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Improved Sensitivity of Whole-Cell Hybridization by the Combination of Horseradish Peroxidase-Labeled Oligonucleotides and Tyramide Signal Amplification

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The substrate fluorescein-tyramide was combined with oligonucleotide probes directly labeled with horseradish peroxidase to improve the sensitivity of in situ hybridization of whole fixed bacterial cells. Flow cytometry and quantitative microscopy of cells hybridized by this technique showed 10- to 20-fold signal amplifications relative to fluorescein-monolabeled probes. The application of the new technique to the detection of natural bacterial communities resulted in very bright signals; however, the number of detected cells was significantly lower than that detected with fluorescently monolabeled, rRNA-targeted oligonucleotide probes.

In recent years, in situ hybridization with nucleic acid probes has been more and more frequently used in microbiology for the detection, enumeration, and localization of specific target sequences in whole fixed cells (2). The high cellular ribosome content of most microorganisms facilitates a culture-independent identification of individual cells based on rRNA-targeted oligonucleotides monolabeled with a fluorescent dye (3, 5). An increase in sensitivity would be advantageous when the number of rRNA molecules drops to low levels or when nucleic acids present at lower copy numbers, such as mRNA or plasmids, should be detected. The two general possibilities are (i) to make the probes more sensitive (2) and (ii) to amplify the target molecules prior to detection, e.g., by an in situ PCR (12, 17).

In earlier studies, we focused on exploiting the signal amplification of probe-conferred enzymes, either by an indirect assay with a digoxigenin-labeled probe and an enzyme-labeled antibody (27) or directly with an enzyme-coupled probe (6). The signal amplification is realized by the enzymatic catalysis of the transformation of multiple substrate molecules to colored precipitates. With the standard, nonfluorescent substrates, several successful applications of indirect approaches have been reported showing the detection of cells even against a strongly fluorescent background (9, 16) or with mRNA as the target (11).

A further increase in sensitivity should be possible with the recently developed fluorescent substrates. By using a combination of 2-hydroxy-3-naphthoic acid-2′-phenylanilide and Fast Red TR, Yamaguchi et al. (26) achieved signal amplifications of up to eightfold. This report deals with the evaluation of fluorescein-tyramide as a substrate for oligonucleotides directly labeled with horseradish peroxidase (HRP).

Strains, cultivation, and cell fixation. The strains used in this study and their phylogenetic affiliations are listed in Table 1. They were grown under the conditions and with the media described in the respective catalogs of strains. The main object of study, an *Escherichia coli* strain recently isolated from cattle (a gift from P. Grimont, Institute Pasteur, Paris, France), was grown aerobically in Luria-Bertani medium at 37°C. To obtain cells with a high ribosome content, overnight cultures were inoculated at 1:100 (vol/vol) into fresh medium from which cells were harvested within 2 hours by centrifugation (5,000 × *g*, 5 min). Stationary-phase cells were harvested from overnight cultures. Cells were fixed as described before (4, 19).

Probes. HRP- (6, 21), and fluorochrome-labeled (5) oligonucleotides were purchased from Interactiva (Ulm, Germany).

In situ hybridization on glass slides. Cells were prepared for in situ hybridization as described by Manz et al. (15). Optionally, cells were treated with lysozyme prior to hybridization. In the optimized protocol for *E. coli*, cells were covered with 50 μl of lysozyme-EDTA solution (0.1 mg of lysozyme [Fluka, Buchs, Switzerland; 66,200 U/mg] per ml in a buffer containing 100 mM Tris-HCl and 50 mM EDTA [pH 8.0]) and incubated at 0°C for 5 min. The enzyme reaction was stopped by rinsing the slide with H_2O and then with a second ethanol series. Hybridizations with fluorescein- and HRP-labeled probes were performed as described before (6, 15). After the stringent washing step, slides were rinsed briefly with distilled water and equilibrated with TNT buffer (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl, 0.05% Tween 20) for 15 min. Excess buffer was removed without drying the slides, and 100 μl of freshly prepared fluorescein-tyramide solution (DuPont, NEN Research Products, Boston, Mass.) was added. After 5 min at room temperature, the slides were rinsed briefly with a few milliliters of TNT buffer and then immersed in 40 ml of the same buffer for 15 min. Finally, the slides were rinsed with distilled water, air dried, and mounted in Citifluor (Canterbury, United Kingdom) AF1. Monochrome photographs were taken on an Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) with Kodak Tmax400 film; color slides were taken with Kodak Panther 1600 film.

