

# Simultaneous oligonucleotide probe hybridization and immunostaining for in situ detection of *Gordona* species in activated sludge

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## Abstract

Oligonucleotide probes targeting ribosomal RNA can be designed with high specificity to target microbial populations at different phylogenetic levels if the cellular abundance of ribosomal RNA is sufficiently high. In contrast, polyclonal antibody probes cannot be produced with the same specificity for various phylogenetic groups, but they have the potential to detect slow growing microorganisms, populations with low metabolic activities, or even non-viable cells. We combined a polyclonal antibody stain with a ribosomal RNA targeted oligonucleotide probe for the single cell detection of species of the genus *Gordona*. *Gordona* species typically require long generation times, often exhibit filamentous growth, and are commonly encountered in activated sludge foams. Our results suggest that the ribosomal RNA content of individual cells of *Gordona* in activated sludge is highly variable. Therefore, the combined use of an immunostain and an oligonucleotide probe targeting ribosomal RNA can determine the identity of single cells and provide an approximation of their activity. This approach should result in improved detection limits while maintaining high specificity. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

*Keywords:* Activated sludge; Antibody stain; *Gordona*; Oligonucleotide probe; rRNA

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## 1. Introduction

The formation of biological foam – a stable, viscous, chocolate colored scum – on the surfaces of activated sludge aeration basins, secondary clarifiers, and sludge digesters poses a serious operational con-

cern to wastewater treatment plants [1–3]. In particular, increased solids levels in final effluents, tipping of anaerobic digester covers, clogging of gas collection and recirculation lines in anaerobic digesters, and greater risk to operating personnel due to impaired footing and possible airborne distribution of pathogens, can significantly increase the cost of operating and maintaining wastewater treatment facilities. Historically, incidents of biological foaming

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have been monitored with classical microscopy including morphological observation and polychromatic staining (e.g. Gram and Neisser staining) [2]. Although the use of these techniques has led to a basic understanding of biological foaming mechanisms, including the identification of some microorganisms suspected to be causative agents (e.g. *Gordona* (formerly *Nocardia*) *amarae* [4,5]), classical techniques suffer many drawbacks when compared to modern molecular signature methods [1,6].

Molecular signature methods are based upon the identification and quantification of molecular signatures unique to specific microbial populations. Two particularly useful molecular signature methods include staining with antibodies and hybridizations with oligonucleotide probes. Both methods can be used to quantify levels of molecular target in extractions of cellular materials subsequently bound to membranes (e.g. Northern, Southern, and Western blots), or to assess the levels of molecular target in whole cells (e.g. fluorescent in situ hybridizations (FISH) and whole cell immunostaining) [7]. Two recent studies reported on the simultaneous use of antibody stains and FISH for the identification of a *Clavibacter* sp. in pure culture and in coculture [8] and for the detection of strains of *Azospirillum brasilense* in the complex community background of rhizospheres [9]. In these two studies, the authors described protocols for the unambiguous identification of whole cells immobilized on microscope slides by combining oligonucleotide probe hybridizations and antibody staining.

Recently, a polyclonal antibody stain [10] and a small subunit (SSU) ribosomal RNA (rRNA) targeted oligonucleotide probe [11] were developed to detect *Gordona* spp. in environmental samples. The *Gordona* specific oligonucleotide probe was demonstrated to be highly specific for *Gordona* spp. in specificity studies using membrane hybridizations [11] and FISH [11,12]. The polyclonal antibody developed with *G. amarae* strain ASF3 [10,13] was found to be somewhat less specific in a limited specificity study. Slight cross reactivity was observed with *Rhodococcus rhodochrous* and *Mycobacterium smegmatis* [12]. On the other hand, the detection of *Gordona* spp. in activated sludge samples from full-scale wastewater treatment plants using the antibody serum or the oligonucleotide probe suggested that the

detectability with the antibody method was much better than with FISH [12]. This is not surprising since antibody stains can detect both viable and non-viable target cells [13,14], while the levels of hybridizable rRNA in target cells can be highly variable depending on growth conditions [15,16].

The current understanding of activated sludge foaming suggests that metabolically active, inactive, and even non-viable filamentous microorganisms may be involved in the formation and stabilization of surface scum [2,3,13]. Therefore, immunostaining and oligonucleotide probe hybridizations can be combined to determine the abundance and the identity of filaments in activated sludge, since the advantages of one method may counteract the disadvantages of the other. In this study, we present the combined application of these two methods to detect *Gordona* spp. in activated sludge from a full-scale wastewater treatment plant.

