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Group-Specific Small-Subunit rRNA Hybridization Probes To Characterize Filamentous Foaming in Activated Sludge Systems

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Foaming in activated sludge systems is characterized by the formation of a thick, chocolate brown-colored scum that floats on the surface of aeration basins and secondary clarifiers. These viscous foams have been associated with the presence of filamentous mycolic acid-containing actinomycetes. To aid in evaluating the microbial representation in foam, we developed and characterized group-, genus-, and species-specific oligonucleotide probes targeting the small subunit rRNA of the *Mycobacterium* complex, *Gordona* spp., and *Gordona* (*Nocardia*) *amarae*, respectively. The use of a universal base analog, 5-nitroindole, in oligonucleotide probe design was evaluated by comparing the characteristics of two different versions of the *Mycobacterium* complex probe. The temperature of dissociation of each probe was determined. Probe specificity studies with a diverse collection of 67 target and nontarget rRNAs demonstrated the specificity of the probes to the target groups. Whole-cell hybridizations with fluorescein- and rhodamine-labeled probes were performed with pure cultures of various members of the *Mycobacterium* complex as well as with environmental samples from a full-scale activated sludge plant which experienced foaming. Quantitative membrane hybridizations with activated sludge and anaerobic digester foam showed that 15.0 to 18.3% of the total small-subunit rRNAs could be attributed to members of the *Mycobacterium* complex, of which a vast majority consisted of *Gordona* rRNA. Several *G. amarae* strains made up only a very small percentage of the *Gordona* strains present. We demonstrated that group-specific rRNA probes are useful tools for the in situ monitoring and identification of filamentous bacteria in activated sludge systems.

The success of the activated sludge process in treating industrial and domestic wastewater hinges crucially on the efficient separation of microbial biomass (i.e., activated sludge or mixed liquor) from the effluent stream. Filamentous foaming, which manifests itself as a "viscous, stable, chocolate-colored" scum layer (17, 41) on the surface of activated sludge aeration basins and secondary clarifiers, prevents this separation, thus reducing effluent quality. In addition, foaming may represent a public health concern because of the possible spread of pathogens in windblown scum (8). Foaming problems are widespread: recent surveys reported that 66% of 114 U.S. activated sludge plants had been affected by foaming (29), and 51% of 129 activated sludge plants in eastern Australia had a foam problem at the time of the survey (37). In addition, foaming problems have been reported for activated sludge plants in France, South Africa, Japan, Germany, Switzerland, and England (10, 17, 41).

Foaming has been associated with the presence of mycolic acid-containing actinomycetes belonging to the family *Nocardiaceae*, as well as other filamentous organisms. In particular, the actinomycete *Gordona amarae* (formerly *Nocardia amarae* [18, 34]) often has been implicated by microscopic and physiological analyses as the dominant species in foam (21). This has led to the term "*Nocardia* foam" or "nocardioform foam," despite the isolation from foam of several genera, including *Nocardia*, *Rhodococcus*, *Gordona*, *Tsukamurella*, and *Mycobacterium*, as well as other gram-positive bacteria such as *Microthrix parvicella* (9, 38–40).

The taxonomy of mycolic acid-containing actinomycetes has undergone tremendous changes in the light of modern phylogenetic methods (12, 36). At present, the actinomycetes that have cell walls of chemotype IV and contain mycolic acids are classified in the *Mycobacterium* complex, a suprageneric group that forms a distinct phyletic line (34) and contains the genera *Corynebacterium*, *Tsukamurella*, *Mycobacterium*, *Gordona*, *Rhodococcus*, and *Nocardia* (24, 34). The name "enlarged *Corynebacterium-Mycobacterium-Nocardia* (CMN)" has also been used for this group (31, 34).

Since many of these filamentous actinomycetes are morphologically similar, reliance on traditional identification and enumeration techniques has contributed to the difficulty in prescribing solutions to foaming problems. Discrepancies between results obtained with culture-dependent methods and total direct microscopic counts for aquatic environments have long been recognized (45). More recently, biases introduced by traditional methods have been pointed out in the context of relating operational problems in activated sludge systems to specific causative microbial populations (25, 41, 48). Identification and classification based on cultivation and morphology are especially challenging for actinomycetes because of their low growth rates, complex life cycles, and spore patterns (42).

Hernandez et al. (16) moved beyond morphological and culture-based methods for identifying filamentous actinomycetes by using fluorescently labeled antibody probes to identify *Gordona* (*Nocardia*) strains in activated sludge and anaerobic digesters. While this immunofluorescent technique is an improvement over conventional methods, the production of antibodies still requires cultivation of target microorganisms. In contrast, the use of rRNA-targeted oligonucleotide hybridization probes allows for the identification and enumeration of

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microorganisms in complex environments without prior cultivation (14). Another advantage of oligonucleotide probes is their ability to target organisms at different phylogenetic levels. Thus, by using such probes, it may become possible to resolve conflicting reports (40, 41) about the causative organisms of nocardioform foaming at the genus or species level. The identification and in situ detection of microbial populations in activated sludge with fluorescently labeled oligonucleotide probes have been reported previously for the subclasses of the proteobacteria (48), gram-negative filamentous bacteria (47), *Acinetobacter* species (49), ammonia-oxidizing bacteria (27, 50, 51), and nitrite-oxidizing bacteria (27, 51).

In this study, we report on the development and characterization of group-, genus-, and species-specific small subunit (SSU) rRNA-targeted oligonucleotide probes for the CMN group and demonstrate the usefulness of these probes in characterizing filamentous foaming in full-scale activated sludge systems.

MATERIALS AND METHODS

Organisms, culture conditions, and nucleic acid extraction. The organisms used in this study are listed in Table 1. Most organisms were obtained from the American Type Culture Collection (ATCC [Rockville, Md.]). Additional strains were obtained from various collections at the University of Illinois, Urbana, and the University of California, Berkeley. Proteobacteria were grown in liquid tryptone-yeast extract medium (10 g of Bacto-tryptone, 5 g of yeast extract, and 7 g of NaCl per liter [pH 7.0]). Gram-positive bacteria with a high DNA G+C content were grown in TGY medium (5 g of Bacto-tryptone, 1 g of glucose, 3 g of yeast extract per liter [pH 7.0]) or yeast-glucose medium (5 g of glucose and 3 g of yeast extract per liter [pH 7.0]). Other strains were grown as recommended by ATCC. The organisms were grown to mid-log phase, and nucleic acids were extracted from cell pellets by a phenol-chloroform-isoamyl alcohol extraction procedure (44). The concentrations of the nucleic acids were measured spectrophotometrically, assuming that 1 mg of RNA per ml is equal to 20 optical density units at A_{260}. The quality of extracted RNA was evaluated by polyacrylamide gel electrophoresis (1). For in situ hybridizations, cells were harvested in mid-log phase and fixed as described below.

Environmental samples. Grab samples were taken from the foam and mixed liquor in the aeration basins and from anaerobic digester foam at the Urbana-Champaign Sanitary District Northeast Treatment Plant (Urbana, Ill.). For membrane hybridizations, 50-ml samples were collected and immediately transported to the laboratory for processing and storage. Nucleic acids were extracted by a phenol-chloroform-isoamyl alcohol extraction procedure (44). For fluorescent in situ hybridizations (FISH), samples were immediately fixed in absolute ethanol (final concentration, 50% [vol/vol]) or in 4% paraformaldehyde (PFA) as described below.

