JOURNAL OF BACTERIOLOGY, Feb. 1988, p. 720–726
0021-9193/88/020720-07$02.00/0

Vol. 170, No. 2

Phylogenetic Group-Specific Oligodeoxynucleotide Probes for Identification of Single Microbial Cells

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Examination of collections of 16S rRNA sequences revealed sequence domains that were unique to (and invariant within) the three primary lines of cellular descent: the archaebacteria, the eubacteria, and the eucaryotes. Oligodeoxynucleotides complementary to these conserved sequence domains were synthesized and used as hybridization probes. Each of the radiolabeled probes specifically hybridized to nylon membrane-bound 16S rRNA from the targeted kingdom. A probe complementary to a universally conserved sequence in 16S rRNAs was used as a positive control, while its complement provided a negative control for nonspecific binding. The abilities of the probes to bind specifically to whole, fixed cells representing a broad array of phylogenetic diversity were tested in whole-cell dot blot assays. Again, all of the probes specifically bound the targeted groups. By microautoradiography, the method was extended to permit phylogenetic identification of single cells microscopically.

Nucleic acid hybridization has long been used to elucidate relationships among cultivated bacteria (9, 10, 17). It has also been used to detect and quantitate microorganisms in mixed microbial populations (11). Nucleic acid hybridization probes have broad applications for microbial identification, medical diagnoses, the detection of genetically altered microorganisms in the environment, and the study of population structure and dynamics in microbial ecosystems. In general, hybridization probes used for microbial identification are highly specific, commonly consisting of cloned genes from particular organisms. Such a specific hybridization probe usually requires cultivation of the organisms of interest in order to produce the probe.

An alternative to hybridization probes that detect specific organisms would be probes that distinguish phylogenetic groups of organisms. Organisms that are evolutionarily related are expected to have common properties, the extent of commonality depending on the phylogenetic breadth of the group. Such probes could be used for characterizing organisms, even in the absence of other preliminary information. In this paper we describe the preparation and use of phylogenetic group-specific probes based on 16S rRNA sequences.

In recent years, rRNA sequences have been used to infer quantitative evolutionary relationships among numerous, diverse, cellular life forms (20). Because of their large size (1,500 to 2,000 nucleotides), the 16S-like rRNAs have been particularly useful. Some segments in the 16S rRNA are invariant in all organisms and therefore are useful as binding sites for oligodeoxynucleotide primers for sequencing protocols (12). Other portions of the 16S rRNAs are unique to particular organisms or related groups of organisms and hence offer targets for hybridization probes with various specificities. The ubiquity of the rRNAs ensures that probes can be designed to identify virtually any organism or group of related organisms.

To illustrate the application of phylogenetic group-specific hybridization probes, we have identified and synthesized oligodeoxynucleotides that are diagnostic for each of the three primary lines of evolutionary descent: the eubacteria, the archaebacteria, and the eucaryotes. The probes are complementary to the rRNAs. Actively growing cells may contain 10^4 ribosomes, each a potential probe target, making it possible to label and identify single microbial cells by in situ hybridization and microautoradiographic procedures. This approach is particularly applicable to the analysis of natural microbial ecosystems, for it is a common theme that only a small fraction of the organisms present can be cultivated and identified by classical techniques. Hybridization probes, such as described here, permit uncultivated organisms to be characterized phylogenetically.

MATERIALS AND METHODS

Probe synthesis and labeling. Oligodeoxynucleotides, 16 to 20 nucleotides long, were synthesized on an Applied Biosystems automated DNA synthesizer. Mixtures of deoxynucleoside phosphoramidites were introduced at steps in the synthesis where nucleotide degeneracy was desired. After deblocking, oligodeoxynucleotides were purified by electrophoresis on 15% polyacrylamide gels and recovered by elution as described previously (12).