## 2. Materials and methods

### 2.1. Cell culturing and fixation

Pure cultures of *Gordona amarae* strains ASF3, NM23, and SE102, *Rhodococcus rhodochrous*, *Mycobacterium smegmatis*, and *Escherichia coli* were grown in batch according to American Type Culture Collection (ATCC) recommendations. During exponential growth ( $OD_{600} \leq 0.4$ ), cultures were fixed in 4% (w/v) paraformaldehyde (4 g paraformaldehyde in 100 ml 1× phosphate buffered saline (PBS) pH 7.2 (1×PBS = 8 g NaCl, 1.44 g  $Na_2HPO_4$ , and 0.24 g  $KH_2PO_4$  in 1 l  $H_2O$ )) for 1 min at room temperature, washed twice with 1×PBS, and stored in 50% (v/v) ethanol in 1×PBS at  $-20^\circ C$  [11].

### 2.2. Optimal antibody dilution

Immunostaining of fixed cells with various dilution factors (1:100 to 1:4000) of rabbit polyclonal whole serum prepared against *G. amarae* strain ASF3 (Rabbit  $\alpha$  *G. amarae*) [13] was conducted in a solution of 1% (w/v) bovine serum albumin (BSA) in 1×PBS in microcentrifuge tubes. After 1 h of staining, unbound Rabbit  $\alpha$  *G. amarae* (primary) antibody was removed by washing the cells twice

with 1% BSA in  $1 \times$  PBS for 15 min each (centrifuged at  $5000 \times g$  then resuspended with BSA solution). Detection of the primary antibody with the manufacturer's recommended 1:200 dilution of an appropriate secondary antibody was conducted in 1% BSA in  $1 \times$  PBS for 1 h. The secondary antibody used was either fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (whole molecule), F(ab')<sub>2</sub> fragment of affinity isolated antibody (FITC-Goat  $\alpha$  Rabbit) (Sigma, St. Louis, MO; F-1262) or Cy3 conjugated sheep anti-rabbit IgG (whole molecule), F(ab')<sub>2</sub> fragment of affinity isolated antibody (Cy3-Sheep  $\alpha$  Rabbit) (Sigma, C-2306). Unbound secondary antibody was removed by washing the cells twice with fresh 1% BSA in  $1 \times$  PBS for 15 min each.

Immunostained cells were resuspended in water, and aliquots were air dried onto Super Cured, heavy teflon-coated microscope slides (Cell-Line, Newfield, NJ). Cells were heat-fixed to the slide surface by passing the slide through a flame. Antibody-conferred fluorescent signal was quantified by digital microscopy as described below.

To test the specificity and cross reactivity of the secondary antibodies, fixed cells of *G. amarae* strains SE102, NM23, and ASF3, and *M. smegmatis* were stained with either a 1:200 dilution of Rabbit  $\alpha$  *G. amarae* in 1% BSA in  $1 \times$  PBS as described above, or 1% BSA in  $1 \times$  PBS without primary antibody. After washing the cells, subsamples were stained in solution with various dilution factors (1:100 to 1:1000) of each secondary antibody in 1% BSA in  $1 \times$  PBS. Unbound secondary antibody was removed as described before, and the immunostained cells were heat-fixed to microscope slides and examined with digital epifluorescence microscopy as described below.

### 2.3. Simultaneous use of oligonucleotide probe and antibody stain

Samples stained in solution with Rabbit  $\alpha$  *G. amarae* and a secondary antibody as described above, were heat-fixed to microscope slides and dehydrated in an increasing ethanol series (50, 80 and 96% (v/v)) for 1 min each. After air drying, 9  $\mu$ l of hybridization buffer (30% (v/v) formamide, 0.9 M NaCl, 100 mM Tris HCl (pH 7.2), 0.1% (w/v) sodium dodecyl sulfate (SDS)) and 1  $\mu$ l (25 ng) of

oligonucleotide probe S-G-Gor-0596-a-A-22 (5'-TG-CAGAATTTACAGACGACGC-3') labeled with tetramethyl rhodamine (TRITC) (Genosys, The Woodlands, TX) were added to each well of the microscope slide [11]. Hybridizations were conducted in moisture chambers for 2 h, in the dark, at 46°C. The slides were rinsed with wash solution (110 mM NaCl, 100 mM Tris HCl (pH 7.2), 0.1% (w/v) SDS, and 5 mM ethylenediamine-tetraacetic acid (EDTA)) and washed for 20 min in 50 ml of wash solution prewarmed to 48°C.