Microscopic quantification of fluorescence intensities. For measuring fluorescence intensities, hybridized samples were recorded with a CF-15/2 charge-coupled device camera (Kappa, Gleichen, Germany) attached to the straight-through port of the epifluorescence microscope and processed as outlined by Trebesius et al. (20). For comparison of data obtained at different exposure times (0.2 to 6 s), it was assumed that the bleaching of fluorescein in the antifading mountant AF1 was negligible and that therefore the recorded intensities would be

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TABLE 1. List of strains and their phylogenetic affiliations

Strain	Source or strain no.	Phylogenetic affiliation
Escherichia coli	P. Grimont, Institute Pasteur	Gamma subclass of *Proteobacteria*
Acinetobacter calcoaceticus	ATCC 23055^T	Gamma subclass of *Proteobacteria*
Burkholderia cepacia	LMG 1222^T	Beta subclass of *Proteobacteria*
Neisseria canis	LMG 8383^T	Beta subclass of *Proteobacteria*
Brevundimonas diminuta	DSM 1635	Alpha subclass of *Proteobacteria*
Bacillus firmus	DSM 12	Gram-positive bacteria with low G+C content of DNA
Bacillus subtilis	DSM 10^T	Gram-positive bacteria with low G+C content of DNA
Corynebacterium glutamicum	DSM 20300	Gram-positive bacteria with high G+C content of DNA
Micrococcus luteus	CCM 169	Gram-positive bacteria with high G+C content of DNA
Methanogenium organophilum	DSM 3596^T	*Euryarchaeota*
Methanosarcina barkeri	DSM 800^T	*Euryarchaeota*

directly proportional to time. Values from shorter exposure times were extrapolated to an exposure time of 6 s.

Hybridization of suspended cells and flow cytometric quantification of fluorescence intensities. For flow cytometric analysis, cells were hybridized in suspension with fluorescently labeled oligonucleotides as described previously (23, 24). For hybridization with HRP-labeled oligonucleotides, the standard protocol was modified as follows. Paraformaldehyde-fixed cells were centrifuged (5,000 × *g*, 5 min), and the supernatant was discharged. Cells were resuspended in the lysozyme-EDTA solution mentioned above, incubated for 5 min at 0°C, washed, centrifuged, and finally resuspended in 1× phosphate-buffered saline (pH 8.4). Ethanol-fixed cells were subjected directly to the hybridization without pretreatment. Hybridizations for 3 h and washing for 30 min were performed at 35°C in the hybridization and washing buffers as referenced above. Cells that had been hybridized with HRP-labeled probes were subsequently washed in TNT buffer, incubated with the fluorescein-tyramide substrate for 5 min, washed in TNT buffer, and finally resuspended in 1× phosphate-buffered saline for the flow cytometric analysis. A FACStar Plus (Becton Dickinson, Mountain View, Calif.) flow cytometer was operated with an argon ion laser adjusted to the 488-nm line (500 mW). System settings, data acquisition, and data analysis were as described before (23, 24).

Initial results. The combination of HRP-labeled, rRNA-targeted oligonucleotide probes and the substrate fluorescein-tyramide, referred to hereafter as the HRP-oligo–tyramide signal amplification (HRP-oligo–TSA) system, was first evaluated on glass slides with an artificial mixture of ethanol-fixed cells from log-phase cultures of *Acinetobacter calcoaceticus* and *E. coli*. The probe ACA (5′-ATCCTCTCCCATACTCTA-3′ [22]) hybridized specifically to the coccoid cells of *A. calcoaceticus* and not to the rod-shaped cells of *E. coli*. The signal conferred by the HRP-oligo–TSA system (Fig. 1A) was much stronger than the signal obtained with the fluorescein-monolabeled oligonucleotide (Fig. 1B). The probe EUB (5′-GCTG CCTCCCGTAGGAGT-3′ [4]) was used as a positive control for the accessibility of the probes to their targets within the cells. Again, the signal conferred by the HRP-oligo–TSA system (Fig. 1C) was much stronger than the one conferred by a fluorescently monolabeled oligonucleotide (Fig. 1D). Control experiments were performed without a probe to test for nonspecific binding of substrate and intrinsic peroxidase activity. An oligonucleotide that was not complementary to bacterial rRNA (EUK516; 5′-ACCAGACTTGCCCTCC-3′ [4]) was used to test for nonspecific probe binding. All control experiments showed only very low levels of nonspecific staining, resulting in black epifluorescence micrographs at the exposure times used for Fig. 1 (data not shown).