Phage T4 polynucleotide kinase (Pharmacia) was used to 5′-end label 0.5 μg of oligonucleotide probe (18). Either 1.0 mCi of [γ-^{32}P]deoxyadenosine-5′-triphosphate (1,073 Ci/mmol; New England Nuclear Corp., Boston, Mass.) or 50 μCi of ^{35}S-labeled 2′-deoxyadenosine-5′-*O*-(γ-thiotriphosphate) (1,372 Ci/mmol; New England Nuclear) was used as the substrate. Labeled probes were purified on C8 reverse-phase Bond Elut columns (Analytichem International, Harbour City, Calif.) as described previously (12).

Organisms and growth conditions. *Chlamydomonas reinhardii* 137C was grown on TAP medium (7). Strains of *Escherichia coli* and *Bacillus megaterium* were obtained from the Indiana University culture collection and grown at 37°C on 2× YT medium containing (in grams per liter) tryptone, 16; yeast extract, 10; and NaCl, 5. *Halobacterium volcanii* was grown at 25°C on Gupta medium containing (in grams per liter) NaCl, 125; $MgCl_2 \cdot 6H_2O$, 45; $MgSO_4 \cdot 7H_2O$, 10; KCl, 15; $CaCl_2 \cdot 6H_2O$, 0.2; yeast extract, 5; and tryptone, 5. *Anacystis nidulans* was grown under cool-white fluorescent illumination on medium BG-11 (15). *Saccharomyces cerevisiae* was obtained from the Indi-

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ana University culture collection and grown at 25°C on 2× YT medium supplemented with glucose (10 g/liter). *Sulfolobus solfataricus* was grown at 80°C in medium containing (in grams per liter) $(NH_4)_2SO_4$, 1.3; KH_2PO_4, 0.3; $MgSO_4 \cdot 7H_2O$, 0.3; KCl, 15; $CaCl_2 \cdot 2H_2O$, 0.1; and yeast extract, 1.0. The pH was adjusted to 3.5 with H_2SO_4. *Drosophila melanogaster* KC cells were grown as described elsewhere (5). The *Methanobacterium* sp. was an undescribed strain kindly provided by David Lane.

Nylon membrane hybridizations. RNA was purified by phenol extraction of French pressure cell lysates (14). 16S and 23S rRNAs were isolated by zonal sedimentation in 5 to 20% exponential sucrose gradients in buffer (10 mM Tris hydrochloride [pH 8.0], 100 mM NaCl, 1 mM EDTA) for 15 h at 30,000 rpm in an SW41 rotor (Beckman) at 4°C.

Solutions of rRNAs in 10 mM Tris hydrochloride (pH 7.4) were applied to strips of nylon membrane (1 by 5 cm; GeneScreen Plus, Du Pont) and dried in vacuo at 80°C for 1 h; each spot contained 50 ng of rRNA. The filters were then incubated in 0.5 ml of hybridization buffer without the labeled probe for 30 min at 37°C, followed by the addition of 10^6 cpm of ^{32}P-labeled DNA probe per filter and incubation overnight. The hybridization buffer contained 1× Denhardt solution (0.2 g of Ficoll [Sigma; average M_r, 400,000], 0.2 g of polyvinylpyrrolidone [Sigma], and 0.2 g of bovine serum albumin [Sigma; fraction V] per liter), 0.02 M sodium phosphate buffer (pH 7.6), 10% dextran sulfate (Sigma; average M_r, 500,000), 0.1 mg of polyriboadenosine (Sigma) per ml, 1 mM dithiothreitol, and 5× SET buffer (1× SET buffer is 150 mM NaCl, 1 mM EDTA, and 20 mM Tris hydrochloride, pH 7.8). Following hybridization, the nylon membranes were washed in four changes of ice-cold 0.2× SET buffer for 10 min each.

