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# Probing Activated Sludge with Oligonucleotides Specific for Proteobacteria: Inadequacy of Culture-Dependent Methods for Describing Microbial Community Structure

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**Bacterial community structures in activated sludge samples from aeration tanks of a two-stage system with a high-load first stage and a low-load second stage were analyzed with oligonucleotide probes. The probes were complementary to conserved regions of the rRNA of the alpha, beta, and gamma subclasses of proteobacteria and of all bacteria. Group-specific cell counts were determined by in situ hybridization with fluorescent probe derivatives. Contributions of the proteobacterial subclasses to total bacterial rRNA were quantified by dot blot hybridization with digoxigenin-labeled oligonucleotides. The activated sludge samples were dominated by proteobacteria from the alpha, beta, or gamma subclass. These proteobacteria account for about 80% of all active bacteria found in the activated sludge. For both samples the community structures determined with molecular techniques were compared with the composition of the heterotrophic saprophyte flora isolated on nutrient-rich medium. Probes were used to rapidly classify the isolates and to directly monitor population shifts in nutrient-amended, activated sludge samples. The rich medium favored growth of gamma-subclass proteobacteria (e.g., enterobacteria) and selected against beta-subclass proteobacteria. The culture-dependent community structure analysis of activated sludge produced partial and heavily biased results. A more realistic view will be obtained by using in situ techniques.**

The treatment of wastewater by activated sludge systems is, in terms of metabolized matter, probably today's most important biotechnological process. A lot of effort has been funneled into process engineering, whereas our current knowledge of microbial community structure-function correlations and consequently a microbiological understanding of the activated sludge process are still very limited. Diversity and dynamics of the microbial consortia in activated sludge have mostly been analyzed by culture-dependent methods (18). However, there is a large discrepancy between the total direct microscopic counts and viable plate counts (usually less than 1% of the former) for many ecosystems. This effect was first observed in oligotrophic and mesotrophic aquatic habitats (12) and is less pronounced in eutrophic water (23). Still, recoveries from activated sludge even with optimized media are only between 5 and 15%. Often the microscopically most prominent microorganisms like the filamentous bacteria in foaming activated sludge cannot be found by standard cultivation procedures (19). This prompted efforts to gain more direct insights.

A key for the microscopic identification of filamentous bacteria based on morphological characteristics was developed by Eikelboom (7, 8). It enables identification of predominant filaments in bulking activated sludge, whereas filaments occurring in low numbers are easily missed (11). The detection and identification of less abundant and morphologically less characteristic bacteria require different techniques. The immunofluorescence approach (5) has successfully been used to identify bacteria in complex environments, e.g., low numbers of *Sphaerotilus natans* in thick flocs of activated sludge (11). However, studies with this approach in activated sludge have been scarce. This is probably due to the severe limitation of the immunofluorescence, i.e., the production of antibodies requires a pure culture of the organism of interest. Today, fluorescent rRNA-targeted oligonucleotide probes are an alternative to fluorescent antibodies in the identification of bacteria (3, 6). A combination of polymerase chain reaction-assisted direct retrieval of rRNA sequences and fluorescent in situ probing enabled the specific detection and identification of hitherto uncultured bacteria (1, 20). Unlike for immunoprobes, specificities of the chemically synthesized oligonucleotide probes can be freely adjusted to different phylogenetic levels ranging from the subspecies to the kingdom level (21). Fluorescent oligonucleotides have been used to analyze the spatial distribution of sulfate-reducing bacteria in multispecies biofilms (4). In this study, for the first time group-specific oligonucleotide probes were applied for in situ analysis of microbial community structure in activated sludge. Special attention was given to the demonstration of cultivation-dependent shifts in community composition.