Oligonucleotide probes. Oligonucleotide probes were designed by comparing the SSU rRNA sequences of 32 organisms of the *Mycobacterium* complex, which were obtained from the Ribosomal Database Project (RDP) (24). The oligonucleotide probes used, their target groups, and the SSU rRNA target sites are listed in Table 2. In addition, a universal probe, S-*-Univ-1390-a-A-18 (53), complementary to the SSU rRNA of most known organisms, and probe S-D-Bact-0338-a-A-18 (4), complementary to the SSU rRNA of bacteria, were used. The probes were synthesized on a DNA synthesizer (Applied Biosystems, Foster City, Calif.) at the University of Illinois Biotechnology Center's Genetic Engineering Facility. A universal base analog, 5-nitroindole (N_5; Glen Research, Sterling, Va.) (22), was incorporated during the synthesis of one of the probes (S-*-Myb-0736-b-A-22). Oligonucleotides used for FISH were synthesized with an aminolinker at the 5′ end (Aminolink 2; Applied Biosystems) and were coupled to tetramethylrhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) (Molecular Probes, Eugene, Oreg.) as previously described (5, 30). A reaction mixture was set up with the following ingredients: 350 μl of oligonucleotide (approximately 200 μg of DNA), 120 μl of sodium carbonate buffer at pH 9.2, 75 μl of fluorochrome dissolved in dimethylformamide (10 mg/ml), and 55 μl of double-distilled H_2O. The reaction mixture was incubated in the dark at room temperature for 12 to 16 h. Free fluorochrome was separated from the oligonucleotide by running the mixture through a Sephadex G-25 DNA-grade NAP10 column (Pharmacia Biotech, Piscataway, N.J.). Unlabeled oligonucleotide was separated from labeled oligonucleotide with an oligonucleotide purification cartridge (OPC; Applied Biosystems) according to the manufacturer's protocol. Purified probes were stored in the dark at −20°C. Oligonucleotides used for membrane hybridizations were purified with the OPC and 5′ end labeled with ^{32}P with T4 polynucleotide kinase (Promega Corporation, Madison, Wis.) and [γ-^{32}P]ATP (ICN Radiochemicals, Irvine, Calif.) (32).

Cell fixation, FISH, and microscopy. To determine the best fixation method for the mycolic acid-containing actinomycetes, the following fixation treatments were evaluated: 4% PFA for 1 min, 4% PFA for 2 h, 50% absolute ethanol for 2 h, and 50% absolute ethanol for 12 to 16 h. Three volumes of fresh (prepared within 24 h of use) PFA fixative (33) or 50% absolute ethanol was applied to cells. After fixation, samples were washed with phosphate-buffered saline (PBS) buffer (130 mM NaCl, 10 mM sodium phosphate [pH 7.2]) and resuspended in 1:1 (vol/vol) PBS-ethanol. Fixed cells were stored at −20°C. Teflon-coated slides (Cel-Line Associates, Newfield, N.J.) were cleaned in ethanolic KOH (10% KOH in ethanol) and coated with gelatin [0.1% gelatin, 0.01% $CrK(SO_4)_2 \cdot 12H_2O$]. Fixed cells were applied to the wells of the slide. The slides were air-dried and dehydrated by serial immersion in 50, 80, and 96% ethanol (3 min each). Subsequently, 9 μl of hybridization solution was mixed with 1 μl (25 ng) of FITC- or TRITC-labeled probe and applied to each well. The slides were incubated at 37°C in a moisture chamber (5) for at least 2 h. After hybridization, the slides were rinsed once with prewarmed (37°C) wash solution and then washed for 20 min at 37°C. The hybridization and wash solution consisted of 0.1% sodium dodecyl sulfate (SDS), 20 mM Tris-HCl, and *X* M NaCl (where *X* varied depending on the probe, as described below). The slides were dipped in a cold solution of 4′,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, Mo.) and then rinsed with cold wash solution corresponding to 0% formamide (FA) (0.9 M NaCl in wash solution). The DAPI solution consisted of 0.1 M Tris-HCl (pH 7.2), 0.9 M NaCl, and DAPI (final concentration of 6.26 μg/ml). The slides were mounted in Citifluor (Citifluor Ltd., London, United Kingdom) and visualized with an epifluorescence microscope (Axioskop; Carl Zeiss, Germany) fitted with filter sets (Chroma Tech. Corp., Brattleboro, Vt.) for TRITC (filter set 41002), FITC (filter set 41001), and DAPI (filter set 31000) and a Zeiss 50-W high-pressure bulb. Images were captured with a liquid-cooled charge coupled device (CCD) camera (Photometrics MXC200L, class 2 Kodak KAF 1400 CCD, 1,317-by-1,035 pixel array, 6.8-μm pixel size; Photometrics Ltd., Tucson, Az.) controlled by a PowerPC 7100 (Apple Computer, Inc., Cupertino, Calif.). Exposure times were varied from 0.05 to 0.2 s for epifluorescence and phase-contrast images. To provide a comparative measure of fluorescence, a constant exposure time of 0.1 s was used for image capture to compare fixation treatments. Images were imported to Adobe Photoshop 3.0 (Adobe, Seattle, Wash.) for printing.

Probe-conferred fluorescence was quantified with IPLab Spectrum image analysis software (Signal Analytics, Vienna, Va.), with semiautomatic image thresholding. Background signals were similarly quantified and subtracted from the mean pixel value for each image. For quantification, at least 100 cells for each hybridization and wash condition were used. The optimal hybridization stringency for the different probes was determined by quantification of probe-conferred fluorescence to target and nontarget reference cells with different NaCl concentrations in the hybridization buffer and wash solution (50).

Determination of optimal wash temperatures. Organisms with zero, one, or two mismatches in the probe target region of their SSU rRNA, as determined from the RDP database, were used in temperature of dissociation (T_d) studies. Nucleic acid samples (30 ng) were slot blotted in triplicate to nylon membranes (Magna Charge; Micron Separation, Inc., Westboro, Mass.). Baked membranes were prehybridized for 2 h at 40°C and hybridized overnight at 40°C (32). Subsequently, the membranes were washed in 100 ml of 1% SDS–1× SSC (0.15 M NaCl, 0.015 M sodium citrate) twice for 1 h at 40°C. The membranes were then cut into separate individual hybridized blots. Each blot was washed in 3 ml of 1% SDS–1× SSC for 10 min at the first temperature (30°C). The blot was then removed and transferred to a new scintillation vial with a wash solution at the next higher temperature, and washes were continued at increasing temperatures until a temperature of 80°C was reached (total of 16 temperatures). The amount of probe released at each wash temperature was quantified by liquid scintillation counting with a model 1600CA liquid scintillation analyzer (Packard Instruments, Downers Grove, Ill.).

Probe specificity studies. The specificity of the oligonucleotide probes was examined by membrane hybridizations (32) with nucleic acids from 67 organisms, representing a broad spectrum of phylogenetic diversity (Table 1). The previously determined T_d for each probe was used as the final wash temperature for the membranes. Hybridization signals were quantified by storage phosphor analysis with a 400 series PhosphorImager and ImageQuant software package (Molecular Dynamics, Sunnyvale, Calif.).

Quantitative membrane hybridization. The oligonucleotide probes were used to quantify the abundances of the microbial groups (group, genus, and species) in activated sludge and anaerobic digester systems. Denatured RNA samples and dilution series of pure culture *G. amarae* SE102 RNA were applied in triplicate to Magna Charge nylon membranes (33). These membranes were hybridized with universal and specific probes, and the resulting hybridization responses were used to determine the relative concentration of target SSU rRNA in the samples (33, 53).

