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Identification of Individual Prokaryotic Cells by Using Enzyme-Labeled, rRNA-Targeted Oligonucleotide Probes

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A method to microscopically detect and identify individual cells of members of the domains *Bacteria* and *Archaea* is presented. rRNA-targeted oligonucleotides were 5′ end labeled with the enzyme horseradish peroxidase and used for whole-cell hybridization. Specifically bound probe was visualized by the enzymatic formation of an intracellular precipitate from the substrate diaminobenzidine. Permeation of the enzyme-labeled probe into whole fixed cells of gram-negative bacteria required their pretreatment with lysozyme-EDTA, whereas permeability of some archaebacterial cells was improved by addition of detergent to the hybridization buffer. Hitherto we had not achieved penetration of enzyme-labeled probe into gram-positive bacteria and yeast cells. This method should be a valuable tool for identification of suitable prokaryotic cells in environments with elevated background fluorescence or in situations in which an epifluorescence microscope is not available.

rRNA-targeted oligonucleotides end labeled with fluorescent dye have successfully been used to detect and identify individual bacterial cells (3, 5, 19). These probes are small enough to freely penetrate whole fixed cells and to hybridize specifically to intracellular rRNA. They are of particular importance to microbial ecological studies since they allow identification of hitherto uncultured organisms in situ (1). However, in many environments this promising technique is hampered by a high background fluorescence of surrounding material like soil, sediment, or plant or animal tissues. Even fixed microbial cells themselves are not necessarily free of interfering levels of autofluorescence. Photosynthetic microorganisms and methanogenic archaea can actually be detected by the characteristic fluorescence of their photosynthetic pigments (14) or coenzyme F_{420} (6); yeast cells and most protozoa show elevated levels of autofluorescence after fixation (unpublished observations). Such intrinsic fluorescence may add up to a noise level that prevents detection of the probe-conferred, specific fluorescence when a standard epifluorescence microscope is used. This limitation is compounded in natural settings in which most microorganisms exist under suboptimal conditions and correspondingly contain fewer ribosomes. The relationship between ribosome content and growth rate was long ago demonstrated (15). More recently, DeLong et al. (5) have shown a direct correlation between growth rate and the strength of the fluorescent hybridization signal (ribosome content) for *Escherichia coli*. In environmental samples, autochthonous cells may grow at a very low rate or even be in a quiescent (dormant) stage (10) and will consequently show only weak probe-conferred fluorescence following hybridization.

As part of ongoing research evaluating methods to increase the sensitivity of whole-cell hybridization, we recently demonstrated the use of digoxigenin (DIG)-labeled oligonucleotides to visualize paraformaldehyde-ethanol-fixed bacterial cells with either fluorescent or enzyme-labeled anti-DIG antigen-binding antibody fragments (Fab) (22). This indirect assay relies on both the specific binding of the nucleic acid probe carrying a reporter molecule to the target rRNA and on the specific detection of the bound reporter by the labeled antibody. However, since the high-molecular-weight enzyme-antibody conjugate must penetrate the cell wall of the fixed target cells, this approach has not served to visualize any gram-positive bacteria so far examined. As an alternative, oligonucleotides can be covalently bound to enzyme molecules via activated linker arms (9, 11, 20). Such directly labeled probes could be made smaller than the enzyme-antibody conjugate and would require fewer steps of signal development. We here present the results of a study evaluating the use of horseradish peroxidase (HRP)-conjugated probes for the identification of single cells by using light microscopy.

MATERIALS AND METHODS

Organisms and growth conditions. *Pseudomonas fluorescens* (DSM 50090) and *Pseudomonas diminuta* (DSM 1635) were chosen as representative gram-negative bacteria, and *Bacillus subtilis* (DSM 10) was chosen as the gram-positive organism. The strains were grown aerobically on YT broth (components in grams per liter: tryptone, 5; yeast extract, 5; glucose, 5; and sodium chloride, 8 [pH 7.2]) at 30°C. Cells of *Archaeoglobus fulgidus* (DSM 4309), *Methanococcus igneus* (DSM 5666), *Pyrococcus furiosus* (DSM 3638), *Pyrodictium occultum* (DSM 2709), and *Sulfolobus acidocaldarius* (DSM 639) were a generous gift from S. Burggraf, Department of Microbiology, University of Regensburg, Regensburg, Germany.