### 2.4. Visualization of cells and quantification

The slides were placed into 100 ml of fresh 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining solution (100 mM Tris HCl (pH 7.2), 0.9 M NaCl, and 25 mg DAPI l<sup>-1</sup> H<sub>2</sub>O) for 2 min, rinsed with cold water, and rapidly air dried [11]. Then, the cells were mounted with Citifluor (UKC, Canterbury, UK) and a cover slip. Visualization of the fluorescence signal was conducted with a Zeiss Axioskop equipped with dichromatic filter sets 41001-fluorescein, 41002-rhodamine (Cy3), and 31000-DAPI (Chroma, Brattleboro, VT). Images were captured and digitized with a CH250 (KAF 1400) charge coupled device camera (Photometrics, Tucson, AZ) using exposure times of 0.5 s (target microorganisms) or 1 s (non-target microorganisms). Data were analyzed with IP Lab Spectrum IO software version 3.0 (Signal Analytics, Vienna, VA).

### 2.5. Environmental samples

Grab samples of mixed liquor were collected from the activated sludge aeration basin of the Urbana-Champaign Sanitary District, Northeast Wastewater Treatment Plant (UCSD, NEWWTP). This plant treats a municipal wastewater with an average daily influent of 110 000 person equivalents using primary clarification, secondary treatment in a contact stabilization activated sludge system, and tertiary nitrification through high rate, synthetic media trickling filters. During an occurrence of seasonal foaming, mixed liquor samples were obtained from the aeration basin and fixed as described above. Immunostaining and FISH were carried out as described above.

### 3. Results and discussion

Initially, various hybridization and staining protocols were evaluated, including: (1) FISH and subsequent immunostaining of samples fixed to microscope slides; (2) FISH and subsequent immunostaining of samples in solution; and (3) immunostaining of samples in solution with subsequent FISH of samples fixed to microscope slides. This evaluation demonstrated that immunostaining in solution with subsequent FISH of samples fixed to microscope slides resulted in the maximum reduction of non-specific background fluorescence (data not shown).

Subsequently, the protocol for solution immunostaining was optimized. The secondary antibodies, Cy3-Sheep  $\alpha$  Rabbit and FITC-Goat  $\alpha$  Rabbit, demonstrated constant staining intensity over a range of dilution factors (1:200 to 1:1000) (data not shown). In addition, both secondary antibodies demonstrated minimal non-specific staining of pure cultures and environmental samples at the manufacturer's recommended dilution factor of 1:200. Therefore, this dilution factor was used for both secondary antibodies in all subsequent experiments.

Fig. 1 shows the quantification of dilution extinction for Rabbit  $\alpha$  *G. amarae* primary antibody over a range of concentrations from 1:100 to 1:4000 and without primary antibody (i.e. using secondary antibody only) for strains of *G. amarae*, SE102, NM23,

and ASF3, and three non-target microorganisms, *M. smegmatis*, *R. rhodochrous*, and *E. coli*. To construct Fig. 1, different exposure times were used for quantification of signal intensity (measured as pixel units) from target and non-target microorganisms. Two phenomena dominated the selection of exposure times for quantitative image analysis. For target signals, the exposure time of 0.5 s was selected such that the maximum signal detected was approximately 75% of the maximum detection threshold of the digital camera. This ensured that no signals would exceed the maximum detection threshold of the digital camera and that a large portion of the dynamic range of the digital camera was utilized. For non-target signals, an exposure time of 1 s was selected. The longer exposure time for non-target cells increased the signal-to-noise ratio for quantification while minimizing fluorescence quenching. A plot of the signal intensity versus the dilution factor of the primary antibody with 1% BSA shows an optimal dilution factor for Rabbit  $\alpha$  *G. amarae* of 1:1000. At this dilution, the levels of Rabbit  $\alpha$  *G. amarae* resulted in excellent specificity while still providing high signals. Thus, specificity and detectability were optimized simultaneously at this dilution.