RESULTS AND DISCUSSION

Probe design. The design of the hybridization probes was based on the phylogeny of the mycolic acid-containing actinomycetes inferred by SSU rRNA sequence analysis from RDP. The mycolic acid-containing actinomycetes under the *Mycobacterium* complex are divided into members of the families

TABLE 1. List of organisms used in the specificity study

Position[a]	Organism
A1	*Rattus norvegicus*
A2	*Dictyostelium discoideum*
A3	*Methanobrevibacter* sp.
A4	*Methanogenium cariaci*
A5	*Methanosarcina acetivorans*
A6	*Chloroflexus aurantiacus* ATCC 29366
A7	*Cytophaga lytica*
A8	*Weeksella virosa* ATCC 43766
A9	*Flavobacterium ferrugineum*
A10	*Bacteroides thetaiotaomicron*
A11	*Azospirillum brasilense* ATCC 29145
A12	*Caulobacter* sp. strain MCS10
A13	*Rhizobium meliloti*
A14	*Chromobacterium violaceum* ATCC 12472
A15	*Alcaligenes eutrophus*
A16	*Alcaligenes faecalis* ATCC 8750
A17	*Oxalobacter formigenes*
A18	*Nitrosomonas* sp.
A19	*Chromatium vinosum*
A20	*Legionella pneumophila*
A21	*Oceanospirillum linum*
A22	*Pseudomonas aeruginosa*
B1	*Vibrio harveyi*
B2	*Aeromonas hydrophila*
B3	*Escherichia coli* K-12
B4	*Proteus vulgaris*
B5	*Desulfovibrio desulfuricans*
B6	*Megasphaera elsdenii*
B7	*Sporohalobacter lortetii*
B8	*Mycoplasma neurolyticum*
B9	*Lactobacillus casei*
B10	*Listeria monocytogenes*
B11	*Staphylococcus aureus*
B12	*Bacillus subtilis*
B13	*Brevibacterium linens* ATCC 9172
B14	*Micrococcus luteus* ATCC 4698
B15	*Micrococcus roseus* ATCC 534
B16	*Amycolata autotrophica* ATCC 19727
B17	*Saccharopolyspora rectivirgula* ATCC 33515
B18	*Mycobacterium komossense* ATCC 33013
B19	*Mycobacterium fortuitum* ATCC 6841
B20	*Mycobacterium smegmatis* ATCC 19420
B21	*Mycobacterium vaccae* ATCC 15483
B22	*Tsukamurella paurometabolum* ATCC 8368
C1	*Corynebacterium xerosis* ATCC 373
C2	*Corynebacterium renale* ATCC 19412
C3	*Nocardia asteroides*
C4	*Nocardia otitidiscaviarum* ATCC 14629
C5	*Nocardia camea* ATCC 6847
C6	*Nocardia paratuberculosis* ATCC 23826
C7	*Nocardia transvalensis* ATCC 6865
C8	*Nocardia nova* ATCC 33726
C9	*Nocardia brasiliensis*
C10	*Dietzia maris* ATCC 35013
C11	*Rhodococcus rhodochrous* ATCC 13808
C12	*Rhodococcus equi*
C13	*Rhodococcus rhodnii* ATCC 35071
C14	*Rhodococcus coprophilus* ATCC 29080
C15	*Gordona terrae* ATCC 25594
C16	*Gordona rubropertinctus* ATCC 14352
C17	*Gordona aichiensis* ATCC 66611
C18	*Gordona sputi* ATCC 29627
C19	*Gordona amarae* SE149B
C20	*Gordona amarae* NM23
C21	*Gordona amarae* SE102
C22	*Gordona amarae* SE6 ATCC 27808
C23	*Gordona amarae* RBI

[a] Location on the hybridization membrane (Fig. 3).

Nocardiaceae (genera *Gordona*, *Nocardia*, *Rhodococcus*, *Tsukamurella*, and *Corynebacterium* [15]) and *Mycobacteriaceae* (genus *Mycobacterium*). A phylogenetic tree with representative organisms from each genus is shown in Fig. 1. Since many studies have implicated *G. amarae* as the dominant foam-causing organism (17, 21, 41), we developed a probe targeting a *G. amarae*-specific region of the SSU rRNA. In addition, we designed a genus-specific probe for *Gordona* and two *Mycobacterium* complex-specific probes. The groups of actinomycetes targeted by these probes are shown in Fig. 1, and probe sequences are given in Fig. 2. Probe design also involved a check of SSU rRNA sequences for nontarget group complementarity, with the CHECK_PROBE program provided by RDP (24) and the Basic Local Alignment Search Tool (BLAST) network service (3).

The *Mycobacterium* complex probe (S-*-Myb-0736-a-A-22), targeting the SSU rRNA of *Gordona*, *Rhodococcus*, *Nocardia*, *Tsukamurella*, *Corynebacterium*, and *Mycobacterium*, perfectly matches all *Gordona* spp. This probe has one A:C mismatch with the target sequences of 9 *Rhodococcus* spp., 3 *Corynebacterium* spp., and 78 *Mycobacterium* spp. and one A:A mismatch with the target sequences of 3 *Nocardia* spp. at position 743 (*Escherichia coli* numbering). Target species with two mismatches in their target sequences include 1 *Rhodococcus* sp., 1 *Corynebacterium* sp., 2 *Tsukamurella* spp., and 11 *Mycobacterium* spp. For all of these organisms, one of the two mismatches occurs in position 743. All nontarget species (nonmembers of the *Mycobacterium* complex) have at least two mismatches with the probe. We designed a new probe (S-*-Myb-0736-b-A-22) by incorporating a new universal base analog, N_5, at position 743 in order to reduce the effect of this mismatch. Loakes and Brown (22) showed that N_5 was superior to other base analogs in providing higher duplex stability and behaving indiscriminately towards four of the five natural bases. This new probe was characterized along with the original probe to evaluate the applicability of N_5 for improving probe design. Thus, probe S-*-Myb-0736-b-A-22 perfectly matches most organisms of the *Mycobacterium* complex and has only one mismatch with a few species in this group.

The *Gordona*-specific probe S-G-Gor-0596-a-A-22 perfectly matches most *Gordona* spp. (including *G. amarae*), but it has one G:G mismatch with *G. terrae* (ATCC 25594) at position 613 (*E. coli* numbering) and a possible mismatch at position 605 (base not reported for this position) with *G. bronchialis*. Nontarget organisms have at least two mismatches in their target sequence with this probe.

Probe S-S-G.am-0192-a-A-18 was designed to be species specific for *G. amarae*. At the time of probe design, only one strain of *G. amarae*, SE6, was available in the database. The probe has no mismatches with *G. amarae* SE6, and nontarget organisms have at least two mismatches (e.g., *Tsukamurella paurometabolum*).

Optimization of wash temperatures. The use of oligonucleotide probes for accurate identification and quantification of target organisms relies on the specific duplex formation between probe and target rRNA. Especially in complex environments such as activated sludge, this specificity must be ensured not only by comparison with existing rRNA sequence databases, but also by optimization of experimental hybridization conditions. An important parameter in this regard is the T_d, defined as the temperature at which 50% of the duplex remains intact during a specified washing period (46). Use of the experimentally determined T_d for perfect duplexes (perfect match between probe and target) as the posthybridization wash temperature generally ensures that duplexes with one or more mismatches are dissociated (51). Table 2 provides a sum-

TABLE 2. Probe names, target groups, target sites, optimized experimental hybridization conditions, and signal intensities of nontarget signals

Probe[c]	Target group	Target site (SSU rRNA positions [*E. coli* numbering])	Exptl T_d (°C)	% Formamide in FISH	Avg nontarget signal intensity (% of target signal intensity)[a]	Range (% of target signal intensity)[b]
S-*-Myb-0736-a-A-22	*Mycobacterium* complex	0736–0757	56	30	0.15	0.08–0.56
S-*-Myb-0736-b-A-22	*Mycobacterium* complex	0736–0757	51	30	0.44	0.17–0.94
S-G-Gor-0596-a-A-22	*Gordona*	0596–0617	54	20	0.56	0.12–5.60
S-S-G.am-0192-a-A-18	*G. amarae*	0192–0209	66	30	0.83	0.29–5.34

[a] Average nontarget signal intensity, % = 100 × (average signal intensity for nontarget)/(average signal intensity for target).
[b] Lower range, % = 100 × (lowest signal intensity for nontarget)/(average signal intensity for target); upper range, % = 100 × (highest signal intensity for nontarget)/(average signal intensity for target).
[c] Probe nomenclature has been standardized according to the oligonucleotide probe database (2).

mary of the experimentally determined T_d values for the four probes characterized in this study.