Preparation of cell smears. In order to obtain maximum cellular rRNA content, cells were harvested in mid-logarithmic phase by centrifugation in an Eppendorf microcentrifuge (5,000 × *g*, 1 min). The pellet was thoroughly resuspended in 1× phosphate-buffered saline (130 mM sodium chloride, 10 mM sodium phosphate [pH 7.2]; PBS), and 3 volumes of paraformaldehyde solution (2) were added. Following incubation at 4°C for 3 to 16 h, the fixative was removed by centrifugation and cells were resuspended in 1× PBS. An equal volume of absolute ethanol was slowly added to prevent freeze damage during storage. The cell suspensions

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were stored at −20°C and could be hybridized for several months without apparent loss of signal strength.

In preparation for hybridization, fixed cells were spotted on clean glass slides on which a hydrophobic coating separated six hybridization wells each with a diameter of 8 mm (Paul Marienfeld KG, Bad Mergentheim, Germany). The slides were then air dried for at least 2 h and dehydrated in 50, 80, and 98% ethanol for 3 min each. Prior to hybridization, wells with immobilized cells were each covered with 30 μl of lysozyme-EDTA solution (0.1 mg of lysozyme [Serva; 20,000 U/mg] per ml in 100 mM Tris HCl-50 mM EDTA [pH 8.0]) and incubated for 10 min at 0°C. The lysozyme was rinsed away with H_2O, and cells were dehydrated in an ethanol series as described above.

Oligonucleotides. The following oligonucleotide probes were used. Eub338 (2), Arch915 (18), and Euk516 (2) are complementary to regions of the small-subunit rRNA specific for members of the domains *Bacteria*, *Archaea*, and *Eucarya*, respectively (21). Probe Pdi23a (5′-TTCCACATA CCTCTCTCA-3′) (16) is complementary to a region of the 16S rRNA of *P. diminuta*. The oligonucleotides were synthesized and modified at the 5′ end with the C6-TFA aminolink [6-(trifluoro-acetylamino)-hexyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite] by MWG-Biotech (Ebersberg, Germany).

Enzyme conjugation of oligonucleotides. The labeling was performed as described by Urdea et al. (20). Approximately 100 μg of crude modified oligonucleotide was dried under vacuum in a 1.5-ml Eppendorf tube. Following resuspension in 50 μl of 0.1 M sodium borate buffer (pH 9.2), 500 μl of a *p*-phenylene-diisothiocyanate solution (20 mg/ml in dimethylformamide; Sigma, Deisenhofen, Germany) was added, and the mixture was thoroughly vortexed. The reaction mixture was incubated at room temperature for 2 h in the dark. Excess *p*-phenylene-diisothiocyanate was removed by extraction with *n*-butanol to a final volume of about 100 μl. HRP (Boehringer, Mannheim, Germany; 10 mg dissolved in 200 μl of 0.1 M sodium borate [pH 9.2]) was added to the activated oligonucleotide and incubated overnight at room temperature in the dark. Oligonucleotide-HRP conjugates were separated from unreacted enzyme and oligonucleotide by electrophoresis on a 7% polyacrylamide gel (12). The oligonucleotide-enzyme conjugate, visible as a slightly brownish band, was cut out with a sterile razor blade and transferred to a 1.5-ml polypropylene tube. Subsequently, the crushed gel was eluted three times with 1 ml of 0.1 M sodium phosphate (pH 7.5) for several hours each. The eluates were pooled, concentrated, and washed twice with 1 ml of 1× PBS in Centricon 30 microconcentrators (Amicon, Beverly, Mass.). The final product was stored at 4°C or frozen in small aliquots at −20°C.