In contrast to previous studies that often reported 'slight' cross-reactivity in immunostaining assays without quantifying the level of cross-reactivity

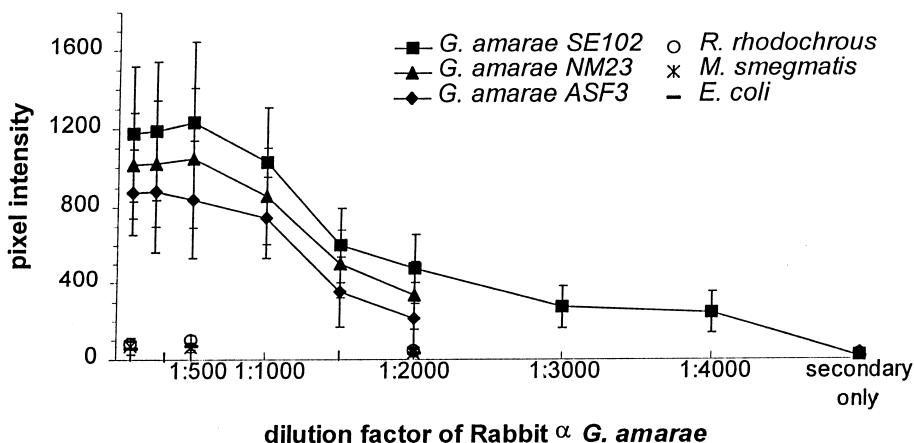


Fig. 1. Dilution extinction curves for Rabbit  $\alpha$  *G. amarae* primary antibody for target and non-target microorganisms. Cy3-Sheep  $\alpha$  Rabbit was used at a 1:200 dilution. An optimal exposure time of 0.5 s (target microorganisms) and 1 s (non-target microorganisms) was used.