Probe S-*-Myb-0736-a-A-22 has a T_d value of 55 to 57°C for three *G. amarae* strains, SE149B, SE102, and SE6 (Fig. 2a). *G. amarae* NM23 has a lower T_d of 51.5°C. This lower T_d suggests a possible mismatch in the target region. We currently are investigating this possibility by SSU rDNA sequencing of the different strains of *G. amarae*. As expected, *T. paurometabolum*, which has two mismatches with this probe, has a lower T_d of 48°C. The relatively large spread in T_d values decreased for the N_5-substituted probe, S-*-Myb-0736-b-A-22 (Fig. 2b). The three strains of *G. amarae* have T_d values of 50.5 to 51.5°C. The T_d for *G. amarae* NM23 is still slightly lower at 49°C, while *T. paurometabolum* has the lowest T_d at 48°C. Thus, substitution with N_5 improved the probe by decreasing the spread in T_d values from 9°C (57 to 48°C) to 3.5°C (51.5 to 48°C). Furthermore, substitution with N_5 decreased the T_d values for the organisms with no mismatches in the target region. These results are consistent with previous studies (22) and ongoing studies in our laboratory which have demonstrated the effectiveness of N_5 as a "universal" nucleotide in probe and primer design (28).

Probe S-G-Gor-0596-a-A-22 showed excellent potential as a genus-specific probe, with a very small T_d spread of 0.4°C (54.2 to 53.8°C). *G. terrae* (ATCC 25594), which has one mismatch with this probe at position 613 (based on RDP), has T_d values similar to those of the other *Gordona* species. The species-specific probe S-S-G.am-0192-a-A-18 exhibits two distinct T_d ranges. Two *G. amarae* strains, SE6 and SE102, have high T_d values (65 to 67°C), while two other strains, SE149B and

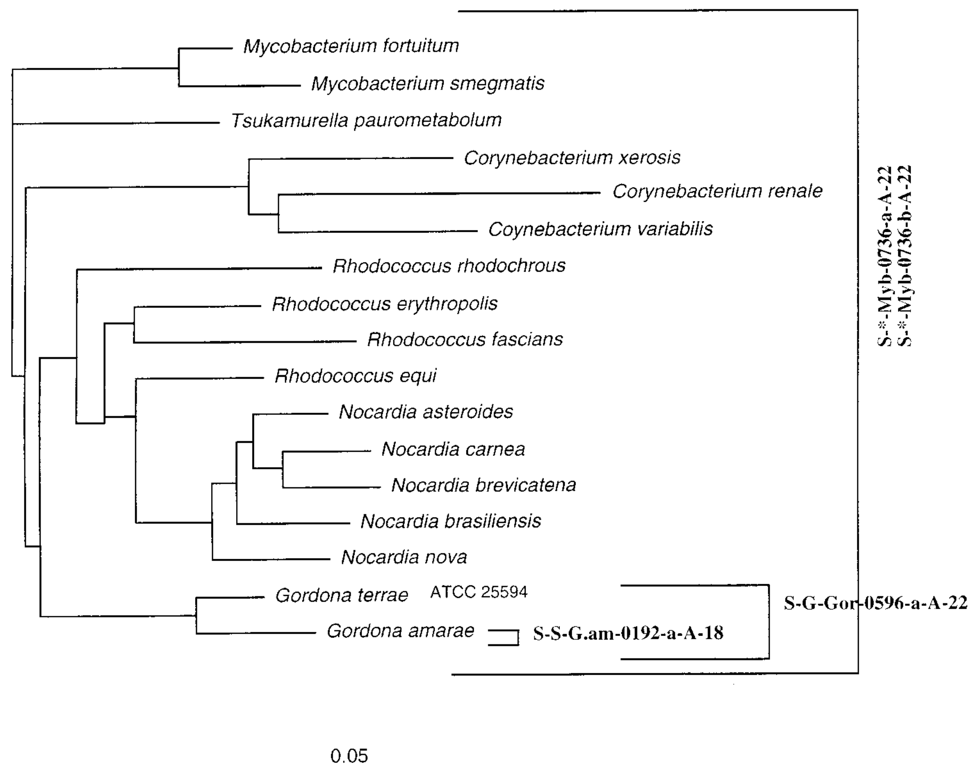
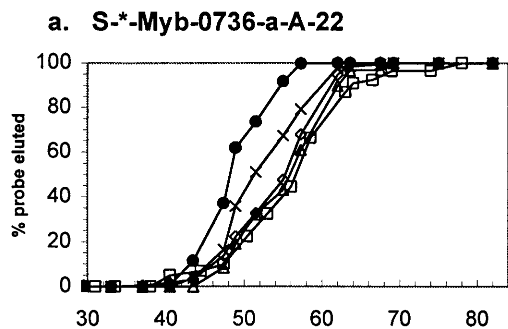
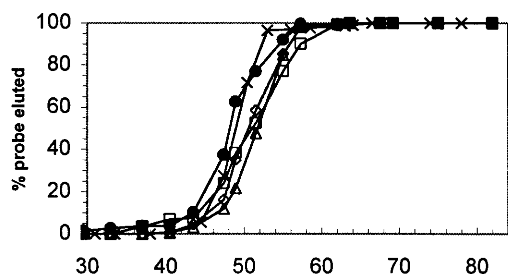
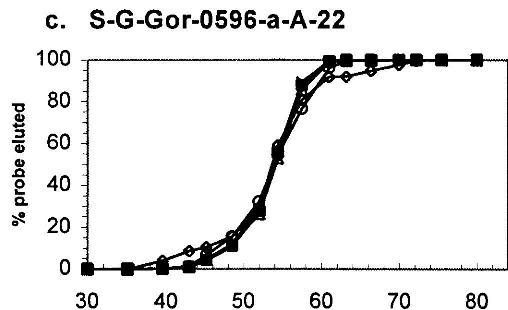
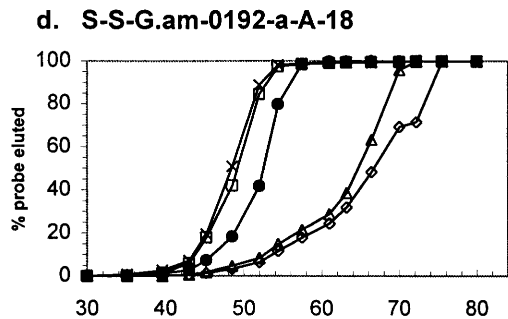


FIG. 1. Phylogenetic tree showing representative mycolic acid-containing actinomycetes, inferred from comparison of the SSU rRNA sequences. The tree was constructed by the neighbor-joining method (35). The bar at the bottom represents 5 estimated changes per 100 nucleotides. The oligonucleotide probes are shown with their respective target groups.