Cell blot hybridizations. The method used for hybridizations was modified from that of Yu and Gorovsky (22). Cells were harvested by centrifugation, suspended at 10^9 per ml in 5 ml of PBS (145 mM NaCl, 100 mM sodium phosphate, pH 7.5), and then stirred on ice as glutaraldehyde was added to 0.5%. After 30 min of stirring on ice, cells were washed twice in PBS and then suspended in 5 ml of 145 mM NaCl–10 mM Tris hydrochloride, pH 7.5. An equal volume of absolute ethanol was added to the suspension while stirring on ice. Fixed cells were stored at −20°C until use. Dilutions of fixed cells were made in a 1:1 mixture of 145 mM NaCl–10 mM Tris-HCl, pH 7.5, and absolute ethanol. Fixed cells (ca. 20 μg [dry weight] per spot) were filtered onto poly-L-lysine-coated glass fiber filters (Whatman 934-AH) with a HYBRI. DOT manifold (Bethesda Research Laboratories). Filters were air dried, and the backs were sprayed with acrylic coating (Krylon 1303, Crystal Clear Acrylic) for mechanical support during subsequent manipulations. Filters were immersed in an aqueous solution containing 0.1 M triethanolamine (pH 8.0 with HCl) for 10 min at room temperature. Acetic anhydride was added to 0.25% (vol/vol), and the filters were incubated an additional 10 min. The filters were then rinsed with 0.2× SET and air dried. Dried filters were subsequently placed in heat-sealable bags with 15 ml of hybridization mix (0.75 M NaCl, 0.15 M Tris hydrochloride [pH 8.3], 10 mM EDTA, 20 mM sodium phosphate [pH 6.8], 1× Denhardt solution, 10% dextran sulfate, 2×10^6 cpm of ^{32}P-labeled probe). Bags were sealed and incubated at 37°C for 6 to 18 h. Filters were washed three times with 0.2× SET for 10 min each at 37°C. After drying, filters were exposed to X-ray film for 6 to 24 h.

Slide hybridizations. Clean glass slides were pretreated to promote cell adhesion by dipping in a solution of 0.1% gelatin, 0.01% $CrK(SO_4)_2 \cdot 12H_2O$ (subbing solution) at 70°C, followed by air drying. Cells suspended in growth medium were applied as 5-μl spots and air dried. Slides were then fixed in 0.5% glutaraldehyde–20 mM sodium phosphate buffer (pH 7.6) for 2 min and transferred through an ethanol series (50%, 2 min; 80%, 2 min; 100%, 2 min) before air drying. Fixed slides were stored for up to 1 year at −70°C in desiccated containers without loss of probe-binding activity. Immediately prior to hybridization, slides were acetylated by immersion in an aqueous solution containing 1% acetic anhydride and 0.1 M triethanolamine (pH 8.0 with HCl) and then rinsed for 5 min in 50 mM Tris hydrochloride (pH 8.5). Background binding of ^{35}S-labeled probes was significantly diminished by reduction of the fixed, acetylated slides with 50 mM sodium borohydride in 100 mM Tris hydrochloride buffer (pH 8.5) for 30 min in darkness. Following borohydride reduction, slides were rinsed in 50 mM Tris hydrochloride buffer (pH 7.6) and air dried.

Prior to hybridization, slides were treated for 30 min with 30 μl of hybridization buffer under a cover slip (22 by 22 mm). The hybridization buffer was the same as that used for nylon screens, except that the polyriboadenosine concentration was 1.0 mg/ml. After pretreatment, the cover slips were removed and the slides were air dried; 30 μl of hybridization buffer containing 10^6 cpm of radiolabeled probe was applied and covered with a cover slip, followed by incubation for 6 h at 25°C. Slides were then gently washed three times in 0.2× SET at 37°C for 10 min each and air dried.

Labeling of individual cells was visualized by microautoradiography. Following the posthybridization wash and drying, slides were dipped in nuclear track emulsion (NTB2; Kodak) diluted 1:1 with water and then allowed to air dry in complete darkness. After 2 weeks of exposure, the slides were developed for 4 min in D-19 (Kodak) diluted 1:1 with water, followed by two rinses in distilled water, 4 min of photographic fixation (Kodak Fixer), and 5 min in distilled water. After development of the autoradiographic emulsion, slides were stained with Giemsa for 15 min (0.75 mg of Giemsa per ml, 5% methanol, 5% glycerol), rinsed gently for 30 min in distilled water, and air dried.