## MATERIALS AND METHODS

**Sampling.** Grab samples of mixed liquor were collected from two successive activated sludge aeration basins (in the following referred to as B1 and B2) of a large municipal sewage plant (München II, Gut Marienhof, Germany). B1 samples were taken from the high-load first stage (food/microorganism [F/M] ratio, 1.8 kg/kg/day), and B2 samples were from the low-load second stage (F/M ratio, 0.1 kg/kg/day). For in situ hybridization, activated sludge samples were fixed with paraformaldehyde solution immediately after the samples were taken (2). They were stored in a 1:1 mixture of phosphate-buffered saline (PBS; 130 mM sodium chloride, 10 mM sodium phosphate buffer [pH 7.2]) and 96%

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ethanol at −20°C. Fixed samples were spotted on precleaned, gelatin-coated [0.1% gelatin, 0.01% $KCr(SO_4)_2$] microscopic slides (Paul Marienfeld KG, Bad Mergentheim, Germany), dried at 46°C for 15 min, and dehydrated in 50, 80, and 96% (vol/vol) ethanol (3 min each). For extraction of total nucleic acids and for plating, samples were transported on ice from the sewage plant to the laboratory.

**Plate counts and cultivation.** Serial dilutions within the range of $10^{-4}$ to $10^{-10}$ were plated in duplicate on solid Luria-Bertani (LB) medium (peptone, 10 g/liter; yeast extract, 2.5 g/liter; sodium chloride, 7 g/liter; glucose, 1 g/liter [pH 7.4]), and plates were scored after an incubation at 30°C for 5 days. Randomly chosen colonies were transferred into liquid LB medium. After 48 h of aerobic incubation at 30°C, cells were harvested by centrifugation (10 min at 18,000 × *g* at 4°C) for the extraction of total nucleic acids.

**Enrichment studies.** Activated sludge samples (5 ml) were mixed with LB medium (5 ml) or diluted LB medium (0.2×; 5 ml) and incubated under vigorous shaking at 21, 30, and 37°C. Samples for whole-cell hybridization were fixed after 0, 2, 4, and 16 h. An identical series was performed with full-strength and diluted LB media containing nalidixic acid (30 μg/ml) to determine viable cells (13).

**Membrane filtration and staining with DAPI.** Polycarbonate filters (pore size, 0.2 μm; Reichelt, Heidelberg, Germany) were counterstained with Irgalan Black (Ciba-Geigy, Wehr, Germany) for at least 2 h. Excess dye was removed by rinsing with 0.2-μm-pore-size filtered water for 5 min. Activated sludge was stained with DAPI (4′,6-diamidino-2-phenylindole; Sigma, Deisenhofen, Germany) (0.33 μg/ml) for 15 to 30 min at room temperature (17) and filtered onto counterstained polycarbonate filters. The filters were mounted in Citifluor (Citifluor Ltd., London, United Kingdom), and fluorescence was detected with a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) fitted for epifluorescence microscopy with a 50-W mercury high-pressure bulb and Zeiss filter set 01.

Dual staining of cells with DAPI and fluorescent oligonucleotides was modified from the method of Hicks et al. (10) so that cells were stained after in situ hybridization with DAPI (0.33 μg/ml) for 5 min.

**Oligonucleotide probes.** Oligonucleotide probes were synthesized with a C6-TFA aminolinker [6-(trifluoroacetylamino)-hexyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite] at the 5′ end (MWG Biotech, Ebersberg, Germany). The following probes were used: (i) EUB338, 5′-GCTGCCT CCCGTAGGAGT-3′ complementary to a region of the 16S rRNA specific for the domain bacteria (21); (ii) ALF1b, 5′-CGTTCG(C/T)TCTGAGCCAG-3′ complementary to a region of the 16S rRNA conserved in the alpha subclass of proteobacteria and some other bacteria (15); (iii) BET42a, 5′-GCCTTCCCACTTCGTTT-3′ complementary to a region of the 23S rRNA specific for the beta subclass of proteobacteria (15); (iv) GAM42a, 5′-GCCTTCCCACATCGTTT-3′ complementary to a region of the 23S rRNA conserved in the gamma subclass of proteobacteria (15).

Labeling with tetramethylrhodamine-5-isothiocyanate (Molecular Probes, Eugene, Oreg.) and 5(6)-carboxyfluorescein–*N*-hydroxysuccinimide ester (Boehringer Mannheim, Mannheim, Germany) and purification of the oligonucleotide-dye conjugates were performed as described by Amann et al. (3). Probes were also labeled with digoxigenin by the protocols of Zarda et al. (24).