Temperature,°C

Organism	Probe/Target
S-*-Myb-0736-a-A-22	3′ GAGACCCATCATTGACTGCGAC 5′
S-*-Myb-0736-b-A-22	3′ GAGACCC**x**TCATTGACTGCGAC 5′
x=5-Nitroindole	
◇ *Gordona amarae* SE6	5′ CUCUGGGUAGUAACUGACGCUG 3′
○ *G. terrae* ATCC 25594	5′ .... 3′
G. sputi	5′ .... 3′
Rhodococcus sp.RHA1	5′ ....C.............. 3′
R. equi	5′ ....C.............. 3′
Nocardia otitidis.	5′ ....A.............. 3′
N. asteroides	5′ ....A.............. 3′
N. brasiliensis	5′ ....A.............. 3′
Corynebacterium xerosis	5′ ....C.............. 3′
Mycobacterium vaccae	5′ ....C.............. 3′
● *Tsukamurella paurometabolum*	5′ .......C........N..... 3′

Organism	Probe/Target
S-G-Gor-0596-a-A-22	3′ CGCAGCAGACACTTTAAGACGT 5′
◇ *Gordona amarae* SE6	5′ GCGUCGUCUGUGAAAUUCUGCA 3′
G. rubropertinctus	5′ .... 3′
G. sputi	5′ .... 3′
G. aichiensis	5′ .... 3′
G. terrae 2	5′ .... 3′
○ *G. terrae* ATCC 25594	5′ ....G.... 3′
G. bronchialis	5′ ....N............ 3′
Rhodococcus rhodochrous	5′ ................C.C... 3′

Organism	Probe/Target
S-S-G.am-0192-a-A-18	3′ GGACGTACCCCCACCCAC 5′
◇ *Gordona amarae* SE6	5′ CCUGCAUGGGGGUGGGUG 3′
● *Tsukamurella paurometabolum*	5′ ....N........U.... 3′

- ◇ G. amarae SE6
- △ G. amarae SE102
- □ G. amarae SE149B
- × G. amarae NM23
- ○ G. terrae ATCC 25594
- ● T. paurometabolum

FIG. 2. T_d studies for probes S-*-Myb-0736-a-A-22, S-*-Myb-0736-b-A-22, S-G-Gor-0596-a-A-22, and S-S-G.am-0192-a-A-18. Adjacent to the probe dissociation results are SSU rRNA sequences of target and nontarget species and probe sequences. The top SSU rRNA sequences for each list of organisms are the target sequences. Periods in the succeeding sequences signify identical nucleotides, and replacement nucleotides indicate differences in the target sequences at the respective positions. *Nocardia otitidis.*, *N. otitidiscaviarum.*

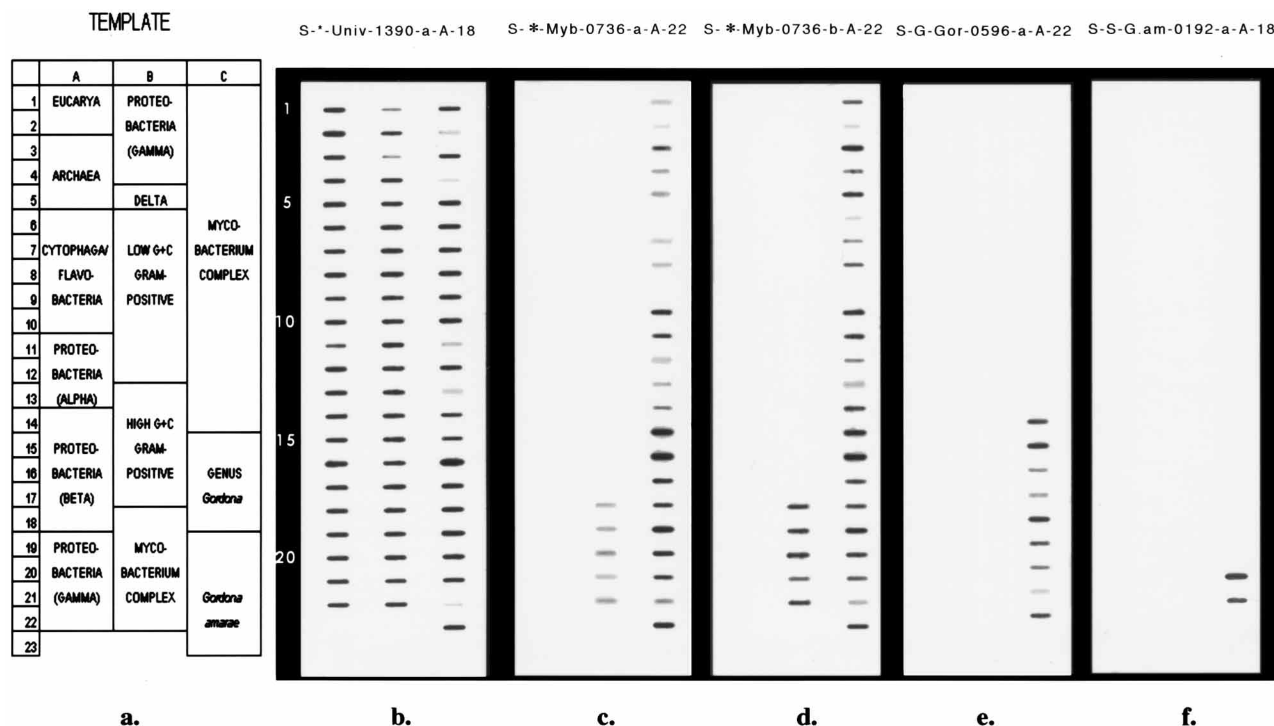


FIG. 3. Probe specificity study. Membrane hybridization results were analyzed with a PhosphorImager and were scanned and printed with Adobe Photoshop 3.0 (Adobe, Seattle, Wash.). (a) Template (names of individual organisms are given in Table 1); (b) probe S-*-Univ-1390-a-A-18; (c) probe S-*-Myb-0736-a-A-22; (d) probe S-*-Myb-0736-b-A-22; (e) probe S-G-Gor-0596-a-A-22; (f) probe S-S-G.am-0192-a-A-18.

NM23, as well as *T. paurometabolum*, have T_d values ranging from 48 to 52.5°C. These results suggest that *G. amarae* SE149B and NM23 have one or more mismatches with probe S-S-G.am-0192-a-A-18. We currently are evaluating this by sequencing the SSU rDNA of the various *G. amarae* strains.

Specificity studies. Table 1 lists the organisms that were used in the specificity study in the order that they were applied to the hybridization membranes, and Fig. 3a shows a schematic representation of the same membrane template with phylogenetic group names. Figure 3b presents the hybridization response with probe S-*-Univ-1390-a-A-18. Since this probe targets almost all known organisms, all 67 RNAs resulted in a positive hybridization response. The lower response for some positions can be attributed to smaller amounts of RNA applied to the membranes. Hybridization with probe S-*-Myb-0736-a-A-22 resulted in a positive signal for all members of the *Mycobacterium* complex, except for *Nocardia paratuberculosis* (C6) and *Nocardia brasiliensis* (C9) (Fig. 3c). Additional hybridizations showed that both organisms bind weakly to this probe (data not shown). However, the number of mismatches of the sequence of *N. paratuberculosis* to the probe cannot be determined, because the SSU rRNA sequence is not available. For *N. brasiliensis*, analysis of its sequence shows that it has one A:A mismatch at position 743. The N_5-substituted probe (Fig. 3d) resulted in a more uniform response for all members of the *Mycobacterium* complex compared to the original version of the probe. Thus, the positive effect of using a universal base analog in the degenerate base position was confirmed in this study. The genus-specific probe (Fig. 3e) was specific for all *Gordona* spp., while the species-specific probe (Fig. 3f) targeted only *G. amarae* SE102 and SE6. These results confirmed results obtained during the T_d studies. In general, the four probes hybridized strongly to nucleic acids from target organisms but not to those of nontarget organisms. Quantification of hybridization signals with a PhosphorImager showed that the highest response for nontarget SSU rRNA was 5.6% of the response for target SSU rRNA. On average, the nontarget response was only 0.5% of the target response (Table 2).