RESULTS

Design of kingdom-specific probes. To explore the utility of phylogenetic group-specific hybridization probes, we focused first on broad groups: the three Ur-kingdoms defined by Woese et al. on the basis of rRNA sequence comparisons (21). Potential probe targets were identified by comparing the complete 16S rRNA sequences of 14 eucaryotes, 7 eubacteria, and 5 archaebacteria. Although only a limited number of sequences were available for comparison at the time the probes were produced, the collection included sequences that spanned substantial diversity. The target sites for the probes were identified as sequences that are invariant, or nearly so, in all members of a particular kingdom but differ significantly in all the representatives of the other two Ur-kingdoms. Oligodeoxynucleotides 16 to 20 residues in length and complementary to the kingdom-specific sequences were synthesized for evaluation as hybridization probes. In the case of the archaebacteria-specific oligonucleotide, degeneracy was introduced at one residue to accommodate variation in known archaebacteria rRNA sequences (Fig. 1). An 18-nucleotide "universal" probe, complementary to a sequence in all cellular 16S-like rRNAs, was used as a positive control to ensure that the cell types inspected were permeable to the group-specific probes. An

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Mouse                          5' UUGACGGAAGGGCACCACCAGGAGUGG-GCCUG 3'
B. subtilis                    5' UUGACGGGGGCCCGC-ACAAGCGGUGGAGCAUG 3'
H. volcanii                    5' UUGGCGGGGGAGCACUACAACCGGAGGAGCCUG 3'
Eubacterial probe                    3' CCCGGGCG-TGTTCGCCA 5'
                                                    |
                                                   343*
Archaebacterial consensus      UUGGCGGGGGAGCACNACAANNGGNGGANCCUG
Eukaryotic consensus           UUGACGGAANNGCACNACNAGNNGUGGNNCNUG

Mouse                     5'        UACCUGGUUGAUCCUGCCAGUAG-CAUAU 3'
B. subtilis               5' UUUAUCGGAGAGUUUGAUCCUGGCUCAGGACGAA  3'
H. volcanii               5'        AUUCCGGUUGAUCCUGCCGGAGGUC-AUU 3'
Archaebacterial probe           3' AAGGCCAACTAGGRCGGCCT 5'
                                                    |
                                                   21*
Eubacterial consensus             GNNGAGUUNGAUCNUNGCUCAGNNNGAA
Eukaryotic consensus              NANNNGGUUGANCCUGCCAGNNNNCAUA

Eukaryotic probe          5' GGGCATCACAGACCTG 3'
                             |
                          1209*
Universal probe           5' GWATTACCGCGGCKGCTG 3'
                             |
                           536*
Control probe             5' GTGCCAGCMGCCGCGG 3'
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FIG. 1. Sequences of archaebacterial and eubacterial probes aligned with corresponding sequences of 16S rRNAs used in filter blots and consensus sequences from nontargeted kingdoms at the homologous positions. Mismatches are in boldface type. N, Variable nucleotide; W, A or T; K, G or T; M, A or C. *, Position numbers refer to the *E. coli* 16S rRNA (3).

oligonucleotide complementary to the universal probe (i.e., it was rRNA-like, as opposed to complementary to the rRNA) was used as a background control; it should be incapable of hybridizing with the rRNA.

Hybridization of probes to filter-bound RNA. We first tested the abilities of the synthetic probes to bind to isolated RNAs in a kingdom-specific manner. This was essential because the probe target sequences, even though present in the rRNA, might not be accessible for the formation of stable hybrids. The rRNA molecules are highly compacted by secondary and tertiary structure, which could preclude the binding of the probes. Moreover, even if bound, a probe might be displaced from hybridization by competitive association of the target sequence with another segment of the rRNA.