**In situ hybridization and probe-specific cell counts.** Hybridizations of fixed activated sludge samples (immobilized on glass slides) were done as described by Manz et al. (15). Slides were examined with Zeiss filter sets 09 and 15. For each sample, more than 5,000 cells staining with probe EUB338 were enumerated. Color photomicrographs were made on Kodak Ektachrom P1600 color reversal film. Exposure times were 0.01 s for phase contrast and 6 to 15 s for epifluorescence micrographs.

**Total nucleic acid extraction and dot blot hybridization.** Cells from activated sludge samples or pure cultures were mechanically disrupted with glass beads (0.17-mm diameter) in combination with sonification. Nucleic acids were purified by phenol extraction and then by ethanol precipitation (modified after the method in reference 16). Nucleic acid concentrations were determined spectrophotometrically by measuring $A_{260}$. Immobilization of nucleic acids on nylon membranes and probing with digoxigenin-labeled oligonucleotides were done as described before (15). Hybridization signals were quantified by video densitometry (Biomed Instruments, Fullerton, Calif.). The mean values of triplicates were determined. The errors were below ±10%.

## RESULTS AND DISCUSSION

**In situ hybridization of activated sludge.** Applicability of fluorescent oligonucleotide probing to the activated sludge samples was tested with probe EUB, complementary to a conserved region of the 16S rRNA of all bacteria. In the first aeration basin, B1, 89.3% ± 7.2% of the microbial cells visualized by staining with DAPI also emitted probe-conferred fluorescence. This showed that the majority of fixed cells were bacteria and that they had sufficient rRNA for detection and were permeable for oligonucleotide probes without additional pretreatments (9). In sample B2, only 69.8% ± 6.6% of all cells were visualized with the bacterial probe. This could be caused by inactive cells staining with DAPI but lacking detectable amounts of rRNA because of environmental changes upon transfer from B1 into B2, where the F/M ratio is only 5.6% of that in B1. Hybridization with probes for the alpha (probe ALF), beta (probe BET), and gamma (probe GAM) subclasses of proteobacteria resulted in clear staining of individual cells. Simultaneous hybridization with two probes—one labeled with rhodamine, the other with fluorescein—was possible (Fig. 1A). The relatively small floc size in samples B1 and B2 after fixation allowed reliable cell counting without further cell dispersal (e.g., sonification). Proteobacteria accounted for about 60 to 75% of the microbial cells visualized by DAPI in the activated sludge samples. Probe ALF, BET, and GAM counts for the two activated sludge tanks B1 and B2 are given in Fig. 2 (lanes I) as fractions of the counts obtained with the bacterial probe. In sample B1, probe ALF hybridized to 10% of the cells hybridizing with probe EUB, probe BET hybridized with 42%, and GAM hybridized with 34%. In sample B2, ALF and BET counts (37% each) dominated over GAM counts (7%). The community structures in the two differently loaded aerated basins B1 and B2 were quite different. This is also reflected in the representative phase-contrast photomicrographs in Fig. 1. Aeration basin B1 contained mostly rods of different sizes hybridizing with the rhodamine-labeled BET probe and filamentous bacteria hybridizing with the fluorescein-labeled probe GAM (Fig. 1A). With the Eikelboom key (8), these filaments are classified as type 1863. In aeration basin B2, probe ALF hybridized to characteristic clusters of coccoid rods (Fig. 1B). It should be stressed that repeated probing of the high-load and low-load basins B1 and B2 always yielded similar distribution patterns