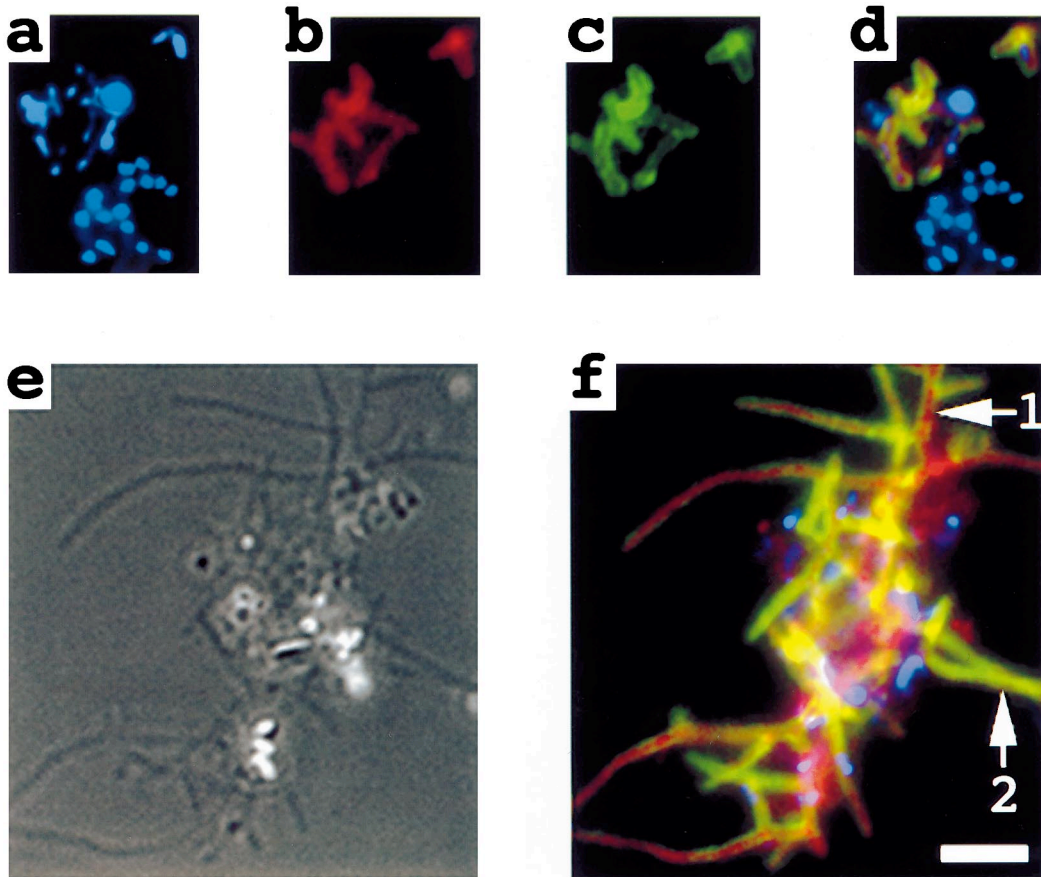


Fig. 2. Representative images of simultaneous immunostaining and in situ hybridization of pure cultures and activated sludge samples. A mixture of pure cultures of *G. amarae* strain SE102, *M. smegmatis*, and *R. rhodochrous* and a grab sample of activated sludge from the UCSD, NEWWTP were immunostained with Rabbit  $\alpha$  *G. amarae* (green), hybridized with S-G-Gor-0596-a-A-22 (red), and stained with DAPI (blue). Cells were visualized with phase contrast (e) and epifluorescence microscopy (a–d and f) and images were captured and digitized with a charge coupled device camera. All images are  $\times 90$ , and the scale bar in f represents 5  $\mu\text{m}$  for all panels. The arrows in f indicate cells demonstrating subcellular localization of immunostaining and hybridization signal (arrow 1) and cells demonstrating positive immunostaining results and minimal hybridization activity (arrow 2).

[13], we quantified target versus non-target signal with digital microscopy (Fig. 1). At the dilution of the primary antibody of 1:1000, the average pixel intensity obtained using the three target microorganisms was 874. Linear interpolation of the average pixel intensities for the three non-target microorganisms at dilution factors of 1:500 and 1:2000 resulted in a pixel intensity of 63 for the 1:1000 dilution. A comparison of these results shows that the signal from target microorganisms was approximately 14-fold greater than the signal from non-target microorganisms. Since the results for target organisms were obtained with a shorter exposure time, the dif-

ference between target and non-target signal intensity would have been even greater if similar exposure times had been used. Thus, although the human eye has the capacity to detect 'slight' cross-reactivity with an epifluorescence microscope, digital quantification of the signal can be used to determine the ratio between target and non-target signal.

After optimizing the protocol for the solution immunostaining of *G. amarae*, simultaneous antibody staining and oligonucleotide probe hybridization was evaluated for mixtures of pure cultures. Fixed cells of *G. amarae* SE102 were mixed with fixed cells of two non-target microorganisms, *R. rhodochrous* and

*M. smegmatis*. This mixture was stained with the primary antibody at a dilution factor of 1:1000 and FITC-Goat  $\alpha$  Rabbit secondary antibody at a dilution factor of 1:200, hybridized with TRITC-labeled S-G-Gor-0596-a-A-22, and stained with DAPI. The results of this experiment are shown in Fig. 2a–d. All three organisms stain with the universal DNA dye, DAPI (Fig. 2a). In contrast, only *G. amarae* can be visualized after hybridization with probe S-G-Gor-0596-a-A-22 labeled with TRITC (Fig. 2b), or staining with Rabbit  $\alpha$  *G. amarae* (1:1000) and FITC-Goat  $\alpha$  Rabbit (1:200) (Fig. 2c). For Fig. 2c, an exposure time of 0.5 s was used for the antibody stain. Under these conditions (a 1:1000 dilution of the primary antibody), the non-target signal was below the signal-to-noise ratio of the digital camera. Therefore, the non-target cells are not visualized in Fig. 2c, although some non-target signal could be quantified when an exposure time of 1 s was used (see above). Combining the blue, red, and green images from Fig. 2a–c generates a color-merged image (Fig. 2d) that shows *G. amarae* in yellow (a combination of green from FITC-Goat  $\alpha$  Rabbit, red from TRITC labeled S-G-Gor-0596-a-A-22, and blue from DAPI) and non-target microorganisms in blue (from DAPI).

The optimized protocol was further examined with samples obtained from the UCSD, NEWWTP. During the summer season, this wastewater treatment plant often experiences severe filamentous foaming on the surfaces of aeration basins and secondary clarifiers. This foam typically dissipates during the fall season and returns during the early summer or later spring. Grab samples of mixed liquor were obtained during an occurrence of seasonal foaming and were immunostained with Rabbit  $\alpha$  *G. amarae* and FITC-Goat  $\alpha$  Rabbit, hybridized with the *Gordona* genus specific oligonucleotide probe, and stained with DAPI. Fig. 2e,f represent representative results from this experiment. Fig. 2e shows a phase contrast micrograph of an activated sludge floc and demonstrates the presence of relatively high levels of filamentous microorganisms that extend significantly outside the floc. Fig. 2f combines images obtained with filter sets for FITC, TRITC, and DAPI and shows that the simultaneous application of the antibody stain, the oligonucleotide probe, and DAPI permits reliable identification of *Gordona* spp. (i.e.,

yellow cells) inside and outside the activated sludge floc.