Effects of cell fixation. A crucial issue in FISH is the fixation of cell samples. The ideal fixative should not only preserve cellular morphology, but should also maximize the diffusion of probe throughout the cytoplasmic matrix. The most common fixation methods for FISH (i.e., treatment with PFA or with ethanol and formaldehyde) have been found to be effective for a wide variety of cells. However, it has been reported that many gram-positive bacteria may be more difficult to permeabilize with these common fixatives (6, 23, 43).

Some actinomycetes, including *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Tsukamurella*, have cell envelopes that contain straight-chain, saturated, and unsaturated fatty acids and tuberculostearic acid in intimate association with high-molecular-mass 3-hydroxy 2-alkyl branched-chain fatty acids (mycolic acids) (23, 26). It has been suggested that the presence of these mycolic acids in the cell envelopes results in increased hydrophobicity and thus leads to difficulties in permeabilization with PFA (7, 23).

Lawrence and Singer (19) showed that fixation with PFA for 1 min was effective for in situ hybridization of chicken embryonic muscle cultures and that cellular retention and hybridization were not improved by increasing fixation times up to 15 min. Macnaughton et al. (23) performed successful hybridizations with mycolic acid-containing actinomycetes with an acid hydrolysis fixation (HCl treatment). However, this fixation procedure did not work for *Gordona bronchialis*, *Mycobacterium fortuitum*, *N. brasiliensis*, *Rhodococcus erythropolis*, *Rhodococcus fascians*, *Rhodococcus rhodochrous*, and *T. paurometab-*

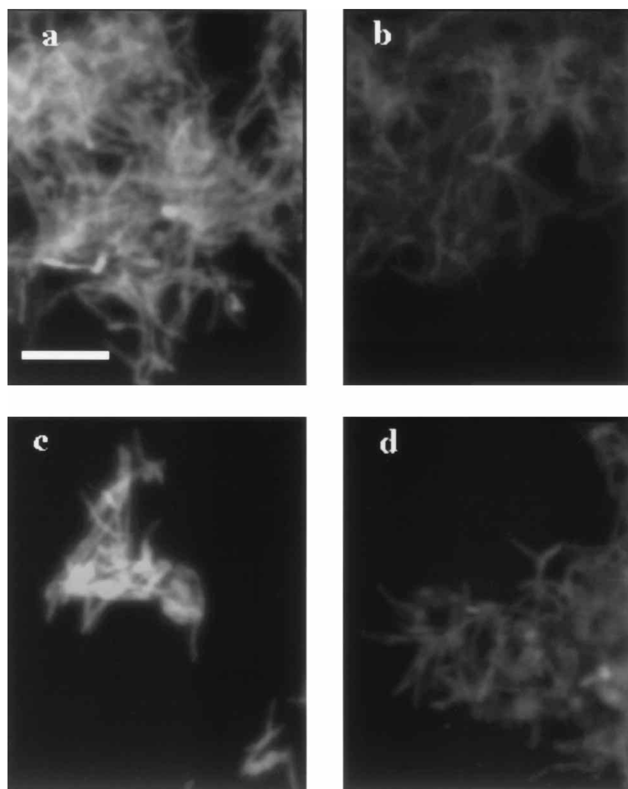


FIG. 4. Effects of fixation treatment on *G. amarae*. (a) *G. amarae* SE102 fixed with PFA for 1 min. (b) *G. amarae* SE102 fixed with ethanol for 12 h. (c) *G. amarae* NM23 fixed with PFA for 1 min. (d) *G. amarae* NM23 fixed with ethanol for 12 h. Bar, 10 μm.

olum. Thus, they concluded that the optimum fixation procedure was strain dependent.

To assess this potential problem, we evaluated four fixation treatments with *G. amarae* SE102, *G. amarae* NM23, *T. paurometabolum*, *R. rhodochrous*, and *Mycobacterium smegmatis* cells. Bacterial probe S-D-Bact-0338-a-A-18, labeled with TRITC, was used in this experiment. The fixation treatments included PFA fixation for 1 min and 2 h and ethanol fixation for 2 h and 12 to 16 h. The shorter fixation time with PFA was hypothesized to be a good compromise to allow retention of cell morphology while avoiding excessive cross-linking of the proteins in the cell wall by PFA. Figures 4 and 5 show that treatment with 4% PFA for 1 min renders *G. amarae*, *R. rhodochrous*, and *M. smegmatis* permeable for labeled probes. *T. paurometabolum* responds best to overnight ethanol fixation. The finding that *T. paurometabolum* is not permeabilized by PFA is consistent with the results from a previous study (23). The differential results obtained with different species indicate that it may be difficult to find a single method for permeabilizing all cells. This observation may be especially problematic in quantitative studies of complex environmental samples. In population characterization studies, this problem could be minimized by side-by-side fixations and use of normalization of cell counts obtained with specific probes to cell counts obtained with a universal probe. Based on the results of these fixation studies, we decided to use 4% PFA fixation for 1 min for further probe characterization studies and the FISH of environmental samples.

Optimization of FISH conditions. To determine the optimal hybridization conditions for FISH, probe-conferred fluorescence was quantified for eight different hybridization conditions, representing 0 to 70% FA in the hybridization and wash solutions. Increasing the FA concentration by 1% has an approximate equivalent effect of increasing the T_d by 0.7°C (43). Thus, various stringencies can be simulated with different FA concentrations. In this study, equivalent FA concentrations were achieved by changing the NaCl concentration in the hybridization and wash solutions, based on empirical formulas for estimating T_d values (43). Representative results of these studies are shown for probe S-S-G.am-0192-a-A-18 in Fig. 6. In general, higher FA concentrations resulted in lower signal intensities (mean pixel value), and signals for target species were consistently higher than those for nontarget species. The optimum FA concentrations were determined to be 30% for both *Mycobacterium* complex probes, 20% for the genus-specific probe, and 30% for the species-specific probe (Table 2 and Fig. 6).

FISH of environmental samples. In situ images of foam obtained from the Urbana activated sludge and anaerobic digester systems taken after FISH with probes for *Gordona* and *G. amarae* are shown in Fig. 7. Initial FISH studies showed that activated sludge samples contained organic matter and cells which autofluoresce; in addition, out-of-focus fluorescence in thick flocs hampered image clarity. Thus, the fluorescent dyes and filter sets that we used, TRITC and FITC, tended to impart some red (TRITC) or green (FITC) even to nontarget cells. This uncertainty can lead to subjectivity in cell identification and can only be resolved by quantification of the fluorescence of all cells in activated sludge. In the absence of a valid quantitative measure, we developed a qualitative method to improve image visualization. This involved inclusion of the dye DAPI in FISH studies with TRITC- and FITC-labeled probes. DAPI, which binds to DNA, has a maximum emission at 460 nm (blue), TRITC has a maximum emission at 610 nm (red-orange), and FITC has maximum emission at 535 nm (green).

