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Identification of Whole Fixed Bacterial Cells with Nonradioactive 23S rRNA-Targeted Polynucleotide Probes

KARLHEINZ TREBESIUS,[1] RUDOLF AMANN,[1]* WOLFGANG LUDWIG,[1]
KLAUS MÜHLEGGER,[2] AND KARL-HEINZ SCHLEIFER[1]

Lehrstuhl für Mikrobiologie, Technische Universität München, D-80290 Munich,[1] and Boehringer Mannheim GmbH, D-82327 Tutzing,[2] Federal Republic of Germany

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Polyribonucleotide probes (ca. 200 to 300 nucleotides in length) carrying multiple reporter molecules were produced by in vitro transcription with labeled UTP derivatives (fluorescein-12-UTP, 7-amino-4-methylcoumarin-3-acetyl-6-UTP, tetramethylrhodamine-6-UTP, or digoxigenin-11-UTP). Despite their length, these molecules penetrated into whole fixed gram-negative cells and hybridized specifically to their target sites on the 23S rRNA. Fluorescence intensities were quantified for target and nontarget cells by the combination of a charge-coupled device videocamera and an image-processing system. Polyribonucleotide probes confer up to 26 times more fluorescence to target cells than oligonucleotide probes do. Probe sensitivity and specificity were strongly influenced by the stringency of hybridization. The use of differently labeled probes allowed the simultaneous detection of three populations. Identification of introduced test organisms in activated-sludge samples proved the applicability of this method for the in situ identification of microorganisms in complex microbial communities.

For most microorganisms the morphology is not sufficiently complex and conserved to allow reliable light-microscopic identification. Cultivation-dependent identification methods are available but are limited to culturable organisms. In many important ecosystems this applies to only a small fraction (<1%) of the cells present (8). The lack of readily available in situ identification methods has been a long-standing problem in microbiology, interfering with our knowledge of the diversity, abundance, and spatial distribution of microorganisms. The phenotypic uniformity of microorganisms is in sharp contrast to their (phylo)genetic diversity. Consequently, in situ hybridization techniques that enable us to identify intracellular target nucleic acids and hence specific microorganisms by using complementary nucleic acid probes have a high potential to address central questions of microbial ecology.

It has been demonstrated that hybridization with fluorescently monolabeled, rRNA-targeted oligonucleotide probes facilitates the in situ identification of whole fixed bacterial cells (5, 10). These probes have successfully been applied to various environments, ranging from the identification of bacterial symbionts (1, 10, 11, 23) and magnetotactic bacteria (9, 22) to the localization of specific cells in multispecies biofilms (2, 19) or activated-sludge flocs (25). However, there have also been reports of failures to detect the majority of cells in, e.g., natural or amended soils (12) or plankton (13). These limitations most probably originate in the relatively low sensitivities of fluorescently monolabeled oligonucleotide probes that enable the detection of only the naturally amplified rRNA molecules. DeLong et al. (10) have observed a direct correlation between the growth rates of *Escherichia coli* cells, the average ribosome contents, and the fluorescence conferred by hybridization probes. With decreasing growth rate, the hybridization signal quickly approaches the detection limit of epifluorescence microscopes (10) or flow cytometers (26). This has prompted studies to develop more sensitive in situ hybridization techniques.

The use of oligonucleotides carrying multiple labels both in the hybridizing sequence and in a noncomplementary tail did not significantly increase the sensitivity of detection (26). Signal amplification by hybridization with multiple monolabeled oligonucleotides is possible (4, 26) but is often restricted to a two- or threefold increase by the limited availability of target sites with identical specificity within the rRNA molecules. Whole-cell identification of fixed bacterial cells was possible with digoxigenin- (DIG) and enzyme-labeled oligonucleotides (3, 27). If visualization takes place (indirectly or directly) via enzymatic transformation of a suitable substrate, both labels can be detected very sensitively. However, applications are hindered by the limited permeability of fixed cells for the relatively large anti-DIG-antibody or oligonucleotide-enzyme conjugate. Here we present results on the use of 23S rRNA-targeted polyribonucleotide transcript probes (17) for hybridization of whole fixed bacterial cells. Biotin-labeled transcript probes targeted to large-subunit rRNAs have already been applied to whole-cell hybridization of *Saccharomyces cerevisiae* (7) and murine leukemia cells (6). These probes were complementary to complete 25S rRNA (*S. cerevisiae* cells) or large parts (2.1 kb) of the 28S rRNA (leukemia cells) and were degraded to an average fragment size of ca. 100 nucleotides before use. Since major regions of the large-subunit rRNA molecules have been conserved in evolution, these probes were not very specific; e.g., the 25S probe bound to all lower eukaryotes (7). In contrast, this study was performed with transcript probes complementary to one variable region in domain III of the 23S rRNA molecules of bacteria (17, 21), which is also the site of significant deletions and insertions (15, 20). Consequently, such probes were expected to be much more specific and were evaluated as tools for in situ monitoring of defined bacterial populations.

