISH probes, we estimated the proportion of AO-stained bacterial cells in the samples which were also labeled by hybridization with the FITC-labeled FZ-1 universal probe. This estimate showed that approximately 80% (±5%) of the cells counted by AO staining were labeled by hybridization with FZ-1. This is comparable to previously reported results for activated sludge (53) but is higher than the 35 to 67% reported for natural bacterioplankton samples (29) and the values for surface-associated and planktonic bacteria associated with drinking water (70 and 40%, respectively) (35). As expected, we observed a high degree of variation in the fluorescence intensity of individual FZ-1 cells in the wetland samples. However, particular cell morphology types observed with phase-contrast microscopy that did not show the label under epifluorescence were not found (data not shown). These observations suggested that the FZ-1 probe was not excluded by any particular cell type. At least 80% (±5%) of the AO-stainable cells in the wetland samples contained sufficient rRNA to permit detection by rRNA-targeted FISH analysis. Furthermore, these results showed that most of the bacterial cells in the wetland samples were permeable to the FZ-1 probe and, therefore, to the PS-1 and PSP-6 probes under the fixation and hybridization conditions we employed. Hybridization with the rRNA-like negative-control probe, FZ-2 (24), or omission of the probe resulted in no detectable fluorescein fluorescence when viewed under the normalized conditions (data not shown).

When the wetland samples were hybridized with the PS-1 probe (see Fig. 5) and PSP-6 probe (see Fig. 6) and washed at stringencies which allowed discrimination at 1 or 2 positions of mismatch between cultured "*L. discophora*" and *S. natans* cells, both probes labeled several types of filamentous sheathed bacteria (see Fig. 5 and 6), but most other bacteria in the sample were not labeled. Filamentous, sheathed cells with the morphology, size, shape, and poly-β-hydroxyalkanoate inclusions characteristic of "*L. discophora*" (37, 46) were detected in all samples (Fig. 5a through f, i, and j), but the "*L. discophora*"-like cells were by no means the most prominent labeled cells.

Many filamentous, sheathed cells hybridized with probe PS-1 and yet were clearly different from the described morphologies of cells of *Leptothrix* spp. For example, larger sheathed filaments composed of rectangular cells approximately 1.5 to 2 μm in diameter were often the most brightly fluorescent cells in a sample. Filaments with this morphology were very common in our samples. These unknown bacteria bear a strong resemblance to descriptions of the sheathed iron- and manganese-oxidizing bacteria in the genus "*Clonothrix*" (30, 32, 42). "*Clonothrix*" has never, to our knowledge, been isolated or grown in laboratory cultures. Therefore, further comparisons of the relatedness of the unknown to the sheathed bacteria discussed in *Bergey's Manual of Systematic Bacteriology* (49) await the isolation of filaments. We also found a significant number of vacant sheaths in the wetland samples (Fig. 5 and 6). A large number of such vacant sheaths is a characteristic associated with *Leptothrix ochracea* (22, 37); however, the vacant *L. ochracea* sheaths are typically heavily encrusted with iron oxide, which was not the case in these samples either before or after mounting in PPD.

One important point illustrated by these results is the different signal intensities observed in the natural sample hybridizations (compare filaments in Fig. 5i and j). In Fig. 5i, there were three sheathed filaments observed by phase-contrast microscopy. Bright fluorescence was associated with filament A (Fig. 5j), while filament B showed only dim fluorescence, and no fluorescence was associated with filament C. Such results indicate that when applying the FISH technique to natural samples or mixed cultures containing bacteria of different sizes and growth rates, this differential signal intensity can be a significant problem with respect to balancing stringency and specificity parameters. The use of a semiquantitative digital fluorescence measurement technique as described in this and an earlier report (23) helped to overcome this problem.