Quantification of the signal amplification. Initial signal quantification was attempted by flow cytometry. The maximum signal amplification for *E. coli* from a log-phase culture with the HRP-oligo–TSA system was found to be up to 20-fold. However, the staining was very heterogeneous, with certain cells showing much lower fluorescence (data not shown). Since the staining appeared to be more homogeneous for cells immobilized on glass slides than for suspended cells that were hybridized, a charge-coupled device camera was used for their quantification. The average fluorescence per cell for paraformaldehyde-fixed, log-phase *E. coli* after hybridization with fluorescein-labeled oligonucleotides was 50.2 ± 16.1 relative units. Cells fixed with ethanol at the same time from the same *E. coli* culture showed, after being hybridized with the HRP-oligo–TSA system, an average fluorescence of 1,043 ± 276 relative units per cell (corrected value; the original value recorded at 0.2 s was 34.8 ± 9.2 relative units). Cells which were fixed in paraformaldehyde and subsequently permeabilized by lysozyme treatment showed an almost identical brightness (1,022 ± 293 relative units). This would indicate a signal amplification by the HRP-oligo–TSA system by a factor of approximately 20. Without a lysozyme treatment, the staining of the paraformaldehyde-fixed *E. coli* cells with the HRP-oligo–TSA system was significantly lower and less homogeneous (710 ± 257 relative units). Interestingly, paraformaldehyde-fixed, lysozyme-treated, stationary-phase cells of *E. coli* showed with the HRP-oligo–TSA system signals (1,025 ± 246 relative units) comparable to those of growing cells, even though probing with fluorescein-labeled probe EUB indicated that the ribosome content of stationary-phase *E. coli* was, with a fluorescence of 12.0 ± 3.7 relative units, significantly lower than that of log-phase cells.

Additional quantifications were performed by flow cytometry on lysozyme-treated, paraformaldehyde-fixed cells. Again, the staining was not homogeneous, and 22% of the cells were not fully stained (Fig. 2). The signal achieved with the HRP-oligo–TSA system for the fully stained population was, at 2,575 ± 302 relative units, approximately 10 times higher than the signal of 264 ± 16 relative units achieved with the standard protocol (paraformaldehyde fixation, no lysozyme treatment, fluorescein-monolabeled probe). The wider dynamic range of the flow cytometer also allowed the measurement of nonspecific staining with identical settings. The incubation of fixed cells with the substrate fluorescein-tyramide alone resulted in a mean signal of 8 relative units, which was only slightly enhanced relative to the autofluorescence of the cells of 4 relative units. The hybridization with a negative control probe (EUK516) followed by a fluorescein-tyramide incubation resulted in a mean signal of 55 relative units. The signal-to-noise