* Corresponding author. Mailing address: Lehrstuhl für Mikrobiologie, Technische Universität München, D-80290 Munich, Germany. Phone: 49 (89) 2105-2373. Fax: 49 (89) 2105-2360. Electronic mail address: amann@mbitum2.biol.chemie.tu-muenchen.de.

TABLE 1. Sources, growth temperatures, and phylogenetic affiliations of strains used in this study

Organism	Source	Strain	Growth temp (°C)
α subclass of proteobacteria (*P. diminuta*)	DSM[a]	1635	30
γ subclass of proteobacteria			
A. calcoaceticus	DSM	586	30
P. alcaligenes	DSM	50342^T	30
P. putida	DSM	6695^T	30
P. pseudoalcaligenes	DSM	50188^T	30
P. stutzeri	DSM	50227	30

[a] German Collection of Microorganisms and Cell Cultures, Brunswick.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Sources and strain numbers of the bacteria used in this study are given in Table 1. All strains were grown aerobically in Luria-Bertani medium (18) and harvested at mid-exponential phase by centrifugation (5,000 × *g* for 5 min) to obtain cells with high rRNA contents.

Cell fixation. Pure cultures and activated-sludge samples were fixed by addition of paraformaldehyde solution (final concentration, 3%) and stored in a 1:1 mixture of phosphate-buffered saline (130 mM sodium chloride, 10 mM sodium phosphate buffer [pH 7.2]) and 96% ethanol at −20°C (4). Prior to hybridization, cells were spotted on precleaned six-well glass slides (Paul Marienfeld KG, Bad Mergentheim, Germany), dried at room temperature, and dehydrated in 50, 80, and 96% ethanol (3 min each). Optionally fixed immobilized cells were further permeabilized by lysozyme treatment. Cell spots were covered with 50 μl of lysozyme-EDTA solution (0.05 mg of lysozyme [Serva, Heidelberg, Germany; 150,000 U/mg] per ml in 100 mM Tris/HCl plus 50 mM EDTA [pH 8.0]) and incubated at 0°C for 7 min. The enzyme reaction was stopped by rinsing the slide thoroughly with H_2O followed by a second ethanol series.

In vitro transcription. Plasmids used for in vitro transcription were the ones described previously (17). Most experiments were performed with plasmid pPst23III, which contains a 255-nucleotide insert coding for a variable region of domain III of the 23S rRNA of *Pseudomonas stutzeri*. Transcription of the *Not*I-linearized plasmid with phage T3 RNA polymerase resulted in synthesis of probe PST23III, which encompasses the 23S rRNA complementary region (255 nucleotides) and a 60-nucleotide nonhybridizing part (between the promoter and the cloning site). The labeled UTP derivatives DIG-11-UTP (DIG-UTP), fluorescein-12-UTP (FLUOS-UTP), tetramethylrhodamine-6-UTP (TMR-UTP), and 7-amino-4-methyl-coumarin-3-acetyl-6-UTP (AMCA-UTP) were tested for enzymatic incorporation. Transcription was performed with an RNA transcription kit (Boehringer GmbH, Mannheim, Germany), as recommended by the manufacturer, at various UTP*/UTP ratios (UTP* is the symbol representing labeled UTP derivative). Transcription products were analyzed by denaturing polyacrylamide gel electrophoresis (18) and spectrophotometrically (Beckman DU650; Beckman Instruments, Munich, Germany) by comparing A_{260} values (maximum absorbance of nucleic acids) with A_{350}, A_{490}, or A_{550} values (absorbance maxima of AMCA, FLUOS, and TMR, respectively). From these values, the efficiency of labeling could be calculated by using the molar absorbance coefficients supplied by the dye manufacturers.

Whole-cell hybridization. Each well containing fixed cells was covered with 10 μl of hybridization mixture (0.01 to 0.1 M sodium chloride, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl [pH 7.2], 150 ng of transcript probe) containing variable amounts of formamide (80 to 95%) and hybridized at 53°C for 4 h in an isotonically equilibrated humid chamber. Working at one fixed temperature, we determined the stringency of hybridization by varying the formamide and sodium chloride concentrations. Hybridization was terminated by immersing slides in distilled H_2O. For comparison, some samples were hybridized with mono-FLUOS-labeled oligonucleotide probes as described previously (4, 19). Samples hybridized with fluorescent probes were mounted in Citifluor solution (Citifluor, London, United Kingdom) and viewed with an epifluorescence microscope equipped with a mercury high-pressure bulb and filter sets 01, 09, and 15 (Axioplan; Carl Zeiss, Oberkochen, Germany). Black-and-white photomicrographs were done on Kodak Tmax 400, and color photomicrographs were done on Kodak Ektachrom P1600. Visualization of hybridized DIG-labeled polynucleotide probes required a further detection step.