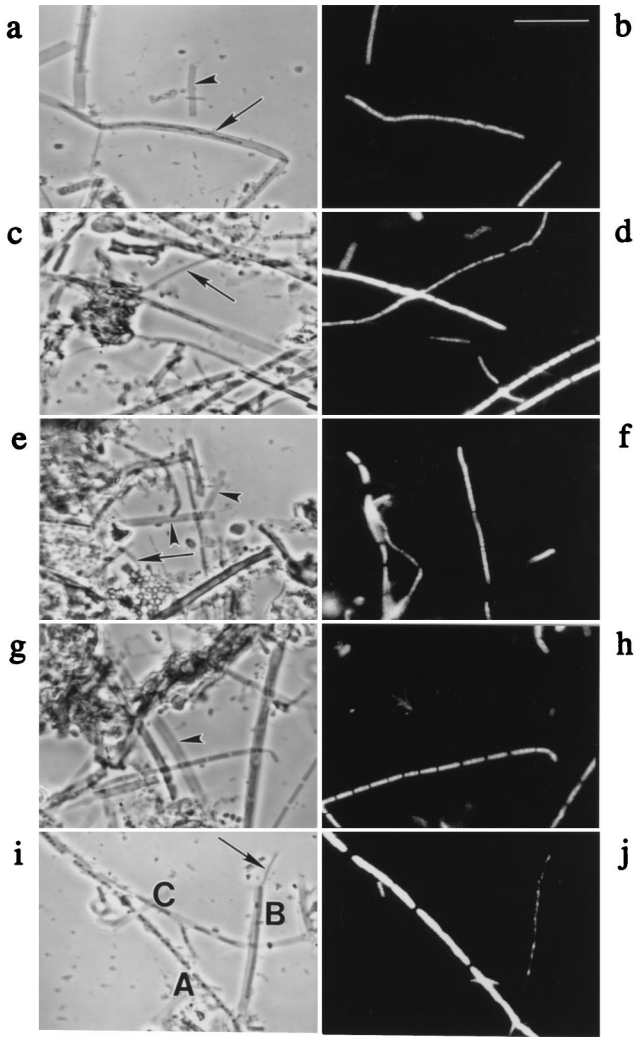

FIG. 5. FISHs with PS-1 probe applied to water samples from Sapsucker Woods Wetland in Ithaca, N.Y. Hybridizations were done as described in the text, and washes consisted of one 10-min wash and two 5-min washes in 1× SET at 48 to 50°C. Native sheathed bacteria which resembled *Leptothrix* spp. in size and shape (indicated by arrows in panels a, c, e, and i) were identified by the PS-1 probe under epifluorescence (b, d, f, and g). Unidentified filamentous sheathed bacteria with cells larger than typical "*L. discophora*" cells (c, e, g, and i) gave strong PS-1 hybridization signals even after optimum stringency washing (d, f, h, and i). Note the numerous empty sheaths (indicated by arrowheads in panels a, e, and g) and differential fluorescence intensities of filaments labeled A, B, and C in panel i. See the text for details. Bar = 15 μm.

Sheathed filamentous cells with a morphology similar to that of "*L. discophora*," but with slightly larger diameters, also hybridized with the PSP-6 probe (Fig. 6). The cells in these larger filaments were longer and wider than *L. discophora* SS-1 or SP-6 cells (e.g., compare Fig. 4 with Fig. 6e and f). Despite their shape and size similarity to *S. natans*, these PSP-6-labeled cells may represent another species of *Leptothrix* in the SP-6 lineage (46). The PSP-6 target sequence contained three po-

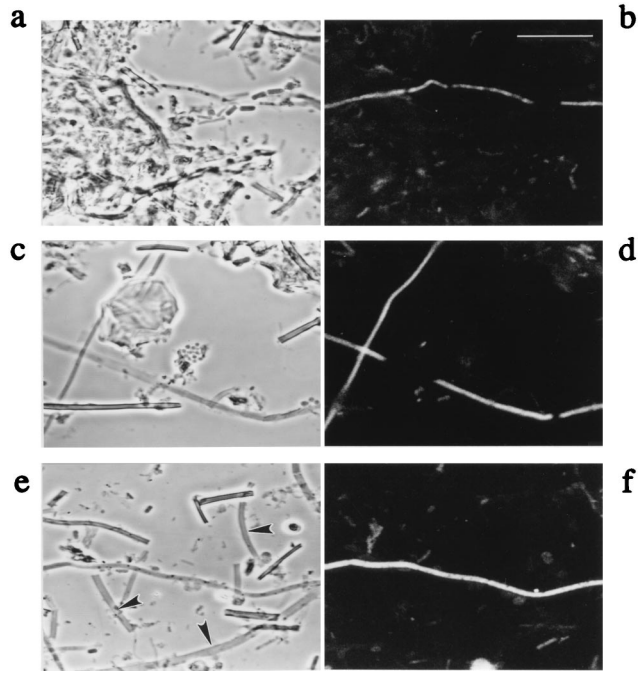

FIG. 6. FISHs with PSP-6 probe applied to water samples from Sapsucker Woods Wetland in Ithaca, N.Y. Hybridizations were done as described in the text, and washes consisted of one 10-min wash and two 5-min washes in 1× SET at 48 to 50°C. Unidentified filamentous sheathed bacteria with cells larger than typical "*L. discophora*" cells (a, c, and e) gave strong PSP-6 hybridization signals even after optimum stringency washing (b, d, and f). Note numerous empty sheaths (indicated by arrowheads in panel e). See the text for details. Bar = 15 μm.

sitions of mismatch in all *S. natans* strains (Table 2) and, therefore, would not be expected to remain hybridized in *S. natans* cells under the stringency conditions employed. We can conclude only that there are unknown filamentous sheathed bacteria in the wetland which have sequence identical to the PSP-6 target sequence but which are morphologically similar to *S. natans.*

Given that the probes used to label cells in the natural samples were designed from sequences of cultured isolates, it is not surprising that previously unidentified or noncultured microorganisms were labeled with the probes. In the most recent edition of *Bergey's Manual of Systematic Bacteriology* (49), some 5,000 species are described but only 25% of these species are present in the 16S rRNA databases (4). Since most of the sequences represent culture collection strains, they do not reflect the true microbial diversity in nature. Our results support this important conclusion.

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