Figures 7a and b present images of activated sludge foam hybridized with probe S-G-Gor-0596-a-A-22 (labeled with TRITC) and counterstained with DAPI. Figure 7a shows the phase-contrast image, while Fig. 7b presents a superimposition of the same image field obtained with TRITC and DAPI filter sets. By this approach, target organisms theoretically should show as a combination of blue and red (purple [TRITC and DAPI]), and nontarget cells should be blue (DAPI only). However, because of the autofluorescence and out-of-plane fluorescence mentioned above, some red showed up in suspected nontarget cells. We found that by optimizing the exposure time with the TRITC filter, the distinction between target and nontarget organisms could be improved. Thus, the greater intensity of TRITC in target cells resulted in a red color for members of the genus *Gordona*, thereby clearly showing the target organisms, while nontarget cells were purple. This technique thus allowed for detection of target organisms despite the presence of autofluorescing organic matter and limited spatial resolution of conventional epifluorescence microscopy. The phase-contrast image showed that several different types of filaments were present in the activated sludge foam. The fluorescence image demonstrated that the *Gordona* probe detected some filament types, but not others, enabling us to distinguish members of the genus *Gordona* from other filamentous types. This is particularly significant, because the morphology (e.g., length and branching of filaments and fragmentation into rods and cocci) of filamentous organisms is affected by different growth rates (20, 41).

We also demonstrated this approach with anaerobic digester foam. Samples were hybridized with probe S-S-G.am-0192-a-

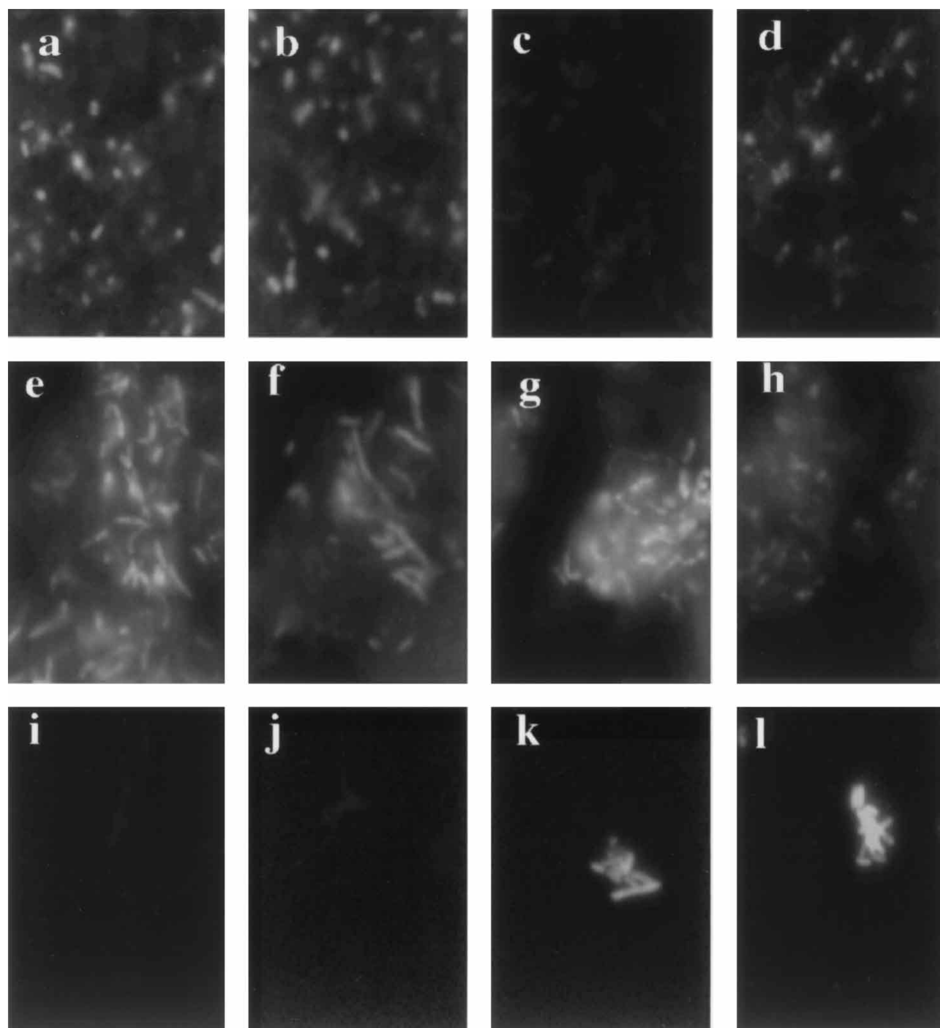


FIG. 5. Effects of fixation treatment on *R. rhodochrous*, *M. smegmatis*, and *T. paurometabolum*. (a to d) *M. smegmatis*; (e to h) *R. rhodochrous*; (i to l) *T. paurometabolum*. (a, e, and i) One-minute PFA fixation; (b, f, and j) 2-h PFA fixation; (c, g, and k) 2-h ethanol fixation; (d, h, and l) 12-h ethanol fixation.

A-18 (labeled with FITC) counterstained with DAPI. Figure 7c shows the phase-contrast image, and Fig. 7d is a superimposition of images obtained with FITC and DAPI filter sets. As described above, autofluorescence in thick flocs can be circumvented by optimizing exposure time for the target organisms. The *G. amarae* cells thus show up as green, while nontarget organisms are light blue. Again, it was possible to identify *G. amarae* cells without regard for morphology, because both filaments and rods were detected. These images show the presence of *G. amarae* in anaerobic digester foam and demonstrate the potential of FISH to study the role of filamentous foaming in anaerobic digesters. In addition to the advantages of FISH discussed above, the demonstration that FISH can be used to study filaments in anaerobic digesters may be of particular practical significance. It has been suggested that *Nocardia* (*Gordona*) filaments lose their Gram-staining property in high-rate anaerobic digesters (16), which makes study of them with traditional staining methods especially problematic.

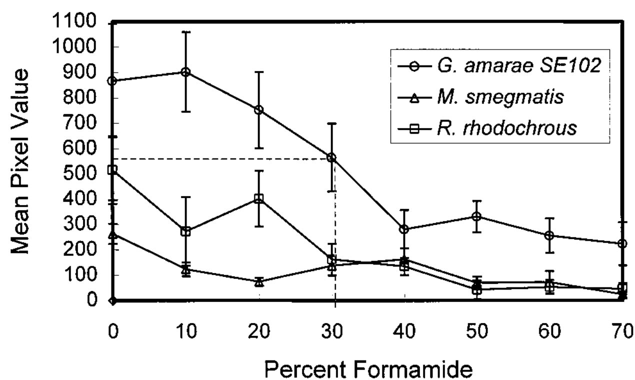


FIG. 6. Optimization of hybridization and wash conditions for FISH with probe S-S-G.am-0192-a-A-18.

The abovementioned difficulties with FISH of activated sludge and anaerobic digester samples made microscopic analysis difficult. Out-of-plane fluorescence, typical for conventional epifluorescence microscopy, and very thick flocs, which led to overlaying of nontarget bacteria in the same microscopic field, added to the lack of image clarity. Filamentous bacteria were observed to cluster together in situ, forming thick fluorescent "balls" and bundles, which were difficult to analyze by