In situ detection of DIG-conjugated polynucleotide probes. DIG was detected by using either anti-DIG antibodies labeled with 1-nm-diameter gold particles (Biocell, Cardiff, United Kingdom) or fluorescently labeled (carboxyfluorescein or carboxyrhodamine 101) anti-DIG Fab antibody fragments (Boehringer). Hybridized cells were covered with 20 μl of detection buffer (20 mM Tris-HCl, 0.7 M sodium chloride, 1% blocking reagent) containing a gold-labeled antibody stock solution (final dilution, 1:1,000) or 25 mg of a polyclonal sheep anti-DIG antibody (Fab)–FLUOS conjugate per ml. After 1 h at 27°C, slides were washed for 10 min at the same temperature in a solution containing 150 mM sodium chloride and 100 mM Tris-HCl (pH 7.5) and finally rinsed in distilled water. Immunogold silver staining was essential for light-microscopic visualization of the 1-nm gold particles and was performed as specified by the manufacturer (Amersham International, Amersham, United Kingdom).

Quantification of fluorescence values. For comparison of fluorescence intensities, hybridized samples were recorded with a charge-coupled device camera (CF-15/2; Kappa, Gleichen, Germany) attached to the straight-through port of the epifluorescence microscope. The Interline charge-coupled device chip with an integrated mosaic-filter consisted of 681 by 582 pixels with a corresponding pixel size of 9.6 μm by 8.4 μm. The analog output signal of the camera was delivered to a color frame grabber (Imaging Technology, Bedford, Mass.) for 8-bit image processing with Optimas software (BioScan, Edmonds, Wash.). Camera parameters were at fixed settings for all measurements. For each picture a correction algorithm was applied to deal with background and shading. Then an intensity threshold was adjusted to mark the regions containing probe-conferred fluorescence. The luminance values in these areas were automatically extracted from the image and further processed with EXCEL 4.0 (Microsoft, Redmond, Wash.). For the different hybridization conditions or probe types, 100 to 320 cells were analyzed.

RESULTS AND DISCUSSION

In vitro transcription. By using T3 RNA polymerase, the UTP derivatives DIG-UTP, FLUOS-UTP, AMCA-UTP, and TMR-UTP could be incorporated into polyribonucleotide probes. Analysis by denaturing polyacrylamide gel electrophoresis showed, for each transcription reaction, one band with approximately the size expected for a full-length product. Labeled transcripts were retarded slightly relative to unlabeled

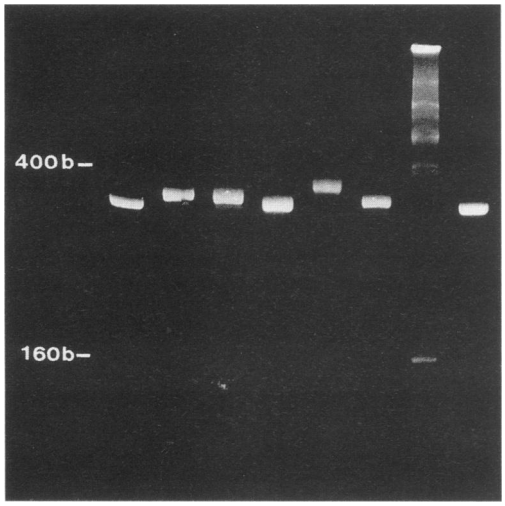

FIG. 1. Analysis of in vitro transcriptions by denaturing polyacrylamide gel electrophoresis. Nucleic acids were visualized by ethidium bromide staining (18). From left to right, the lanes contain PST23III-TMR (TMR-UTP/UTP ratio, 6.5:3.5), PST23III-AMCA (AMCA-UTP/UTP ratio, 6.5:3.5), PST23III-FLUOS (FLUOS-UTP/UTP ratio, 6.5:3.5), PST23III-FLUOS (FLUOS-UTP/UTP ratio, 3.5:6.5), PST23 III-DIG (DIG-UTP/UTP ratio, 6.5:3.5), PST23III-DIG (DIG-UTP/UTP ratio, 3.5:6.5), RNA length standard, and PST23III (unlabeled).

transcript (Fig. 1). This effect was strongest for incorporation of DIG-UTP and less pronounced for fluorescently labeled UTP derivatives. The efficiencies of incorporation of fluorescently labeled UTP and RNA yields were determined spectrophotometrically (Table 2). They were different for the different UTP derivatives and dependent on the molar ratios of UTP* to UTP. For a FLUOS-UTP/UTP ratio of 3.5:6.5, only 1 in 40 nucleotides in the synthesized probe carried a FLUOS molecule whereas approximately 1 in 15 nucleotides was labeled if the ratio was shifted to 6.5:3.5. Simultaneously, the yield of RNA transcript decreased by 25% from 8 to 6 μg/μg of template. Incorporation of AMCA-UTP was more efficient whereas incorporation of TMR-UTP was significantly lower (1 in 380 at 3.5:6.5; 1 in 110 at 6.5:3.5) under identical conditions. Incorporation of DIG-UTP was investigated in detail previously (14). Relative labeling was 1 in 25 for a DIG-UTP ratio of 3.5:6.5.