Figure 2 shows the results of hybridizing ^{32}P-labeled probes to dot blots of various RNAs immobilized on nylon membranes. Hybridizations were carried out at a series of temperatures, followed by washing at lower temperatures (lower stringency). This method inspects the hybridization of the probes under equilibrium conditions, providing an estimate of the dissociation temperature (T_m) of any hybrids formed. This approach to evaluating the stability of oligonucleotide hybrids is preferable to the use of high-temperature washes following hybridization at low stringency, which assesses stability under nonequilibrium conditions. With the latter, nonequilibrium technique, dissociation may occur at temperatures substantially below the nominal melting point of the hybrids. This is because the dissociation temperatures of oligonucleotide hybrids, as opposed to polynucleotide hybrids, depend on the concentrations of the complements (19). For each of the kingdom-specific probes, it was possible to identify a temperature range at which binding to the target rRNA was selective. Although we have not evaluated the T_ms of the probe-rRNA hybrids in detail, they were about as expected (Fig. 2), 50 to 70°C (4). None of the probes hybridized with *Bacillus subtilis* 23S rRNA, providing further assurance of their specificity.

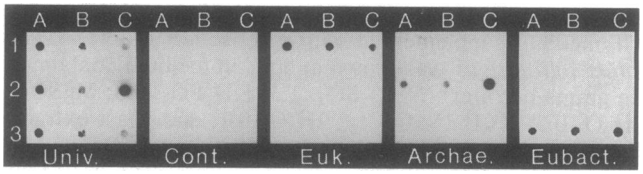

FIG. 3. Hybridization of ^{32}P-labeled oligodeoxynucleotides to microbial cells immobilized on glass fiber filters. Each spot contains 20 μg (dry weight) of glutaraldehyde-fixed cells. Hybridization conditions are discussed in Materials and Methods. Cells: 1A, *Saccharomyces cerevisiae*; 1B, *Drosophila melanogaster* KC cells; 1C, *Chlamydomonas reinhardii*; 2A, *Halobacterium volcanii*; 2B, *Sulfolobus solfataricus*; 2C, *Methanobacterium* sp.; 3A, *Escherichia coli*; 3B, *Bacillus megaterium*; 3C, *Anacystis nidulans*. See Fig. 2 legend for probe abbreviations.

Hybridization of probes to fixed cells. The binding of the probes to a broad diversity of fixed, intact cells collected on glass fiber filters was studied also. As shown in Fig. 3, the specificity of the hybridization of the probes to intact cells was the same as seen for RNA bound to the nylon membranes. For example, the eucaryotic probe bound to the eucaryotes tested, but it did not hybridize to the representatives of the other kingdoms. All of the organisms inspected bound the universal probe; none bound the negative-control (rRNA-like) oligonucleotide. The universal and eucaryotic probes both hybridized readily to yeast spores (not shown),

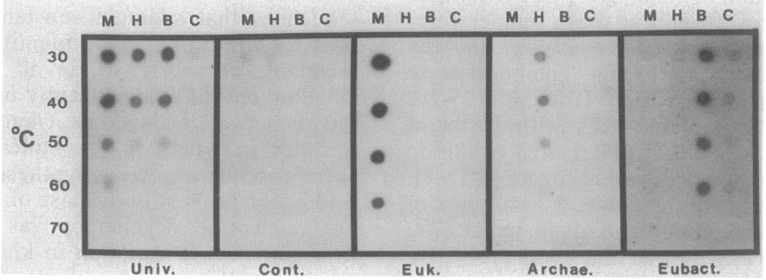

FIG. 2. Temperature dependence and sequence specificity of probe hybridization to nylon membrane-bound rRNAs. Each spot contained 50 ng of RNA. Blots were hybridized to 10^6 cpm of ^{32}P-labeled DNA probe, as discussed in Materials and Methods. Abbreviations: M, mouse 18S rRNA; H, *Halobacterium volcanii* 16S rRNA; B, *Bacillus subtilis* 16S rRNA; C, *B. subtilis* 23S rRNA; Univ., universal probe; Cont., control probe; Euk., eucaryotic probe; Archae., archaebacterial probe; Eubact., eubacterial probe.

indicating that the fixation protocol rendered even dense spore coats permeable to molecules the size of these oligonucleotides (5,200 to 6,500 daltons).