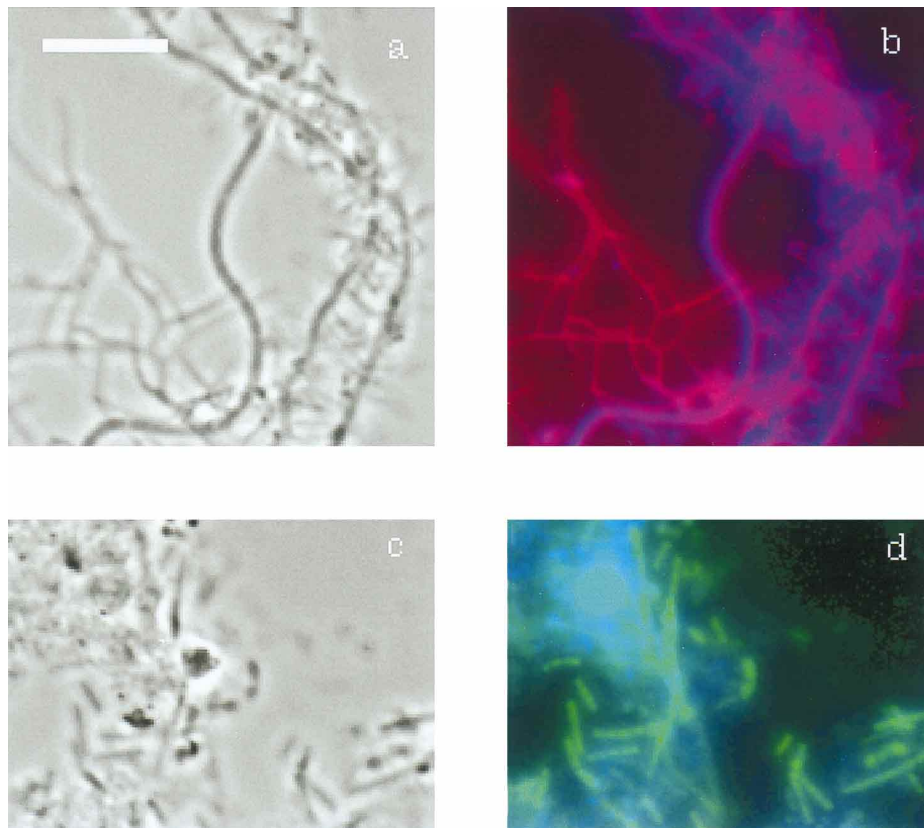


FIG. 7. Phase-contrast and epifluorescence images of FISH of environmental samples. (a and b) Activated sludge foam; (c and d) anaerobic digester foam. Magnification, ×630. Bar, 10 μm. (a and b) Phase-contrast and epifluorescence micrographs, respectively, with S-G-Gor-0596-a-A-22 labeled with TRITC and dual staining with DAPI; (c and d) phase-contrast and epifluorescence micrographs, respectively, with S-S-G.am-0192-a-A-18 labeled with FITC and dual staining with DAPI.

two-dimensional epifluorescence microscopy. Thus, it was easier to visualize target organisms at floc edges rather than within flocs. Although beyond the scope of this paper, some possibilities for minimizing these problems include the use of confocal laser scanning microscopy, which would minimize out-of-focus fluorescence due to the three-dimensional nature of flocs, and the use of competitor probes, which would minimize nonspecific binding within the floc. Various sample preparation techniques must also be explored to reduce the floc density and to separate filaments.

Quantitative membrane hybridization. The group-, genus-, and species-specific probes also were used to characterize the microbial community structure of foam and mixed liquor from the Urbana activated sludge and anaerobic digester systems, which experienced foaming problems at the time of sampling. The abundances of different microbial groups were expressed as a percentage of the total SSU rRNA in the sample (Table 3).

The N_5-substituted *Mycobacterium* complex probe consistently detected higher fractions of target organisms than the original probe, demonstrating the improved detection of all target organisms. The results show that a relatively high percentage (15.0% ± 2.7%) of the rRNA in activated sludge foam can be attributed to members of the *Mycobacterium* complex, of which the vast majority consisted of *Gordona* rRNA. rRNAs from *G. amarae* SE6 and SE102 made up only small fractions of the *Gordona* rRNA present (the stringency of the species-specific probe was controlled to target *G. amarae* SE6 and

TABLE 3. Quantitative hybridization results, expressed as percentages of total SSU rRNA in samples

Target group and probe	% of total SSU rRNA in sample (SD)			
	Activated sludge		Anaerobic digester	
	Foam	Mixed liquor	Foam	Sludge
Mycobacterium complex				
S-*-Myb-0736-a-A-22	12.9 (2.2)	7.0 (2.6)	15.6 (2.1)	3.0 (1.6)
S-*-Myb-0736-b-A-22	15.0 (2.7)	8.8 (3.1)	18.3 (2.6)	3.8 (2.0)
Gordona, S-G-Gor-0596-a-A-22	12.6 (2.0)	8.6 (2.4)	14.6 (1.9)	3.6 (1.4)
G. amarae SE6 and SE102, S-S-G.am-0192-a-A-18	0.8 (0.8)	0.6 (1.0)	0.8 (1.2)	0.5 (0.5)

SE102 only [Fig. 2d]). The relative abundance of *Mycobacterium* complex rRNA in anaerobic digester foam was even higher than that in activated sludge foam (18.3% ± 2.6%). However, as expected, the percentage in activated sludge mixed liquor (8.8% ± 3.1%) was higher than that in anaerobic digester sludge (3.8% ± 2.0%). As for activated sludge foam, most of the *Mycobacterium* complex rRNA in activated sludge mixed liquor, anaerobic digester foam, and anaerobic digester sludge consisted of *Gordona* rRNA; rRNAs from *G. amarae* SE6 and SE102 were minor contributors to the *Gordona* rRNA. Even though *G. amarae* SE6 and SE102 were present, the hybridization results suggest that the foaming problem in the Urbana wastewater treatment system may be linked to *Gordona* strains other than *G. amarae* SE6 or SE102. SSU rDNA sequencing of other *Gordona* strains and development of strain-specific probes should resolve such questions.

The observation that *Gordona* species were predominant among the mycolic acid-containing actinomycetes (*Mycobacterium* complex) was made possible through a "probe nesting" approach and illustrates the advantage of using probes for groups representing different phylogenetic levels. We also emphasize that relative rRNA concentrations do not necessarily correspond to filament numbers, but should be used to evaluate target group activity relative to total microbial activity (43).

Significance for control of filamentous foaming. Control of foaming problems in activated sludge plants can only be furthered through a thorough understanding of the mechanisms that cause foaming. The role of microbiological factors, such as the growth of filamentous bacteria, must be understood. Identification and quantification of important organisms are thus integral components of treatment plant operation and laboratory-scale studies (52). The filamentous organism identification keys of Eikelboom (13) and later modifications by Jenkins et al. (17) have been critical in addressing this need. However, these techniques, which are based on staining characteristics and other morphological criteria, may not be able to identify and quantify causative microorganisms in all cases. The use of hybridization probes allows for the in situ detection of foam-causing bacteria regardless of morphological characteristics. This advantage may be important in laboratory- and full-scale studies in which microbial populations must be monitored over periods of time across treatment processes or for the detection of morphologically diverse nocardioform actinomycetes (fugacious mycelia may break up into rod-shaped or coccoid elements [20]). In some cases, total counts of filaments may not be adequate to represent the abundance of *Gordona* spp. For example, Jenkins and coworkers compared the *Nocardia* (*Gordona*) filament counts from two treatment plants employing different methods for dealing with foam and recycle streams (11, 17, 29). They hypothesized that seeding of the influent with *Nocardia* (*Gordona*) organisms from different process recycle streams would explain the differences in *Nocardia* (*Gordona*) filament counts. However, attempts to confirm this by detecting *Nocardia* (*Gordona*) filaments in the activated sludge influent were not successful (17, 29). A method that does not rely on morphology may have been beneficial in this study.

In conclusion, we developed and characterized a "nested" set of oligonucleotide probes for filamentous bacteria which are thought to be important in activated sludge foam as an alternative to conventional identification methods. The use of hybridization probes allowed for the identification and quantification of filamentous microbial populations in activated sludge and anaerobic digester foam. Further probe development is necessary to fully assess the microbial community structure in foam. At the same time, current limitations of FISH and membrane hybridization methods must be addressed. For example, we demonstrated the importance of optimizing cell fixation methods, as well as the applicability of a universal base analog in probe design. The development of methods that allow for microbial characterization of foams and mixed liquor is an essential step in understanding the biological basis of foaming. When combined with chemical analyses and carefully designed reactor studies, the use of hybridization probes may provide insights into the causes and control of foaming.

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