TABLE 2. Efficiency of incorporation of different UTP derivatives and RNA transcript yield

UTP derivative	UTP*/UTP ratio[a]	Incorporation[b]	Yield[c]
FLUOS-UTP	3.5:6.5	1 in 40	8.0
	6.5:3.5	1 in 15	6.6
TMR-UTP	3.5:6.5	1 in 380	7.5
	6.5:3.5	1 in 110	6.9
AMCA-UTP	3.5:6.5	1 in 20	8.0
	6.5:3.5	1 in 6	5.9
DIG-UTP	3.5:6.5	1 in 25[d]	8.3
	6.5:3.5	ND[e]	5.1

[a] Molar ratio of labeled UTP derivative (UTP*) and unlabeled UTP in the nucleotide mixture used for in vitro transcription.
[b] Average frequency of incorporation of UTP derivative in the transcripts as determined spectrophotometrically by comparing A_{260} values with A_{350}, A_{490}, and A_{550} values.
[c] Yield of transcript given as micrograms of transcript per microgram of template.
[d] As determined in reference 14.
[e] ND, not done.

Potential differences in sensitivity between polynucleotide probes and monolabeled oligonucleotide probes are significant. Probe PST23III (315 nucleotides) labeled with FLUOS-UTP at a ratio of 6.5:3.5 should confer ca. 20 FLUOS molecules to 1 target rRNA molecule. If the probe is labeled with DIG-UTP used in a ratio of 3.5:6.5 and subsequently detected with anti-DIG Fab antibody fragments carrying three FLUOS molecules, up to 36 (12 × 3) labels could be conferred to each target molecule. For a DIG-UTP/UTP ratio of 6.5:3.5, the labeling efficiency was not determined, but the higher retardation of the resulting transcript on polyacrylamide gels (Fig. 1) indicated an even higher density of DIG in the probe.

Whole-cell hybridization. Our initial observation indicated a remarkable influence of cell fixation and pretreatment on the result of whole cell hybridization. Paraformaldehyde-fixed cells of *P. stutzeri* hybridized specifically with DIG-labeled probe PST23III, and the hapten could be detected with FLUOS-labeled antibody fragments. However, the fluorescence was unevenly distributed over the cell. Brightly fluorescent rings marked the cell peripheries, whereas the cell centers were only weakly fluorescent (Fig. 2A). Such a halo appearance is characteristic for identification of cells by using fluorescently labeled antibodies binding to surface epitopes. Also, in our experiment, the halo effect indicated that the polynucleotide probes bound preferentially to target structures in the cell periphery. The mechanism of this phenomenon remains unclear, but penetration of the probe was already sufficient for specific hybridization, as evident from the binding of probe to *P. stutzeri* and not to the smaller *P. diminuta* cells. A lysozyme treatment prior to hybridization permeabilized the cell walls and resulted in evenly stained target cells (Fig. 2B). For comparison, the fluorescence conferred by FLUOS-monolabeled oligonucleotide probe Gam42a, specific for bacteria of the gamma subclass of proteobacteria (19), was documented by using identical camera settings (Fig. 2C). Obviously a much stronger signal is produced by the multilabeled probe.

To investigate whether parts of the bound polynucleotide probes, probably the nonhybridizing parts, extend into or beyond the cell walls, we tried to detect probe-conferred DIG with gold-labeled anti-DIG antibodies followed by silver enhancement. It had been reported that complete anti-DIG antibodies are too large to penetrate into paraformaldehyde-fixed bacterial cells (27). Nevertheless, the combination of probe PST23III and the gold-labeled antibody resulted in specific silver staining of the larger rods of *P. stutzeri* and not the smaller *P. diminuta* cells (Fig. 3). This detection of intracellular targets with extracellular and therefore possibly very large reporters could significantly increase the sensitivity and may allow for a probe-based physical enrichment of whole fixed cells. These options are currently being evaluated. Cells of *P. stutzeri* that had been added to activated sludge from a municipal sewage plant could be identified in this diverse collection of bacteria (Fig. 4A). This suggested that the specificities of these polynucleotide probes could be high enough for studies in complex ecosystems.

Simultaneous detection of several bacterial species in one microscopic field is possible with differently labeled probes. This was illustrated by hybridizing a mixture of cells of *P. stutzeri*, *P. diminuta*, and *P. aeruginosa* with AMCA-labeled probe PST23III, FLUOS-labeled probe PDI23III, and DIG-labeled probe PAE23III (PAE23III is specific for *P. aerugi-*