All of the cell dots shown in Fig. 3 contained the same nominal cell masses (dry weights, after extraction with 50% ethanol and water), yet the extents of binding of the various probes clearly varied severalfold. For instance, the *Methanobacterium* sp. bound substantially more of both the universal probe and the archaebacterial probe than did equivalent dry weights of *Halobacterium volcanii* or *Sulfolobus solfataricus*. This result could be due to variations in cellular RNA contents or different permeability to the probes. The specific ribosome content of bacteria is known to be inversely proportional to doubling time over a wide range of growth rates (8). However, in some cases different levels of binding of the oligodeoxynucleotide probes by the various cell types appeared to be related to the particular probe. For example, *Anacystis nidulans* bound more of the eubacterial probe than the other eubacteria tested, but it bound the least universal probe. This result cannot be due to the permeability of the fixed cells or to cellular RNA contents. The variation may result from variable accessibility of the different rRNA target sequences in the structure of the fixed ribosomes.

Detection of probe-binding to single cells. Because of inevitable background binding, the use of oligonucleotide probes to determine phylogenetic group representation in mixed microbial populations by hybridization to cells collected on a filter could be unreliable if the target organisms constitute a minor fraction (a few percent or less) of the population. Moreover, the simple hybridization of a group-specific probe to a mixed population of organisms does not provide information on the complexity of the target phylogenetic group. Consequently, we were interested in extending the resolution of the method to the level of individual cells. If this could be accomplished, it would be possible to enumerate cells that bind the hybridization probes and to gain some perspective on the diversity of morphotypes that constitute the phylogenetic group detected.

The oligonucleotide probes were readily labeled to specific activities of about 40 μCi/μg with [γ-^{35}S]ATP and polynucleotide kinase as detailed in Materials and Methods. ^{35}S emits β particles at an energy of 0.167 MeV, providing a sufficiently localized exposure of photographic emulsion to detect the deposition of the radioactive probes in individual cells (16). Initial attempts at labeling glutaraldehyde-fixed cells on microscope slides with oligodeoxynucleotides containing the [^{35}S]phospothionate group were frustrated by nonspecific background labeling, possibly a consequence of reactions between the phosphothionate groups and unstable imines resulting from aldehyde cross-linking within the cells. This spurious binding of the probes was rectified by reducing the fixed cells with sodium borohydride prior to hybridization. Group-specific binding of the probes could then be visualized. The results paralleled those obtained with rRNAs bound to nylon membranes and fixed cells collected on glass fiber filters.

As described in Materials and Methods, hybridizations were carried out with glutaraldehyde-fixed cells attached to glass slides. Following washing to remove unbound probes, labeling was detected by autoradiography, and cells were stained with Giemsa. Figure 4 illustrates the fidelity of the kingdom-specific probes. Eucaryotes were labeled by the eucaryotic probe but not the eubacterial probe, and vice versa. Neither cell type bound the archaebacterial probe, which in turn specifically hybridized to the archaebacteria tested (not shown). The results in Fig. 4 were obtained with homogeneous populations of organisms, but the probes could also identify a particular phylogenetic type in mixtures of organisms, as shown in Fig. 5.

DISCUSSION

The probes described here allocate the inspected organisms to the primary lines of evolutionary descent: the archaebacteria, the eubacteria, and the eucaryotes. This is a very broad classification, but it provides substantial information about the fundamental properties of the cells: the primary kingdoms differ profoundly in many fundamental cellular properties. Probes with finer phylogenetic resolution can be derived by using the existing collections of 16S (or 23S) rRNA sequences. For instance, we have designed a hybridization probe, not described here, that specifically binds to cyanobacteria. Probes selective for other phylogenetic groups could be designed and synthesized from currently available sequence information. rRNA target sequences appropriate for species-specific probes can also be identified (6). As the data base of rRNA sequences expands, the phylogenetic identification of organisms, especially microorganisms, will become increasingly useful. Furthermore, the coupling of phylogenetic group-specific probes with in situ hybridization techniques provides a means of assessing natural microbial ecosystems by phylogenetic criteria.

