In Situ Visualization of High Genetic Diversity in a Natural Microbial Community

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Simultaneous in situ visualization of seven distinct bacterial genotypes, all affiliated with the phylogenetically narrow group of beta-1 *Proteobacteria*, was achieved in activated sludge. This finding indicates that the high diversity found in the same sample by direct rRNA sequence retrieval was indeed present in this complex community. By the combination of comparative rRNA sequence analysis, in situ hybridization with fluorescently labeled, rRNA-targeted oligonucleotides and confocal laser scanning microscopy several microbial populations can be analyzed for abundance, relative spatial distribution and phylogeny directly at their site of action without prior cultivation.

It is today generally accepted that our knowledge of bacterial diversity has been severely limited by the need to obtain pure cultures prior to characterization (4, 26). Although fewer than 4,000 bacterial species are known, several million plant and animal species have been described (9). Bacterial diversity can today be more directly accessed by the cultivation-independent comparative rRNA analysis (4, 26). One common observation in the so-called rRNA approach (20) to analysis of complex environmental samples is that diversity is high and that the large majority of retrieved sequences do not match the approximately 6,000 prokaryotic 16S rRNA sequences stored in public databases (18, 22). There are two main categories of diversity, (i) sequences with similarities of approximately or below 80% to all other hitherto-determined sequences and (ii) clusters of closely related sequences with similarities between 93 and 99% (6, 7, 12, 15, 27).

The question has been raised whether this high "microdiversity" is indeed present in nature or just the result of inaccuracies in the acquisition of data. There are several factors that could cause artificial sequence diversity in rRNA gene libraries. It is well-known that the PCR (21), today applied in most studies, can cause artifacts. For example, there is no proofreading function for the thermostable DNA polymerase from *Thermus aquaticus*, resulting in an error frequency of about 1 in 1.7×10^4 (5). If this frequency is not increased in the amplification of ribosomal DNA (rDNA) from environmental DNA, only 1 of 10 clones containing almost full-length rDNA (approximately 1,600 bp) would have one error. A second potential source of inaccuracies might be more serious. Again, depending on the quality of the DNA used for PCR in vitro recombinations of two or more wild-type rRNA genes, so-called chimeric sequences can be formed at frequencies of several percent (16, 17). Furthermore, a third, natural source might contribute to an overestimation of biodiversity. Many prokaryotes have several rRNA operons and even though the rRNA coding regions are usually highly similar, they are not necessarily identical. Small differences (microheterogeneities) between them have been found; e.g., *Rhodobacter capsulatus* has three rRNA operons with similarities between 99.3% (*rrnB*

to *rrnA* and *rrnC*) and 99.8% (*rrnA* to *rrnC*) (13). Differences are about equally frequent in the intergenic spacers and in the structural genes. The archaeon *Haloarcula marismortui* reportedly has a significantly higher degree of heterogeneity of approximately 5% between the two 16S rRNA genes (19). The cloning step in rRNA analysis separates not only the rRNA genes of different organisms but also the different genes in one strain. Slightly different gene fragments could therefore originate from one strain and would not indicate the presence of closely related organisms. The impact of this potential source of microdiversity on the rRNA approach is currently difficult to judge because of limited sequence information. Heterogeneities between rRNA genes might still be an exception. The complete sequencing of the *Haemophilus influenzae* genome revealed that the coding regions of the six rRNA operons are completely identical and that differences are present only in the intergenic spacers (14).

The objective of this study was to examine in one complex environmental sample, a municipal activated sludge, from which such highly related sequences had been retrieved whether microdiversity was indeed present in the sample or whether it was an experimental artifact. We used fluorescently labeled, rRNA-targeted oligonucleotide probes to link the retrieved genotypes to distinct cell populations (1, 3, 11). Simultaneous in situ hybridization with three probes targeted to three sites with little evolutionary conservation in one of the retrieved rDNA clones proved that at least seven closely related populations with different types of rRNA were present in the examined sample.