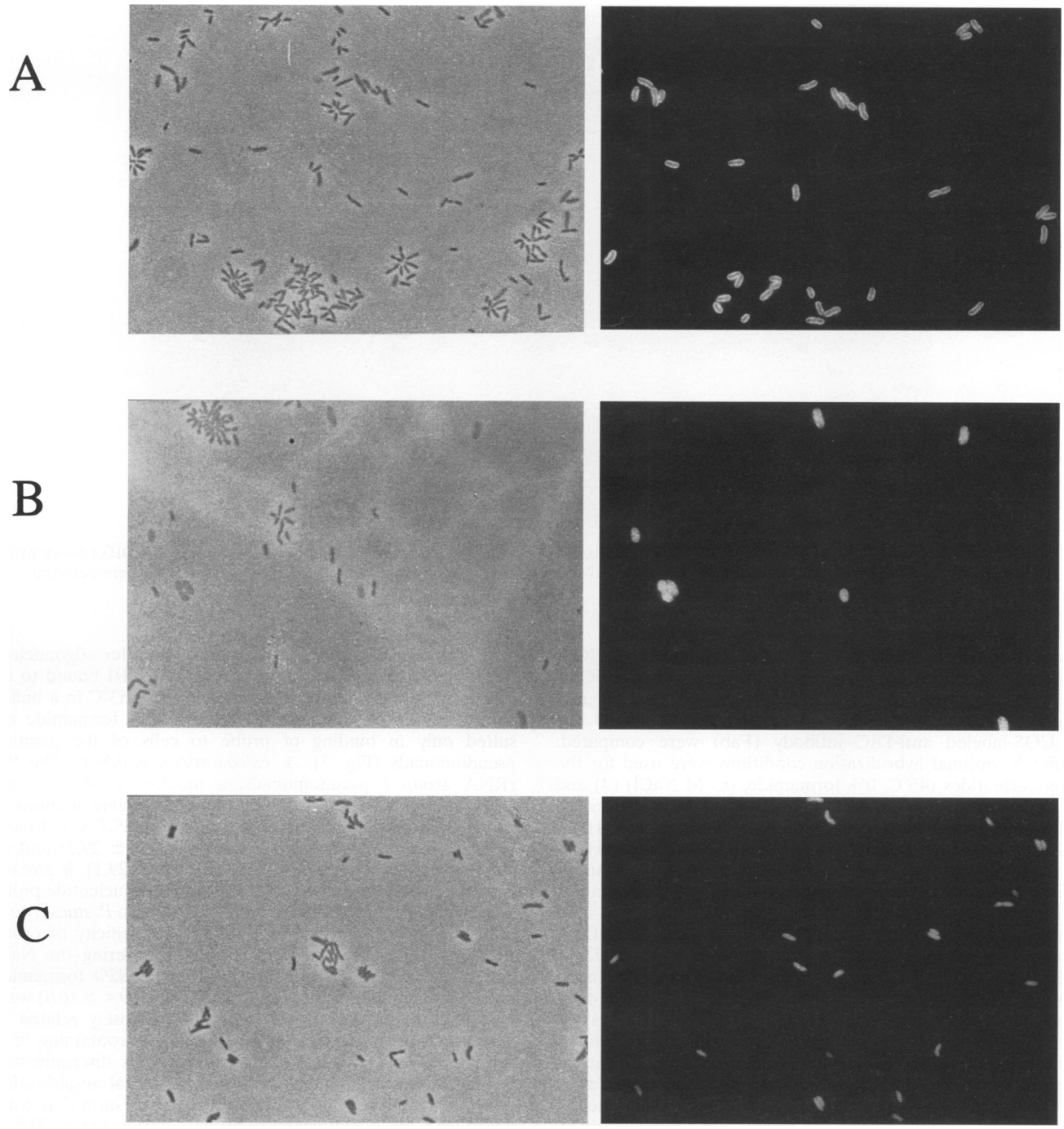

FIG. 2. Whole-cell hybridization of fixed cells of *P. diminuta* and *P. stutzeri*. Phase-contrast (left) and epifluorescence (right) photomicrographs are shown for identical microscopic fields. (A) Paraformaldehyde-fixed cells were hybridized with DIG-labeled probe PST23III and detected with FLUOS-labeled anti-DIG antibodies (Fab). (B) Paraformaldehyde-fixed cells were treated with lysozyme prior to hybridization with DIG-labeled probe PST23III and detection with FLUOS-labeled anti-DIG antibodies (Fab). (C) Hybridization with oligonucleotide probe Gam42a.

nosa). After detection of DIG with rhodamine-labeled anti-DIG Fab antibody fragments, three separate cell populations were visualized by using filter sets 01, 09, and 15 (Fig. 4B). The fact that *P. stutzeri* cells could be visualized with an AMCA-labeled probe also demonstrated the increased sensitivity of polynucleotide probes. Oligonucleotides monolabeled with this fluorescent dye, which has a molar absorbance inferior to that of FLUOS or rhodamine (16), were not sensitive enough for reliable single-cell detection (data not shown). Polynucleotide probes labeled with AMCA make a third color available for whole-cell hybridization of bacteria, thereby allowing the detection of three different populations in one sample.