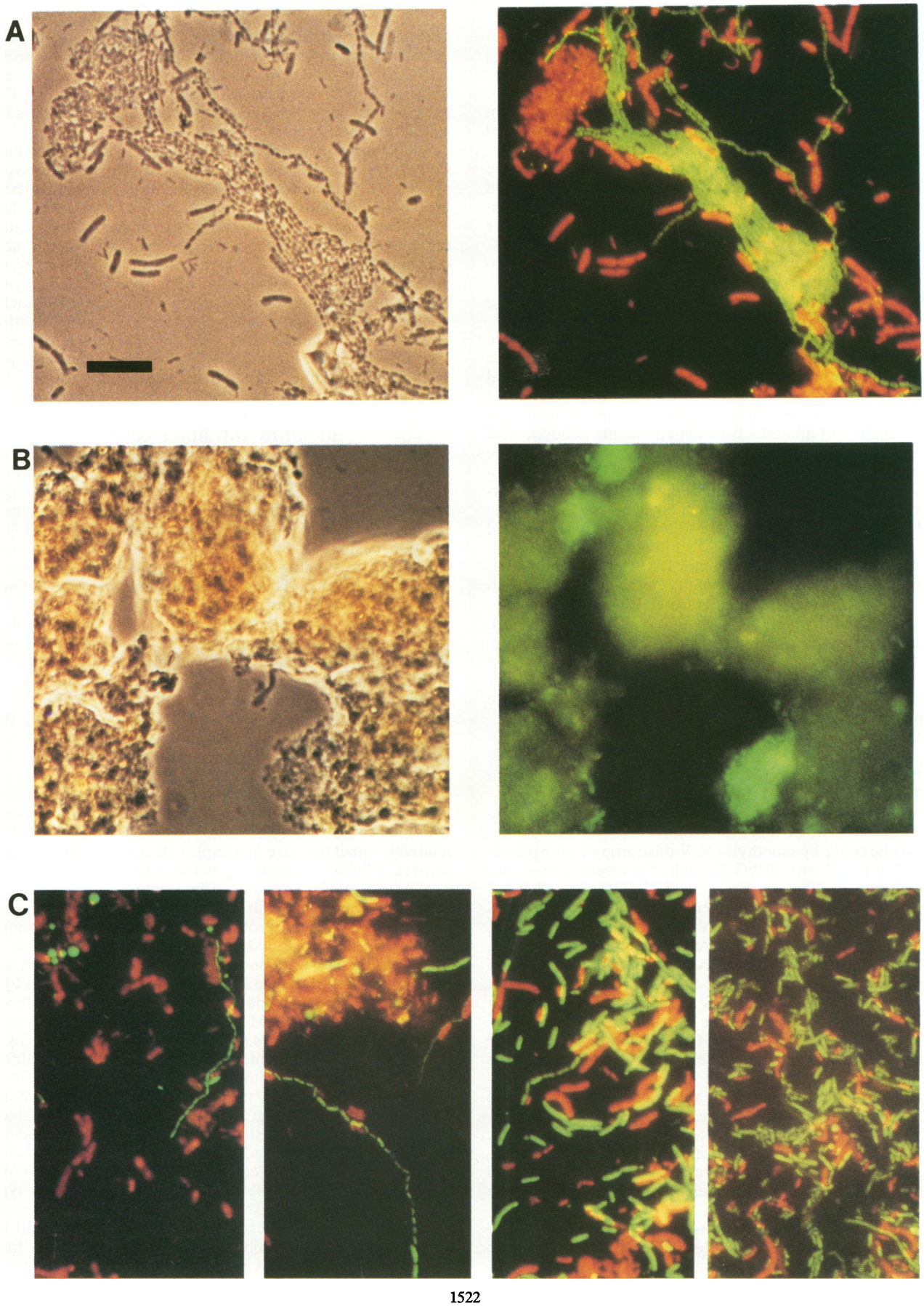
A
B
C

FIG. 1. In situ hybridization of activated sludge samples B1 (A) and B2 (B). Phase-contrast (left) and epifluorescence (right) micrographs are shown for identical microscopic fields. (A) Simultaneous hybridization with fluorescein-labeled probe GAM and tetramethylrhodamine-labeled probe BET. (B) Hybridization with fluorescein-labeled probe ALF. (C) In situ hybridization of a nutrient-amended activated sludge sample incubated aerobically at 30°C for 0, 2, 4, and 16 h (left to right). Epifluorescence micrographs visualize fluorescein (green) and rhodamine (red) molecules conferred by probes GAM and BET, respectively. Yellow regions are caused by overlapping of fluorescein- and rhodamine-stained cells. All photomicrographs were done at a magnification of ×1,000. Bar = 10 μm (panel A, left) and applies to all photomicrographs.

of proteobacterial subclasses. Beta- and gamma-subclass proteobacteria formed the characteristic flora in B1, whereas B2 was dominated by alpha subclass proteobacteria.