A close comparison of Fig. 2b–d shows the sub-cellular localization of the molecular targets. Probe S-G-Gor-0596-a-A-22 hybridizes to SSU rRNA located throughout the cell (Fig. 2b), while the Rabbit  $\alpha$  *G. amarae* antibody preferentially stains the cell wall (Fig. 2c). This produces a ring of yellow fluorescence (a combination of Rabbit  $\alpha$  *G. amarae*, S-G-Gor-0596-a-A-22, and DAPI) surrounding a core with a purple hue (a combination of S-G-Gor-0596-a-A-22 and DAPI) (Fig. 2d). The color-merged image of the activated sludge floc (Fig. 2f) demonstrates a similar pattern. The two arrows in Fig. 2f indicate specific signal patterns observed in the activated sludge samples. Some cells show a ring of yellow fluorescence (a combination of Rabbit  $\alpha$  *G. amarae*, S-G-Gor-0596-a-A-22, and DAPI) corresponding to cell walls, surrounding a core of purple/reddish fluorescence (a combination of S-G-Gor-0596-a-A-22 and DAPI) (Fig. 2f, arrow 1). Other cells show intense antibody staining, but lack an internal purple core (Fig. 2f, arrow 2). Based on the combined use of the antibody and the oligonucleotide probe, it is obvious from the micrograph that these cells are *Gordona* spp. that contain the molecular target for Rabbit  $\alpha$  *G. amarae*, but lack detectable levels of SSU rRNA (a lack of strong hybridization signal from S-G-Gor-0596-a-A-22). If only SSU rRNA hybridization would have been performed, the absence of significant signal for most of the filaments would not have allowed for the unambiguous identification of these filaments as *Gordona* spp. Here, we can derive that the individual cells of *Gordona* spp., which compose the filaments, appear to have variable levels of rRNA while maintaining relatively constant levels of antigen.

One caveat to this discussion is the complications presented by non-specific immunostaining of non-target microorganisms in activated sludge. In fact, if some microorganisms demonstrate non-specific, non-target staining with signal intensity similar to *Gordona* spp., then discrimination between target and non-target microorganisms would be impossible. As discussed above, with a ratio of target to non-target signal intensity of 14 (i.e. using a dilution factor of 1:1000 for the primary antibody), the identification and quantification of *Gordona* spp. in acti-

vated sludge is possible. Nevertheless, the inability to quantitatively test non-target cross-reactivity for all possible non-target microorganisms is still a source of potential error. However, this problem is not specific to immunostaining, but extends to all molecular signature methods. A full discussion of the consequences of misidentifying non-target microorganisms using molecular signature methods is beyond the scope of this work. Instead, we focus on the additional confidence in identifying target microorganisms when employing two molecular signature methods. Although both immunostaining and FISH are prone to errors due to non-specific signal from non-target microorganisms, the possibility of two independent methods misidentifying the same non-target microorganisms should be low. Since it is likely that filaments that are metabolically inactive or non-viable, and thus contain low cellular rRNA levels, contribute to the formation and stabilization of activated sludge foam [2,3,13], the combined use of an antibody and an oligonucleotide probe provides additional confidence for filament identification and quantification in the complex community background of activated sludge.

In conclusion, the simultaneous application of a polyclonal antibody serum and an oligonucleotide probe targeting SSU rRNA offers the advantage of detecting slow growing or metabolically less active microorganisms while maintaining high phylogenetic specificity in complex environments such as activated sludge. The solution antibody staining procedure outlined herein provides clear results with high specificity and detectability thereby facilitating the dual immunostaining and hybridization approach. The combination of Rabbit  $\alpha$  *G. amarae* with S-G-Gor-0596-a-A-22 can be used to reliably quantify *Gordona* spp. in activated sludge, providing an additional tool for correlating the presence and activity of specific microorganisms with the occurrence of biological foaming in wastewater treatment systems.

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