Quantification of probe-conferred fluorescence. The fluorescences conferred by a FLUOS-monolabeled oligonucleotide

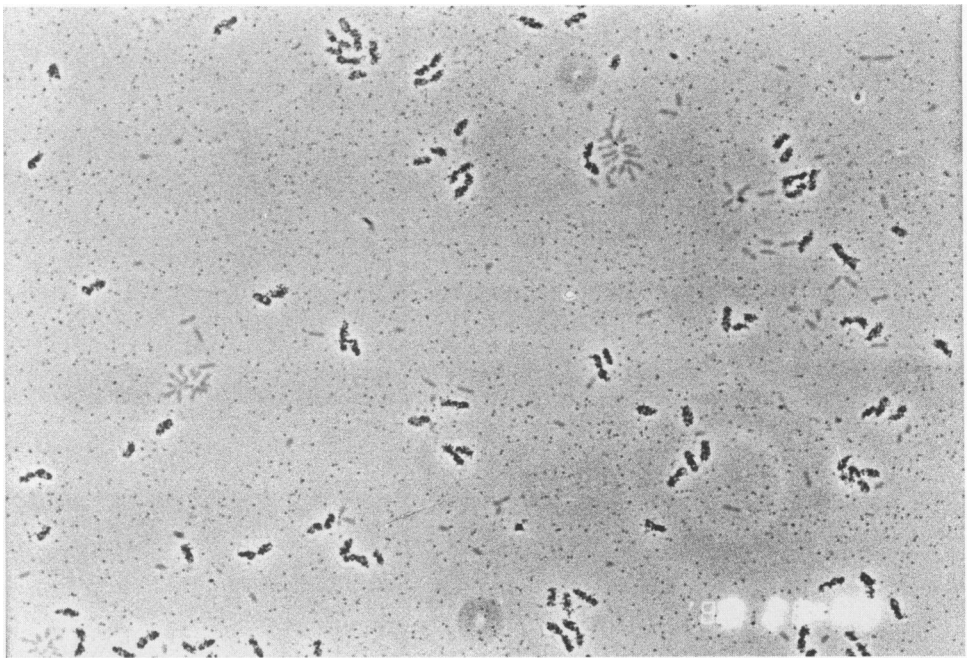

FIG. 3. Phase-contrast photomicrograph showing lysozyme-treated *P. stutzeri* and *P. diminuta* cells after hybridization with DIG-labeled probe PST23III, incubation with gold-labeled anti-DIG antibodies, and silver staining. Note that only *P. stutzeri* cells (larger rods) were detected.

probe binding to all bacteria (Eub-FLUOS) (4), the FLUOS-polylabeled transcript probe PST23III (PST23III-FLUOS), and the indirect systems with DIG-labeled oligo- and polynucleotides (Eub-DIG, PST23III-DIG) detected by FLUOS-labeled anti-DIG-antibody (Fab) were compared. Different optimal hybridization conditions were used for the oligonucleotides (46°C, 0% formamide, 0.9 M NaCl) (4) and polynucleotides (53°C, 92% formamide, 0.1 M NaCl). Since after hybridization with probe PST23III, lysozyme-treated cells of *P. stutzeri* showed a higher average fluorescence per cell (112 ± 38) than did untreated cells (99 ± 13), all quantifications were performed on pretreated cells. Quantifying between 316 and 422 cells, the average intensities (expressed as total fluorescence/1,000) per cell for Eub-FLUOS, Eub-DIG, PST23III-FLUOS, and PST23III-DIG were 3.5 ± 1.3, 7.9 ± 2.3, 28.2 ± 9.4, and 89.4 ± 43.4, respectively, corresponding to respective increases in intensity per cell of 2.3-, 8-, and 26-fold. The sensitivities and specificities of nucleic acid probes are strongly dependent on the hybridization conditions (for a recent review, see reference 24). Consequently, quantifications were also performed for target and nontarget cells at different hybridization stringencies (summarized in Fig. 5). For each of the individual datum points, 100 to 320 cells were quantified. The standard deviations were between 25 and 35% of the mean values. The variance between different hybridizations under fixed conditions was on average 10% and did not exceed 30%. First, cells of *P. stutzeri*, *P. alcaligenes*, *P. pseudoalcaligenes*, *P. putida* (all genuine pseudomonads [17]), and *Acinetobacter calcoaceticus* were hybridized under optimal conditions with the bacterial probe Eub-FLUOS. Probe-conferred fluorescence values per cell were between 3.5 ± 1.1 (*P. stutzeri*) and 17.1 ± 5.3 (*P. putida*) for the fixed-cell preparations of the different species, reflecting different cell sizes and rRNA contents. The fact that *P. stutzeri* cells had the smallest number of ribosomes ensured that discrimination against nontarget cells by probe PST23III was not caused by lower cellular ribosome contents. Under conditions optimal for oligonucleotide probe Eub, polynucleotide probe PST23III bound to all cell preparations examined. Hybridization at 53°C in a buffer containing 0.1 M sodium chloride and 95% formamide resulted only in binding of probe to cells of the genuine pseudomonads (Fig. 5). *A. calcoaceticus*, which is, like the rRNA group I pseudomonads, a member of the gamma subclass of proteobacteria, did not bind detectable amounts of probe. Some discrimination of *P. alcaligenes* (39.2 ± 16.0) and *P. putida* (22.0 ± 9.8) from *P. stutzeri* (74.5 ± 29.1) and its closest relative, *P. pseudoalcaligenes* (74.9 ± 29.1), is already measurable. Under these conditions, the polynucleotide probe conferred on average 22 times more FLUOS to *P. stutzeri* cells than an oligonucleotide probe did. The specificity of probe PST23III could be further increased by lowering the NaCl concentration (Fig. 5). In a buffer containing 95% formamide and 0.06 M sodium chloride, *P. stutzeri* cells (60.4 ± 16.9) were more fluorescent than were cells of the closely related *P. pseudoalcaligenes* (36.6 ± 13.9). In a buffer containing 95% formamide and 0.03 M sodium chloride, this discrimination became more pronounced; however, the signal amplification achieved by the polynucleotide probe with *P. stutzeri* as compared with the oligonucleotide probe deteriorated to 32.5 ± 11.3. Furthermore, the probe still bound to the nontarget cells *P. pseudoalcaligenes* (12.3 ± 4.8) and slightly to *P. alcaligenes* (3.3 ± 1.7). This nonspecific binding could be avoided by lowering the NaCl concentration to 0.01 M (Fig. 5). Under these extremely stringent conditions, the fluorescence values of *P. stutzeri* cells were further lowered (4.8 ± 2.0) and were only 1.4 times higher than the values obtained with the oligonucleotide probe. A further increase in stringency completely prevented hybridization of probe PST23III to *P. stutzeri* cells.