The relatively small size of oligonucleotide hybridization probes minimizes problems of cellular permeability and access to binding sites. Other methods of constructing probes rely on enzymatic syntheses of complementary RNA or DNA molecules from cloned genome fragments. In general, these approaches produce probes of greater length than those described here. Nick translation and end-labeling methods produce symmetric (double-stranded) DNA probes which can self-anneal as well as bind to target sequences (1). Larger, asymmetric RNA probes may be transcribed from genes of target RNAs cloned adjacent to a phage T7 RNA polymerase promoter (1). Asymmetric probes also may be made by reverse transcription from RNA templates. Some of these types or probes, hundreds of nucleotides in length, have been successfully hybridized to intact, fixed cells (E. DeLong, unpublished). However, questions about the relative permeability of cells to these larger probes remain unanswered.

The sequences that we selected as target sites for the kingdom-specific probes were not unique at all of the sequence positions. For instance, the archaebacterial probe was 45% similar to the eubacterial consensus sequence and 65% similar to the eucaryotic consensus sequence at the homologous positions. In these cases, binding specificity remained uncompromised; even a single base pair mismatch in an oligodeoxynucleotide hybrid of 17 base pairs can result in an 11°C decrease in the melting point of the hybrid duplex (19). Thus, oligonucleotides that differ in sequence at only one position are potentially useful as sequence-specific probes.

The design of probes for specific phylogenetic groups is dependent on analyses of adequate sequence data bases. As more data become available, consensus sequences for specific phylogenetic groups become more accurate. Examination of 16S and 18S rRNA sequences which have been published since these probes were designed revealed organisms with slight variations from the signature sequences described here. For example, the archaebacterial probe