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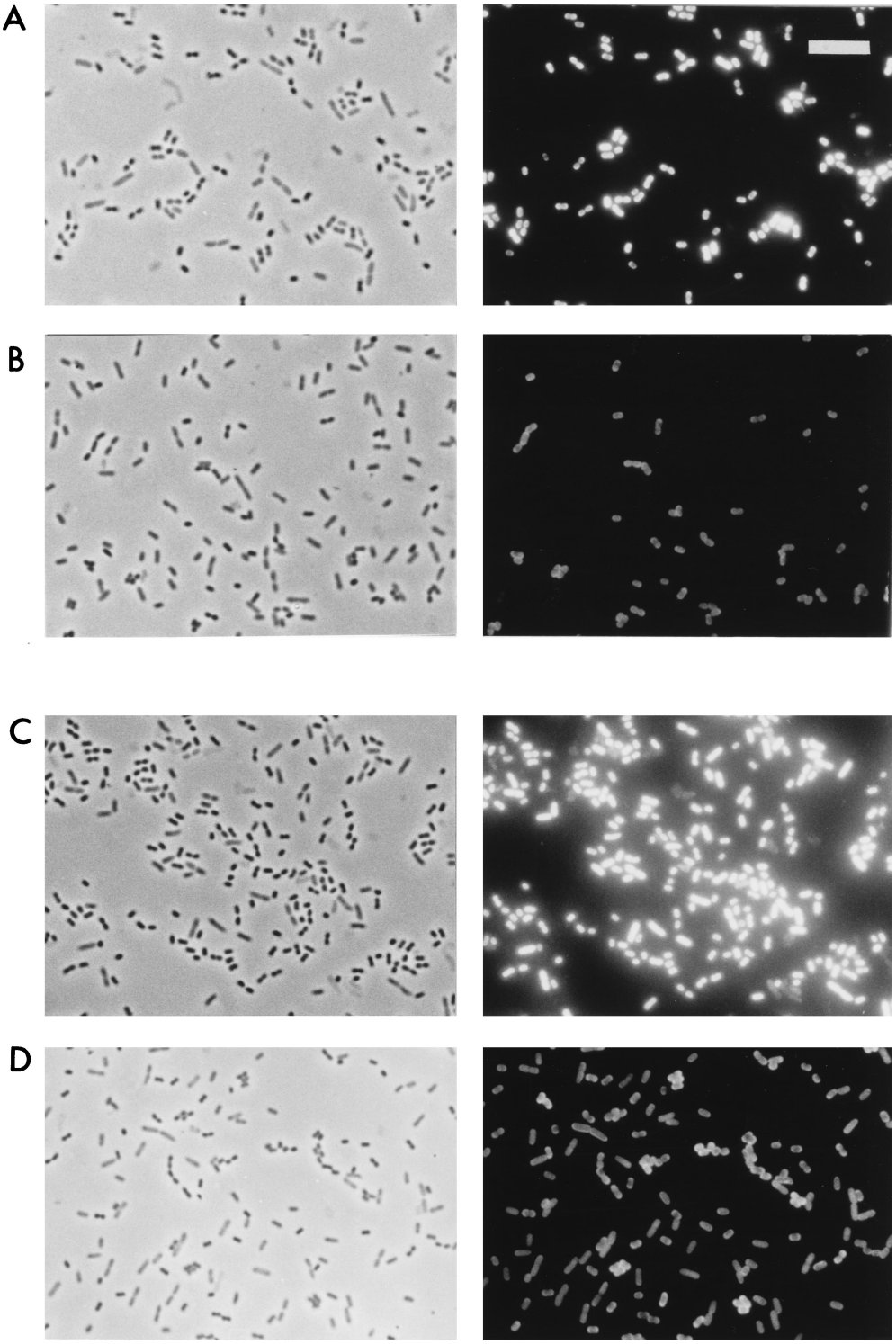

FIG. 1. Monochrome micrographs of an artificial mixture of *A. calcoaceticus* and *E. coli*. Bar, 10 μm. (A) HRP-oligo–TSA system hybridization with probe ACA; (B) hybridization with fluorescein-monolabeled probe ACA; (C) HRP-oligo–TSA system hybridization with probe EUB; (D) hybridization with fluorescein-monolabeled probe EUB. Phase-contrast (left) and epifluorescence (right) micrographs are shown for identical microscopic fields. Exposure times for epifluorescence micrographs were 4 s (A and B) and 2 s (C and D).

ratio with the HRP-oligo–TSA system was calculated to be 47 (2,575/55), compared to 66 (264/4) for the standard system.