MATERIALS AND METHODS

Amplification and cloning of 16S rRNA gene. Mixed liquor from activated sludge basin 1 of a large municipal wastewater treatment plant (München-I, Großlappen, 2,000,000 population equivalents) was used directly for PCR amplification of almost full-length bacterial 16S rRNA gene fragments. The nucleotide sequences of the primers were 5′-AGAGTTTGATYMTGGCTCAG-3′ (*Escherichia coli* 16S rDNA positions [8] 8 to 27) and 5′-CAKAAAGGAGGTG ATCC-3′ (*E. coli* 16S rDNA positions [8] 1529 to 1546). Amplification was performed with a Hybaid OmniGene temperature controller (MWG-Biotech, Ebersberg, Germany) as follows. A mixture of 1 μl of cell suspension, 50 pmol each of the appropriate primers, 200 μmol of each deoxyribonucleoside triphosphate, 10 μl of 10× PCR reaction buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 15 mM MgCl₂, 0.1% [wt/vol] gelatin), 10 μl of 25 mM MgCl₂, and 3 U of thermostable *Taq* polymerase (Promega, Madison, Wis.) was added to a 0.5-ml test tube. The total volume was adjusted to 100 μl with sterile water and overlaid with 70 μl of mineral oil (Sigma, Deisenhofen, Germany). The mixture was initially heated to 94°C for 3 min and then subjected to 30 cycles consisting of

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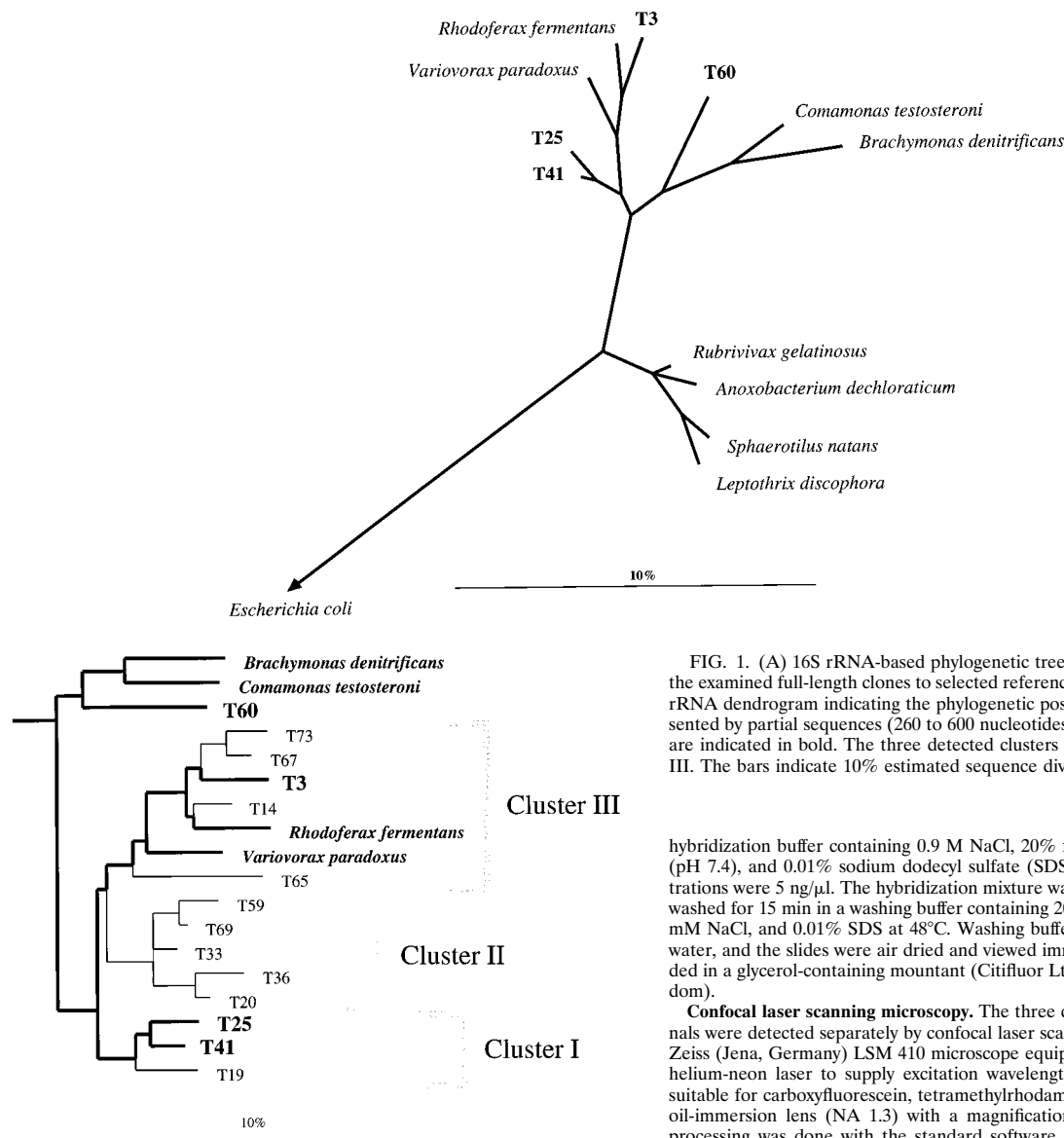
FIG. 1. (A) 16S rRNA-based phylogenetic tree showing the relationships of the examined full-length clones to selected reference sequences (18, 22). (B) 16S rRNA dendrogram indicating the phylogenetic position of the organisms represented by partial sequences (260 to 600 nucleotides). Strains with full sequences are indicated in bold. The three detected clusters are numbered with I, II, and III. The bars indicate 10% estimated sequence divergence.