**Dot blot hybridization of total nucleic acids extracted from activated sludge.** Organism abundance can also be estimated from the fractional contribution of its specific rRNA molecules to the total ribosome population (22). Here, hybridizations with the three subclass-specific probes were quantified relative to probe EUB signals determining the contributions of the alpha, beta, and gamma subclasses of proteobacteria to the total bacterial rRNA. In sample B1, rRNA molecules hybridizing with probes BET (21%) and GAM (25%) were more frequent than rRNA molecules hybridizing with probe ALF (4%). Sample B2 contained significantly more rRNA hybridizing with BET (59%) than with ALF (17%) or GAM (9%). The relative distribution among the alpha, beta, and gamma subclasses of proteobacteria determined by dot blot (Fig. 2, lanes II) and in situ hybridization (Fig. 2, lanes I) were more similar for B1, but there was an obvious discrepancy for sample B2. Whereas in situ cell counts with probes ALF and BET were nearly identical, the dot blot hybridization signal obtained with probe BET was approximately 3.5 times the signal obtained with ALF. This could be due to better nucleic acid extraction efficiencies or a higher average cellular ribosome content of beta-subclass bacteria compared with those of alpha-subclass bacteria. The mechanical cell lysis method used in this study was originally chosen to rule out such extraction artifacts. In situ hybridization of B2 with the subclass-specific probes showed that the average beta-subclass cell was indeed larger than the alpha-subclass cells (dominated by small coccoid rods [Fig. 1B]) and consequently might have a higher cellular ribosome content. Therefore, relative cell numbers cannot reliably be extrapolated from the rRNA abundance values determined by dot blot hybridization. However, as already suggested by Stahl et al. (22), these values could be valid measures for the contribution of a defined group to the total metabolic activity.

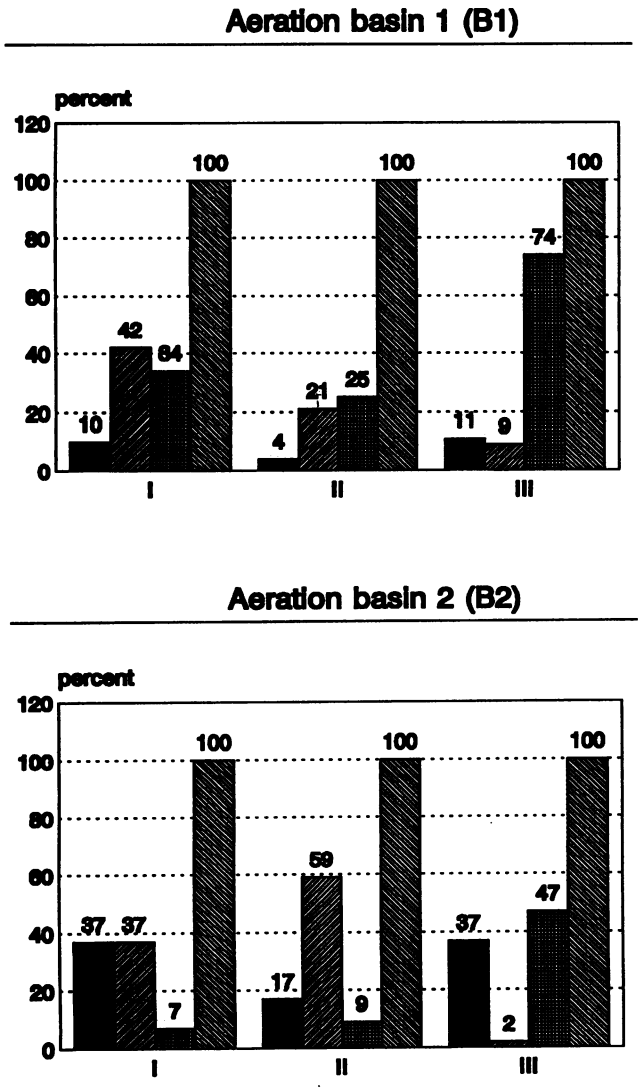


FIG. 2. Comparison of community structure analyses in activated sludge samples B1 and B2 as determined by in situ hybridization (lanes I), dot blot hybridization of extracted nucleic acids (lanes II), and classification of colonies after cultivation (lanes III). In all three panels the values obtained with the subclass-specific probes (ALF [■], BET [▨], and GAM [▦]) were standardized over the values obtained with the bacterial probe EUB (▧; 100%).