Applicability of the HRP-oligo–TSA system. The general applicability of the HRP-oligo–TSA system for in situ identification of whole fixed bacterial cells was evaluated by hybridization of various gram-negative and gram-positive bacteria and two strains of *Euryarchaeota* (Table 1). The tested representatives of the alpha, beta, and gamma subclasses of *Pro-*

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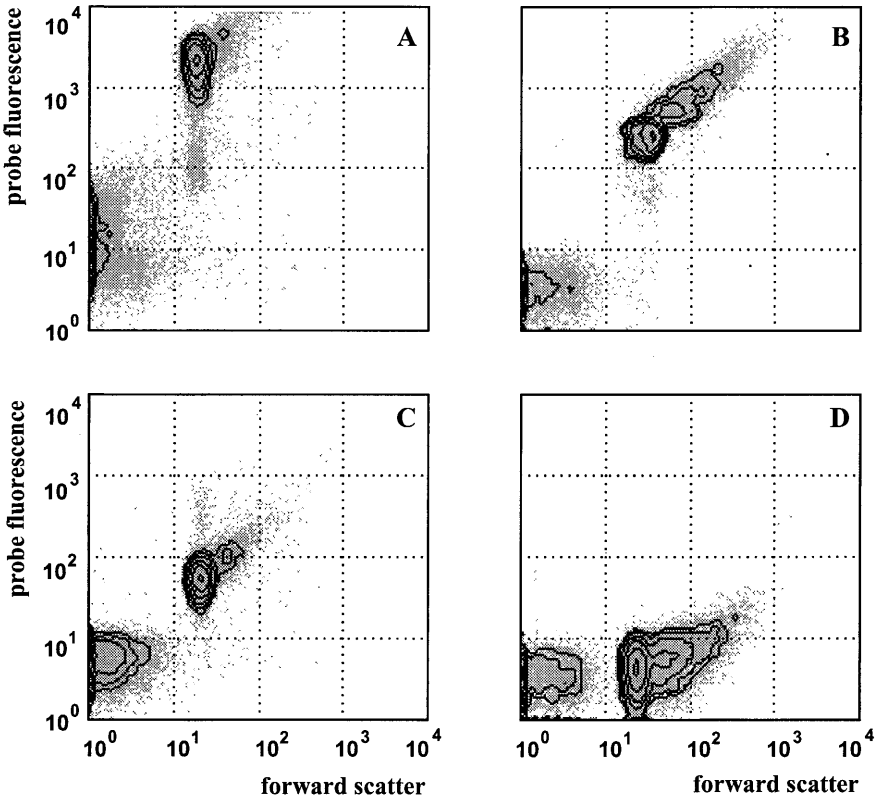

FIG. 2. Distribution of forward scatter (*x* axis) and probe-conferred fluorescence (*y* axis) in pure cultures of *E. coli* hybridized with probe EUB specific for *Bacteria* (A and B) or probe EUK specific for *Eucarya* (C and D). (A and C) HRP-oligo–TSA system; (B and D) fluorescein-monolabeled probes. Each dot represents a cell. Frequencies can be estimated from the density of the dots and the overlaid contour lines (3, 6, 12, 24, 48, and 95% of maximum frequencies [outer to inner lines]).

teobacteria were accessible by the HRP-oligo–TSA system after ethanol fixation even though the staining was quite uneven. The combination of paraformaldehyde fixation and lysozyme treatment generally resulted in a more homogeneous staining. On the other hand, the HRP-oligo–TSA system did not yield strong homogeneous signals with log-phase cultures of four examined gram-positive species, namely, *Bacillus subtilis*, *Bacillus firmus*, *Corynebacterium glutamicum*, and *Micrococcus luteus*. The majority of ethanol-fixed cells remained unstained, and even upon lysozyme treatment of paraformaldehyde-fixed cells, only a few cells showed strong signals. Of the two examined *Archaea*, *Methanosarcina barkeri* was accessible by the HRP-oligo–TSA system upon ethanol fixation whereas *Methanogenium organophilum* was inaccessible. For this experiment, the archaeal probe ARCH 915 (5′-GTGCTCCCCCGCCAA TTCCT-3′ [2]) was used as a positive control.