94°C (1 min), 50°C (2 min), and 72°C (3 min). The retrieved amplificates were analyzed by electrophoresis in 1% (wt/vol) agarose gel (Bio-Rad, Munich, Germany) and stained with ethidium bromide. The amplificates were purified by using the Magic Prep kit (Serva, Heidelberg, Germany) and subsequently cloned into the pGEM-T vector (Promega).

rDNA sequencing. 16S rDNA clones were sequenced with a direct blotting electrophoresis system (GATC 1500; MWG-Biotech). A cycle sequencing protocol of the chain termination technique (10) was applied according to the manufacturer's instructions (Boehringer, Mannheim, Germany), with digoxigenin-labeled primers.

Tree construction. A 16S rRNA-based phylogenetic tree showing the relationships of the examined full-length clone sequences to selected reference sequences (18, 22) was reconstructed by the maximum likelihood method (18), including only alignment positions that are invariant in at least 50% of the sequences available for beta-subclass *Proteobacteria*. The partial sequence data were integrated in a dendrogram according to the maximum parsimony criterion (18); however, these data were not allowed to change the topology of the tree as established with full sequences.

Sampling and in situ hybridization. The activated sludge sample was fixed in paraformaldehyde and ethanol as previously described (24). Amino-linked oligonucleotide probes (MWG-Biotech) were labeled with the activated fluorescent dyes carboxyfluorescein (Boehringer), tetramethylrhodamine (Molecular Probes, Eugene, Oreg.), and Cy5 (Biological Detection Systems, Pittsburgh, Pa.) following standard protocols (3). In situ hybridization was performed at 46°C in a

hybridization buffer containing 0.9 M NaCl, 20% formamide, 20 mM Tris-HCl (pH 7.4), and 0.01% sodium dodecyl sulfate (SDS) for 90 min. Probe concentrations were 5 ng/μl. The hybridization mixture was removed, and the slide was washed for 15 min in a washing buffer containing 20 mM Tris-HCl (pH 7.4), 180 mM NaCl, and 0.01% SDS at 48°C. Washing buffer was removed with distilled water, and the slides were air dried and viewed immediately after being embedded in a glycerol-containing mountant (Citifluor Ltd., Canterbury, United Kingdom).