It is evident from these results that polynucleotide probes do indeed offer increased sensitivity compared with oligonucleotides. The signal amplifications achieved with DIG-labeled probe PST23III and FLUOS-labeled anti-DIG antibody frag-

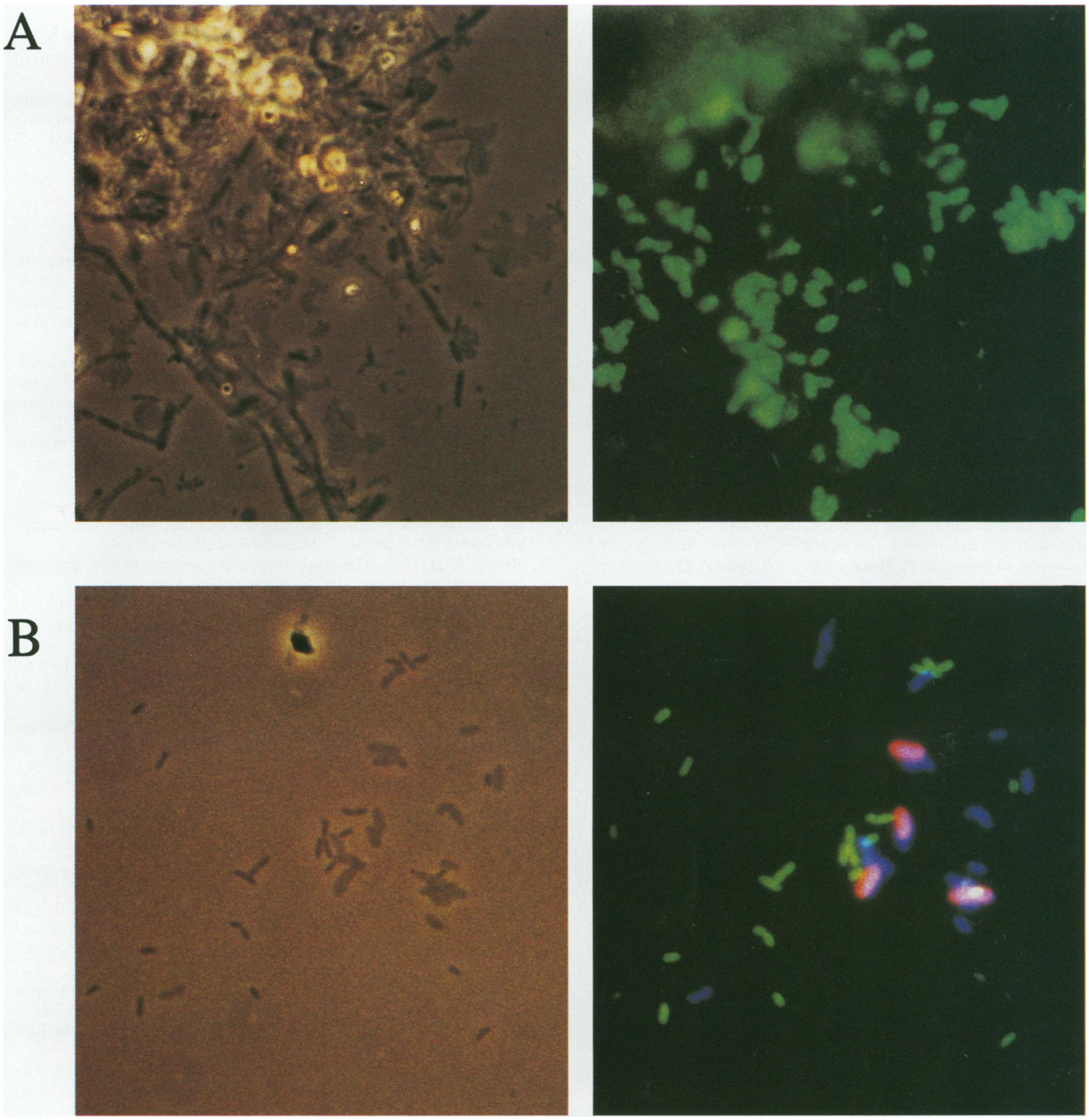

FIG. 4. (A) Phase-contrast (left) and epifluorescence (right) micrographs demonstrating the detection of individual cells in a mixture of activated sludge and a pure culture of *P. stutzeri*. Hybridized DIG-labeled probe PST23III was detected with FLUOS-labeled anti-DIG antibody fragments. (B) Whole-cell hybridization of an artificial mixture of *P. stutzeri*, *P. diminuta*, and *P. aeruginosa* with AMCA-labeled probe PST23III, FLUOS-labeled probe PDI23III, and DIG-labeled probe PAE23III. Phase-contrast (left) and epifluorescence (right) micrographs are shown for an identical field. After detection of DIG with rhodamine-labeled anti-DIG Fab antibody fragments, three separate cell populations were visualized by using specific filter sets and documented as a triple-exposure epifluorescence photomicrograph. The blue fluorescence of AMCA is restricted to *P. stutzeri*, the green fluorescence of FLUOS is restricted to *P. diminuta*, and the red fluorescence of rhodamine is restricted to *P. aeruginosa*.

ments (26- and 22-fold in two consecutive experiments) fell short of the theoretical value, which should exceed 36-fold, being the product of an average labeling of PST23III with 12 or more DIG-UTP molecules and a molar ratio of 3 to 4 fluorescein molecules per anti-DIG Fab fragment as determined by the manufacturer. Potential explanations are limited cell permeabilities for the polynucleotide probe or the antibody fragments. The increase in fluorescence of only 2.3-fold conferred by the short oligonucleotide probes Eub-FLUOS and Eub-DIG indicated a problem in the antibody step. Earlier flow-cytometric quantifications of fluorescence conferred by FLUOS- and DIG-labeled oligonucleotide probes with the same FLUOS-labeled antibody fragments also showed a factor of 2 at most and not the expected value between 3 and 4 (27).