Environmental samples. To further evaluate the applicability of the HRP-oligo–TSA system, ethanol-fixed water samples from a eutrophic pond and activated sludge were analyzed and the results were compared to those obtained with Cy3-labeled oligonucleotide probes. In both cases, the HRP-oligo–TSA system yielded very strong green signals only with part of those bacteria that hybridized with the Cy3-labeled probe (Fig. 3). In the pond, hybridization with the HRP-oligo–TSA system resulted in a detection rate, as defined by the fraction of DAPI (4′,6-diamidino-2-phenylindole)-stained cells that were detected with the general bacterial probe EUB, of 25% compared to 75% with Cy3-labeled probes. In the activated sludge sample, detection rates were 37% for the HRP-oligo–TSA system compared to 77% for Cy3-labeled oligonucleotides.

Conclusions. The catalyzed reporter deposition was originally designed for enzyme immunoassays. In the original configuration (7, 8), the analyte-dependent reporter enzyme HRP catalyzed the deposition of biotin-labeled tyramine, a low-molecular-weight phenol derivate, on nitrocellulose membrane-bound proteins. This system found successful applications to immunohistochemistry when used in combination with biotin-labeled antibodies (1) and nucleic acid probes (14). Recently, tyramides labeled with fluorescein, Texas red, and aminomethylcoumarine were marketed by DuPont, NEN Research Products, in so-called TSA-Direct systems. In these kits, probe-conferred biotin is detected via streptavidin-HRP conjugates and the bound HRP is subsequently directly visualized by the catalytic deposition of the fluorescent dye-labeled tyramine. The TSA-Direct system has already been used in ultrasensitive fluorescent in situ hybridization (18). In our study, the use of HRP-labeled oligonucleotide probes results in an even more direct detection, since the biotin-streptavidin step could be omitted. The combination of HRP-labeled oligonucleotide probes and the substrate fluorescein-tyramide actually yielded a strong increase in sensitivity of whole-cell hybridization when compared to that of oligonucleotide probes labeled with fluorescein or even with the currently brightest dye (25), the carbocyanin Cy3. The detection was highly specific. The very satisfactory signal-to-noise ratio of 47 might be due in part to the direct mode of detection since every additional step could contribute to nonspecific binding. Furthermore, the procedure is quite fast and can be completed in less than 3 h from the start of the hybridization to the microscopic examination. The similar strengths of signals obtained with the HRP-oligo–TSA