Confocal laser scanning microscopy. The three different probe-conferred signals were detected separately by confocal laser scanning microscopy (25) with a Zeiss (Jena, Germany) LSM 410 microscope equipped with an argon ion and a helium-neon laser to supply excitation wavelengths of 488, 543, and 633 nm suitable for carboxyfluorescein, tetramethylrhodamine, and Cy5, respectively. An oil-immersion lens (NA 1.3) with a magnification of ×100 was used. Image processing was done with the standard software package, Zeiss LSM version 3.54B, delivered with the instrument. Sequences of images were taken along the optical axis with 0.7-μm increments in the fluorescein (excitation wavelength, 488 nm; dichroic mirror, 510 nm; and band pass, 515-565 nm), tetramethylrhodamine (excitation 543 nm; dichroic mirror, 560 nm; long pass, 590), and Cy5 (excitation wavelength, 633 nm; neutral-teiler, 80/20; emission filter RG 665) channels. The signal-to-noise ratio was improved by averaging the results for 16 scans. From the available data, one optical plane was selected. The artificial colors green, red, and blue were assigned to the monochrome images acquired in the fluorescein, tetramethylrhodamine, and Cy5 channels, respectively. The three images were subsequently overlayed by using the shift correction mode of the standard software. The standard additive color system was used when cells were detected in two or all three channels. The data were converted to a postscript file with the software package Corel Draw! 5.0 (Corel Corp.) and transferred to a 100 ASA diapositive film (Ektachrome; Kodak, Rochester, N.Y.) with an Agfaforte slide exposure device (Agfa, Munich, Germany).