**Cultivation and classification of colonies.** The rich culture medium used in this study had been compared with several other media and yielded maximum plating efficiency (14a), e.g., for sample B1, 13.86% ± 1.26% of the total microscopic counts ($5.05 \times 10^8 \pm 0.454 \times 10^8$) formed colonies ($7.0 \times 10^7$ CFU/ml). Randomly chosen colonies (35 from B1 plates and 43 from B2 plates) were classified by dot blot hybridization of extracted nucleic acids, and the results were in all cases independently confirmed by in situ hybridization (Fig. 2, lanes III). A total of 26 (74%) of the B1 colonies hybridized with the GAM probe, only 4 (11%) hybridized with ALF, and 3 (9%) hybridized with BET. Nucleic acids extracted from B2 colonies mainly bound probes ALF (37%) and GAM (47%). These results obtained after cultivation are dramatically different from the direct in situ counts. The original community compositions in B1 and B2 are hardly reflected in the colonies present on the plates. The rich medium favored gamma-subclass bacteria present in the activated sludge samples, but it strongly selected against beta-subclass bacteria and to a lesser degree against alpha-subclass bacteria. Fatty acid analysis from different samples of B1 showed that the cultured bacteria cluster mainly with enterobacteria,

aeromonads, and *Acinetobacter* spp., all members of the gamma-subclass of Proteobacteria (14a).

**Fluorescent probing of enrichments.** The effects of cultivation in rich medium on the community structure of activated sludge could directly be visualized by fluorescence probing. Activated sludge samples were amended with LB medium and incubated aerobically at different temperatures with and without nalidixic acid. Subsamples taken after 0, 2, 4, and 16 h of incubation at 30°C and probed with rhodamine-labeled BET and fluorescein-labeled GAM are shown in Fig. 1C. Independent of the temperature, there was a clear shift over time from aggregated communities dominated by beta-subclass proteobacteria to cell suspensions dominated by the gamma subclass. Addition of nalidixic acid (13) resulted in the formation of elongated cells mostly hybridizing with probe GAM. Subsamples were also hybridized with a probe for enterobacteria (20a); whereas no enterobacteria could be detected in the original sample, their number increased over the incubation time to approximately 10% of the total counts (16 h).

**Future perspectives.** A thorough knowledge of the bacterial populations responsible for a functioning activated sludge process can only originate from the combination of different approaches. In this study the principal applicability of in situ hybridization with fluorescently labeled, rRNA-targeted oligonucleotides was demonstrated. Group-specific nucleic acid probes facilitated monitoring of the majority of cells. Changes in community composition have been followed not on the population (species) level but on a rougher scale. It should now be evaluated if defined functional changes in the activated sludge process are linked to community shifts on this level.

Autecological studies of specific bacterial species in activated sludge are nevertheless necessary (14). Population shifts could serve as early indicators for upcoming malfunctions (e.g., filamentous bacteria as indicators for sludge bulking) so that corrective measurements could be made in time. Keeping in mind the biases caused by cultivation, future studies should rely on in situ identification of individual cells with immuno- or nucleic acid probes. The possibility of producing specific oligonucleotide probes without prior cultivation of the organisms of interest (1, 20) makes many more species in activated sludge accessible for autecological studies. The comparative rRNA sequence analysis required for the construction of a specific probe will at the same time yield valuable information on the phylogenetic affiliation of these microorganisms. Knowledge of the closest culturable relatives could give hints for more directed enrichment and cultivation attempts. Pure cultures are a prerequisite for a detailed analysis of an organism's physiology and consequently its function. Thus, interdisciplinary studies promise new insights into the biology of the activated sludge process.

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