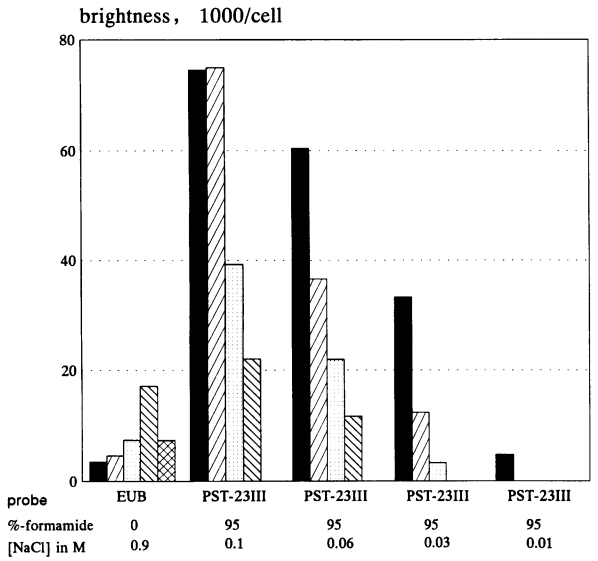

FIG. 5. Brightness per cell for *P. stutzeri* and selected reference species after hybridization with probes Eub-FLUOS and PST23III-DIG, using different hybridization stringencies. Symbols: ■, *P. stutzeri*; ▨, *P. pseudoalcaligenes*; ▩, *P. alcaligenes*; ▧, *P. putida*; ▩, *A. calcoaceticus*.

The discrepancies could originate in the inaccessibility of bound DIG molecules for anti-DIG antibodies or in self-quenching of fluorescence emitted from the FLUOS molecules on one or between different antibody molecules.

The fact that probe specificities of this new class of 23S rRNA-targeted whole-cell hybridization probes are strongly dependent on the hybridization conditions makes them quite flexible tools. For example, probe PST23III can serve for identification of all genuine pseudomonads under one set of hybridization conditions and as a specific probe for *P. stutzeri* under a different set of conditions. Of course, this also means that a reasonable interpretation of in situ identifications in complex environments requires careful control of the hybridization conditions. Furthermore, results obtained with polynucleotide probes will be strongly influenced by the permeability of the fixed cells. This will probably complicate the detection of gram-positive cells with this type of probes. However, accidental target identities are more unlikely for longer probes than for oligonucleotide probes. Therefore, hybridization of a cell with a 250-nucleotide probe will indicate its phylogenetic affiliation even more reliably (17).

In summary, the usefulness of polyribonucleotide probes for in situ detection of whole fixed cells even in complex environmental samples was demonstrated. Depending on the hybridization stringency, these multiply labeled probes are already offering significantly higher sensitivity than monolabeled oligonucleotide probes. They should facilitate the in situ detection of microbial cells with low cellular rRNA contents as they occur in oligotrophic environments.

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