In situ hybridization with HRP-conjugated oligonucleotide probes. Each hybridization well was covered with 10 μl of hybridization solution (0.9 M sodium chloride, 0.01% sodium dodecyl sulfate [SDS], 20 mM Tris HCl [pH 7.2]) (1) containing 0.002 optical density (260 nm) unit of HRP-labeled probe. For effective hybridization of fixed cells of *M. igneus*, the SDS concentration in the hybridization buffer had to be increased to 1%. Slides were incubated at 46°C in isotonically equilibrated humid chambers. After 2 h of incubation, the slides were washed in hybridization solution for 20 min at 48°C.

In situ detection of HRP-conjugated oligonucleotide probes. Probe-delivered enzyme was visualized by intracellular formation of a colored precipitate. The substrate diaminobenzidine (Sigma) was dissolved in 150 mM sodium chloride–50 mM Tris HCl at a concentration of 0.5 mg/ml and then made 0.003% H_2O_2 (8). Each hybridization well was covered with 50 μl of this freshly prepared substrate solution and incubated in a humid chamber at room temperature for 30 to 120 min. If necessary, the visibility of the brownish precipitate was enhanced by gold-silver intensification (13, 16, 17): wells were covered with gold chloride solution (0.1% in H_2O; Fluka AG, Neu-Ulm, Germany), rinsed twice for 10 min each with H_2O, air dried, and finally incubated for 3 min at room temperature with Gallyas developer (7). The reaction was stopped by immersing the slide in 1% acetic acid for several minutes. Cells were viewed with an Axioplan microscope (Zeiss, Oberkochen, Germany) equipped with a Plan-Neofluar 100× objective. Phase-contrast illumination was used to visualize unstained and stained cells; probe-conferred intracellular staining of cells was specifically detected with bright-field illumination. Photomicrographs were taken with Kodak Tmax 400 (Eastman-Kodak) or Agfapan 25 (Agfa-Gevaert AG) film.

RESULTS AND DISCUSSION

Specific in situ hybridization of HRP-labeled, rRNA-targeted oligonucleotide probes. Amino-linked oligonucleotides were effectively labeled with HRP by the method of Urdea et al. (20). Up to 50% of the oligonucleotides were conjugated with HRP (data not shown). The conjugates hybridized specifically to intracellular rRNA in whole fixed bacterial cells and were detected by the formation of an insoluble diaminobenzidine precipitate within the target cells (Fig. 1). Photomicrographs are shown of cells hybridized with probes distinguishing between the domains *Archaea* and *Bacteria* (Fig. 1A and B) and a probe for the specific identification of *P. diminuta* in a mixture of gram-negative bacteria (Fig. 1C). Compared with fluorescent-oligonucleotide probing, bright-field illumination substitutes for epifluorescence as a means of specific detection. The same hybridization parameters (e.g., temperature, salt concentration, and time) as those previously used for fluorescently or DIG-labeled derivatives could be applied to HRP-labeled oligonucleotides. This suggests that the relatively large HRP molecule has little influence on the stability of the oligonucleotide-rRNA duplex. In our hands this assay using a probe covalently bound to HRP yielded a higher signal-to-noise ratio than did the indirect assay, which involved the use of DIG-labeled oligonucleotide probes and their detection with enzyme-labeled anti-DIG Fab fragments (data not shown). Avoiding the immunoreaction not only saves time but also reduces the number of steps required for indirect detection. Thus, the potential for nonspecific binding of the antibody (fragment) is eliminated.

Penetration of HRP-labeled oligonucleotides into whole fixed cells. The molecular weight of horseradish peroxidase (40,000) is approximately 100 times greater than that of fluorescein or tetramethylrhodamine, the two most common labels of rRNA-targeted oligonucleotide probes for single-cell identification. This increases the overall molecular weight of a probe molecule from approximately 6,000 to about 50,000, and penetration of enzyme-labeled probe through the cell periphery might be expected to hinder whole-cell identification. However, we were encouraged by our previous studies demonstrating detection of DIG-labeled oligonucleotides with HRP-labeled anti-DIG Fab fragments (22). These conjugates have a molecular weight of at least 100,000, but nevertheless probe-conferred, HRP-mediated intracellular formation of a substrate precipitate could be