RESULTS AND DISCUSSION

Sequence analysis. When analyzing 16S rDNA fragments retrieved from activated sludge from the large municipal wastewater treatment plant München-I, we found in high frequency (29 of 66 examined clones) a group of closely related sequences with similarity values between 96.5 and 99.2% (accession numbers X95836 to X95839 and X97590 to X97599) (Fig. 1). Comparative analysis of four full-length sequences
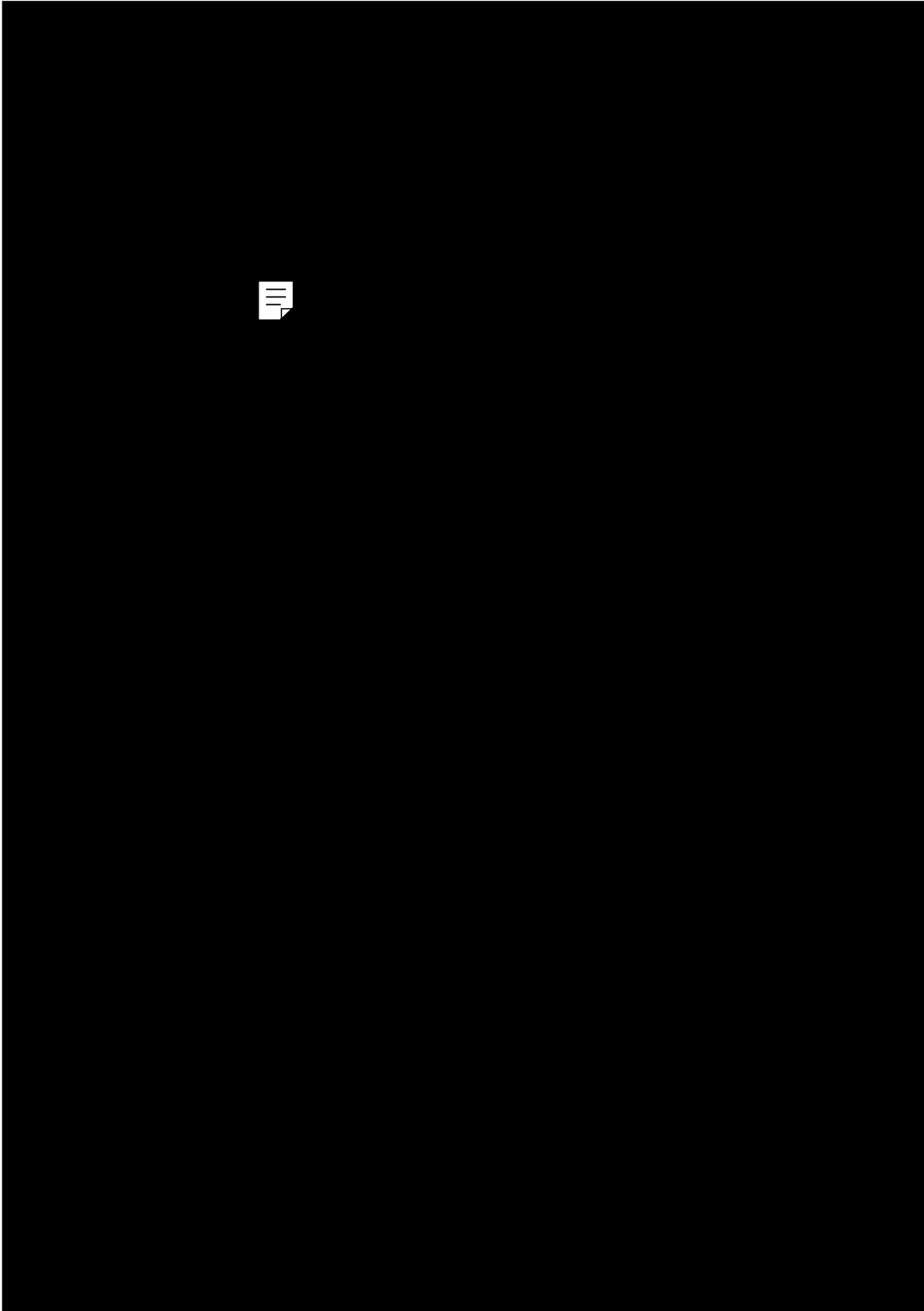
FIG. 2. (A) Additive color table for the combinatory application of three differently labeled probes. The dual combinations of the three colors blue, red, and green are yellow, as a product of red and green; purple, as product of red and blue; and turquoise, as a product of blue and green. The combination of all three colors yields white. (B) Confocal laser scanning image of an activated sludge sample after in situ hybridization with tetramethylrhodamine-labeled probe T3-86, fluorescein-labeled probe Sna8b, and Cy5-labeled probe Sna23a. The artificial colors red, green, and blue were assigned to the data recorded in the tetramethylrhodamine, fluorescein, and Cy5 channels, respectively. The additive color table shown in panel A was used to indicate the simultaneous binding of two or three probes to one cell.

(clones T3, T25, T41, and T60) revealed close affiliation with the sequences of *Proteobacteria* of the beta-1 group (28) (Fig. 1A). Members of the beta-1 *Proteobacteria*, e.g., the filamentous bacterium *Sphaerotilus natans* and bacteria formerly classified as pseudomonads (*Comamonas* spp. and *Acidovorax* spp.), are frequently isolated from activated sludge. When partial sequences of 10 more clones (260 to 600 nucleotides) were integrated into this tree, three sequence clusters were evident (Fig. 1B). None of the sequences was identical to an earlier published sequence. Both the presence of closely related sequences and the lack of identity with already-sequenced strains have frequently been reported for other rRNA gene libraries from complex environmental samples (6, 7, 12, 15, 27).

In situ hybridization. Characterization of the activated sludge sample by in situ hybridization with group-specific probes (24) revealed that approximately 30% of the cells were detected by a fluorescently labeled, rRNA-targeted oligonucleotide specific for beta-1 *Proteobacteria* (5′-GAATTCCATCCCC CTCT-3′; *E. coli* 16S rRNA positions [8] 663 to 679). The high abundance of this group is consistent with the high frequency of beta-1 proteobacterial sequences identified in the rRNA gene library.