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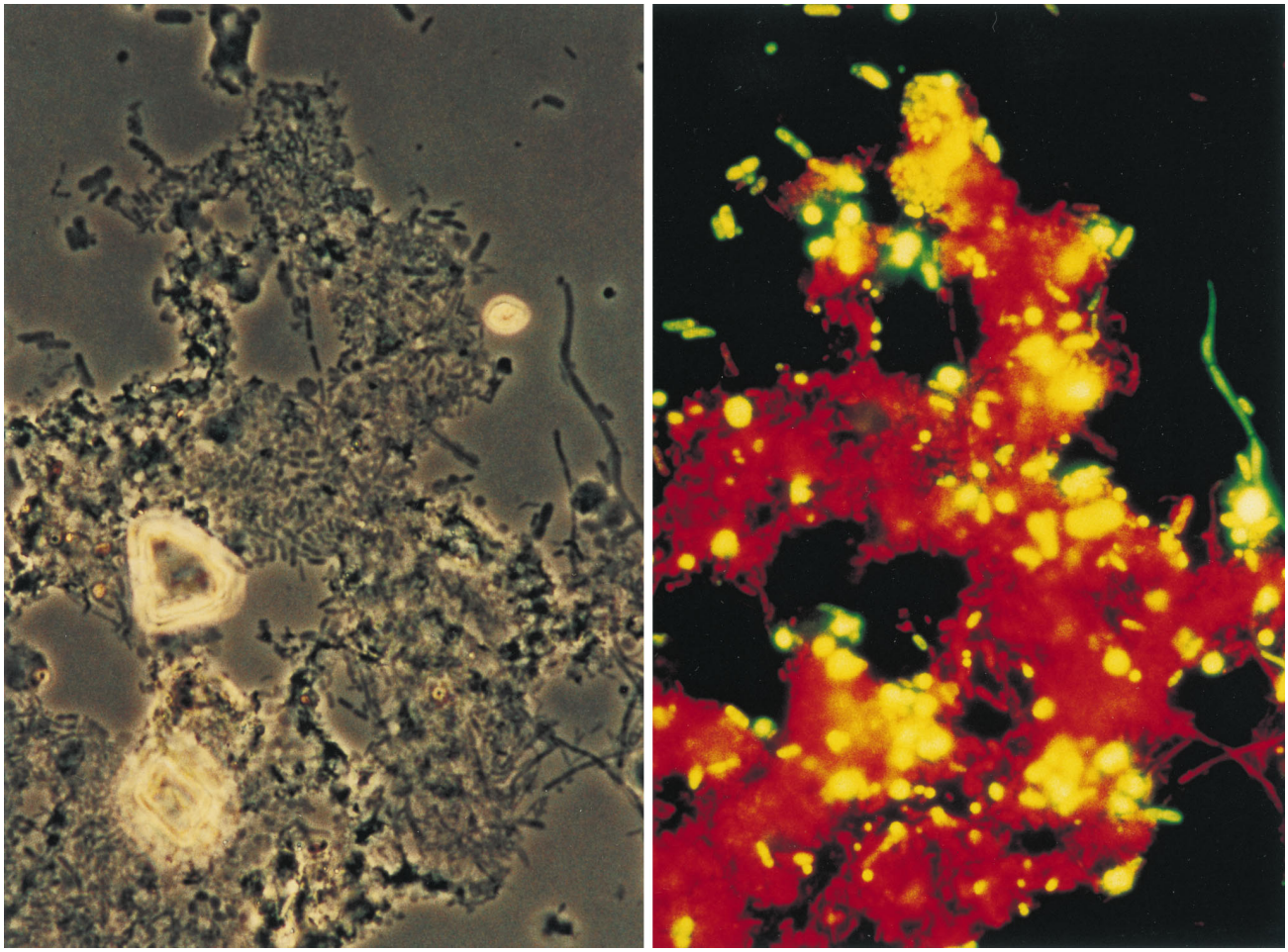

FIG. 3. Simultaneous hybridization with Cy3- and HRP-labeled oligonucleotides (probe EUB) in activated sludge. Phase-contrast (left) and epifluorescence (right) micrographs are shown for identical microscopic fields. A red-green dual-band filter set (Chroma, Brattleboro, Vt.) was used for visualization of fluorescein and Cy3.

system for log-phase and stationary cells of *E. coli* indicate that this detection system might not be quantitative.

The signal amplification of between 10- and 20-fold represents a significant increase in the sensitivity of whole-cell hybridization, and preliminary results (13a) of work with the regulatory RNA II of ColE1 plasmids (13) in *E. coli* indicate that the HRP-oligo–TSA system enables the detection of nucleic acid molecules that are present at copy numbers between 100 and 1,000. A further increase in sensitivity should be possible by using the tyramide of the currently brightest fluorochrome, Cy3 (25), as a substrate. Cy3-tyramide was recently synthesized and used for immunofluorescence (10) but is not yet commercially available.

However, it is clear from the screening of various bacteria and the application to environmental samples that the HRP-oligo–TSA system, as implemented here, lacks general applicability. The basic problem of many signal amplification systems is that they require a diffusion of large-molecular-weight molecules such as enzymes, antibodies, or (strept)avidin into whole fixed cells. The permeabilization gets more difficult for larger molecules since the margin between the accessibility of target molecules and the loss of target molecules or complete cell lysis becomes very narrow. Consequently, the use of the HRP-oligo–TSA system is recommended preferentially for well-defined target species for which optimal permeabilization protocols can be developed but not for the detection of larger groups which encompass species with different cell walls. However, even if the HRP-oligo–TSA system is applied as a tool for studies of the autecology of a defined species, it should be kept in mind that the probe permeability might be different for the different growth phases and differentiation forms of one strain. In our experiments with *E. coli*, no such differences were evident. After fixation in paraformaldehyde and lysozyme treatment, growing and stationary cells both stained well. This should be a good basis for a highly sensitive in situ hybridization assay for the detection of this important indicator species in environmental samples.

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