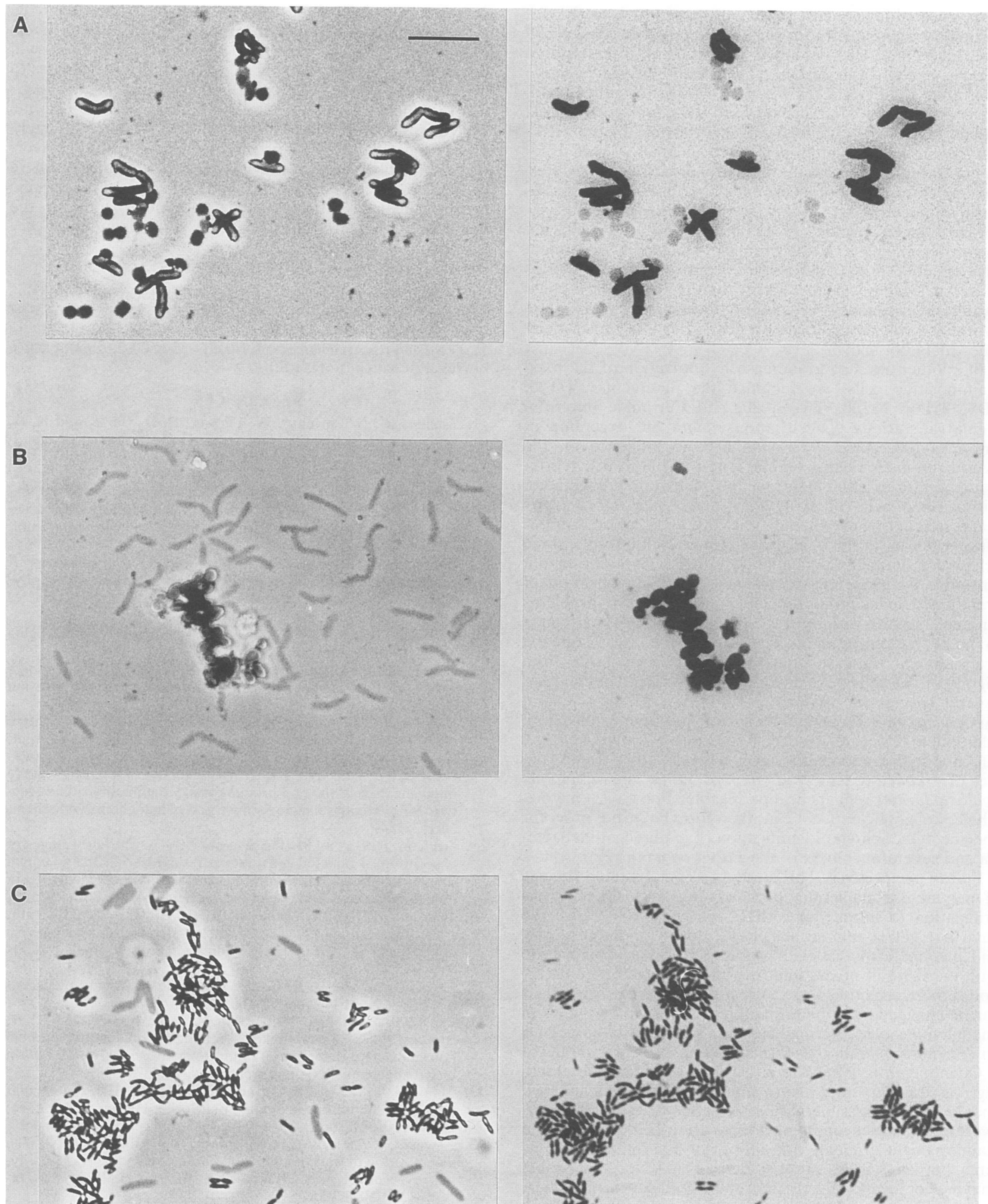

FIG. 1. Specific identification of whole fixed cells with enzyme-labeled oligonucleotide probes. Bar, 10 μm. HRP was visualized by intracellular diaminobenzidine precipitation and subsequent gold-silver intensification. Phase-contrast (left) and bright-field (right) micrographs are shown for each field. (A and B) Mixtures of *M. igneus* and *P. fluorescens* hybridized with the *Bacteria*-specific probe Eub338 (A) and the *Archaea*-specific probe Arch915 (B). (C) Mixture of *P. diminuta* and *P. fluorescens* hybridized with the specific probe Pdi23a.