Specific oligonucleotide probes were identified by comparison of the clone sequences and all published rRNA sequences (18, 22). In situ hybridization with one of these oligonucleotides, T3-86 (5′-GCCACACGCCACCAGGAT-3′; *E. coli* 16S rDNA positions [8] 86 to 103), complementary to clone T3, resulted in the detection of cells with a variety of different morphologies even under the most stringent hybridization conditions (results not shown). Under highly stringent conditions, oligonucleotides can discriminate closely related target sites. A single central destabilizing mismatch might effectively prevent probe binding. Therefore, the binding of probe T3-86 to different morphotypes could indicate the presence of additional unknown populations that contain the T3-86 target site (or at least a very similar site). The binding could, however, also be a consequence of pleomorphism of cells within one population. To unequivocally identify the cells of interest, we made use of the possibility of applying several differently labeled oligonucleotide probes simultaneously (2). Binding of a second and a third specific probe increases the reliability of probe-based identification significantly. After hybridization the binding of the different probes can be analyzed by specific visualization of the different markers by using different excitation wavelengths and specific filter sets. Here, tetramethylrhodamine-labeled probe T3-86 was combined with two other oligonucleotides, Sna23a (5′-CATCCCCCTCTACCGTAC-3′; *E. coli* 16S rDNA positions [8] 656 to 673) and Sna8b (5′-CCGCTCCAATCG CGCGAG-3′; *E. coli* 16S rDNA positions [8] 215 to 232), originally designed to be specific for *Sphaerotilus natans* (23) but also matching the sequence of clone T3. These two probes were labeled with the fluorochromes Cy5 and fluorescein, respectively. After simultaneous hybridization with all three oligonucleotides, the fluorescent signals were individually recorded in the fixed activated sludge sample by confocal laser scanning microscopy (25). For visualization they were assigned the colors green (fluorescein-labeled Sna8b), red (tetramethylrhodamine-labeled T3-86), and blue (Cy5-labeled Sna23a). Each cell binding more than one probe would be visualized

according to the additive color table shown in Fig. 2A, with white identifying those cells simultaneously detected by all three oligonucleotides. Few cells were visualized in white, and thereby with high probability assigned to the sequence type T3 (Fig. 2B). Much to our surprise, however, all seven possible probe-binding patterns and consequently all seven colors were realized, even in one 0.7-μm optical section of an individual activated sludge floc measuring less than 100 μm in diameter. This finding demonstrates that at least seven different rRNA sequence types, all closely related and characteristic of beta-1 group *Proteobacteria*, are present in a small area of the investigated sample. The specificity of the hybridization was confirmed by the turquoise (green and blue) color of cell filaments with the typical morphology of *Sphaerotilus natans*, as the previously determined sequence (18) of this organism is complementary to the fluorescein (green)- and Cy5 (blue)-labeled probes, Sna8b and Sna23a, respectively, but not to the tetramethylrhodamine-labeled probe T3-86. The visualization of red, purple (red and blue), and yellow (red and green) cells indicates the occurrence of at least three additional genotypes that have the probe T3-86 target region. rRNA sequences with the corresponding probe binding patterns are currently not present in the available public databases nor for the activated sludge clones analyzed so far. The 16S rRNA sequences of these populations have yet to be determined.

Conclusions. Our results clearly demonstrate that in the examined activated sludge, the high diversity within a relatively narrow phylogenetic group indicated by direct rRNA sequence retrieval is indeed present in the environment. We have achieved a high-resolution structural analysis of seven populations in a complex microbial community. The distribution of cells with different rRNA sequence types seems to be random in certain areas but highly organized, with small, homogeneous microcolonies in other regions. In situ hybridization with multiple probes not only facilitates the identification and phylogenetic placing of yet-uncultured organisms (4) but also represents the ideal method to assist in the directed isolation of these organisms. Further multidisciplinary studies will be necessary for an in situ characterization of the functions and interactions of the different populations.

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Sna23a-Cy5
Sna8b-FLUOS
T3-TRITC

Bar = 10μm