demonstrated for gram-negative cells after lysozyme-EDTA treatment. Thus, it was not surprising that an enzyme-probe conjugate could also penetrate all strains of gram-negative bacteria examined in this study. But again this was effective only after lysozyme-EDTA treatment. On the other hand, the gram-positive bacteria examined (*B. subtilis* and several strains of lactococci) remained impermeable to the HRP-labeled probe even after prolonged incubation with lysozyme, lysostaphin (Sigma), or mutanolysin (Sigma) at different concentrations. Following extensive digestion, the gram-positive bacteria were only partly stained while gram-negative bacteria, added as a control, were lysed (data not shown). Proteinase K (Boehringer Mannheim) treatment was effective but could not be controlled in a way to preserve morphological integrity of the cells. They could hardly be visualized by phase-contrast microscopy and hybridized weakly probably because of loss of target molecules (data not shown).

In another attempt to increase hybridization of the fixed gram-positive cells, we included the detergents EDT20, SDS, Triton X-100, Tween 20, and Cetrimide (all from Sigma) at various concentrations. With the exception of Triton X-100, these detergents had no influence on the hybridization. Inclusion of 1% Triton X-100 in the hybridization buffer resulted in intracellular substrate precipitation within fixed cells of *B. subtilis* and *Lactococcus lactis* (harvested during exponential growth), using probe Eub338. Since even in an actively growing pure culture only some cells were stained, enzyme-labeled oligonucleotides cannot currently be used for specific single-cell identification of gram-positive bacteria. We encountered similar problems with cells of *Saccharomyces cerevisiae*, and again attempts to make the intracellular RNA accessible by enzymatic pretreatment (lyticase and β-glucoronidase; both from Sigma) of the cells or detergent addition failed. In contrast, several species of *Archaea* (*A. fulgidus*, *M. igneus*, and *Pyrococcus furiosus*) could be sufficiently permeabilized by addition of 1% SDS to the hybridization buffer to allow effective hybridization (Fig. 1B), whereas other tested species of *Archaea* (e.g., *Pyrodictium occultum* and *Sulfolobus acidocaldarius*) remained impermeable. For now, enzyme-labeled oligonucleotides can therefore be used only for specific detection and identification of gram-negative bacteria and several members of the *Euryarchaeota* (21).

Future perspectives. This study has demonstrated the use of enzyme-oligonucleotide probe conjugates for specific hybridization to intracellular rRNA within whole cells. We have not yet compared sensitivity limits for fluorescently and enzymatically labeled probes. However, comparison with the work of others indicates that enzyme-based detection is more sensitive, especially if the enzyme assay format can be changed from the formation of colored precipitates to chemiluminescent intermediates (4). We are also looking for an enzyme substrate that forms a fluorescent precipitate.

Quantification of cellular rRNA contents via probe binding, as demonstrated using fluorescent oligonucleotides, should also be possible with enzyme-labeled probes. However, one should keep in mind that cell-to-cell differences in staining might originate not only from differing rRNA contents but also from variable permeability for the probe-enzyme conjugates. Furthermore, the gold-silver intensification used in our assay is very sensitive but has been difficult to control in a way necessary to obtain quantitative results. Studies of cellular rRNA content should therefore be performed with fluorescence-labeled oligonucleotides.

We are continuing to evaluate alternative approaches to permeabilize whole fixed cells. Although we are optimistic that methods can be tailored for single strains under investigation, we do not expect to find a universal method that will permeabilize the whole array of different microorganisms to a comparable degree. This means that general probes (e.g., for the three domains) should not be used as enzyme derivatives for the characterization of environmental samples because they will likely produce biased results. Nevertheless, highly specific enzyme-probe conjugates used in combination with suitable methods to permeabilize specific cells of interest should provide a valuable tool for the detection and identification of individual cells in situ.

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