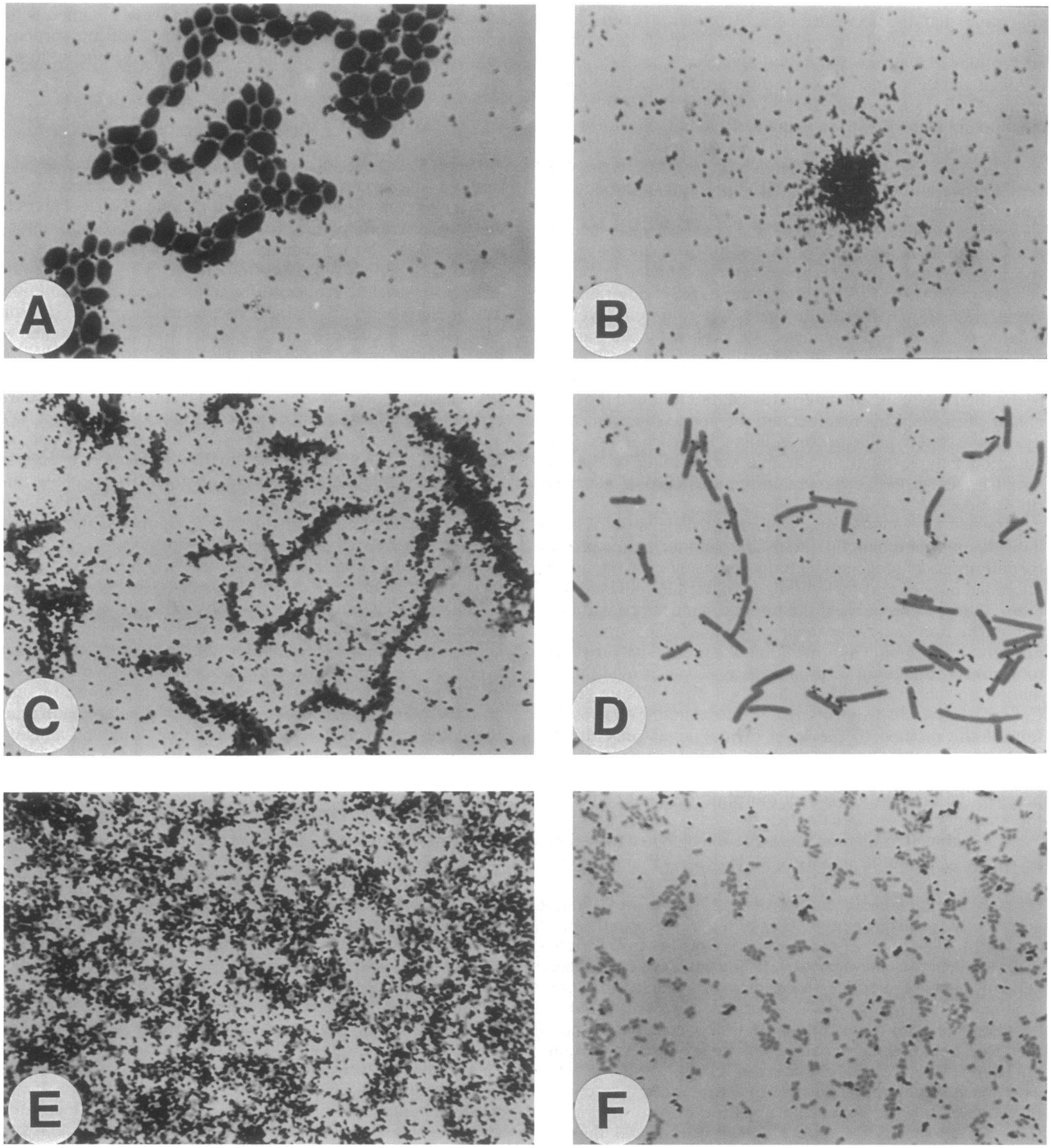

FIG. 4. Microautoradiographs of single cells after hybridization to ^{35}S-labeled DNA probes, as described in Materials and Methods. Hybridization cocktails contained 10^6 cpm in a volume of 30 μl. Cells were stained with Giemsa and photographed by bright-field microscopy. Panels: *S. cerevisiae* (spores) hybridized with the eubacterial (A) and eucaryotic (B) probes; *B. megaterium* hybridized with the eubacterial (C) and eucaryotic (D) probes; *E. coli* hybridized with the eubacterial (E) and eucaryotic (F) probes.

described here has mismatches at two positions to homologous sites in the 16S rRNAs of *Methanospirillum hungatei* and *Thermoproteus tenax*. Additional probes could be designed to bind specifically organisms with these sequence variations, or else inosine, which base pairs relatively nonspecifically, could be incorporated at variable positions.

It can be seen in Fig. 4 that the use of ^{35}S as an isotopic label compromised the resolution of the in situ hybridizations when applied to small cells. Because of the emission energy of ^{35}S, the resolution (the distance from a point source at which the grain density falls to one-half that directly over the point source) is 2 to 5 μm. Thus, grains are

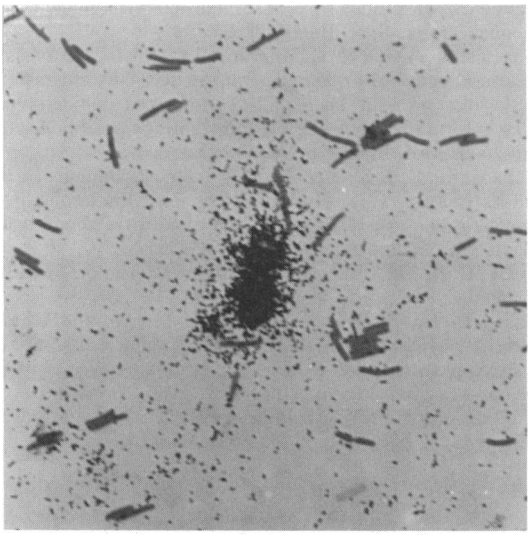

FIG. 5. Microautoradiograph of a mixed field containing *S. cerevisiae* and *B. megaterium* cells after hybridization to ^{35}S-labeled eucaryotic probe, as described in the legend to Fig. 4. The cells were stained with Giemsa and photographed by bright-field microscopy.

clearly localized over large cells, such as *Bacillus megaterium* or *Saccharomyces cerevisiae*; however, the binding of the probes to small cells, such as *Escherichia coli*, is not well defined. In principle, this problem could be improved by using a less energetic isotopic label than ^{35}S, for instance, ^{3}H, which has a resolution of 0.5 to 1.0 μm. However, longer exposure times would be required to visualize the labeled cells. It also may prove possible to use fluorescent probes, visualizing their binding in the fluorescence microscope. This approach would be more rapid and have better spatial resolution than autoradiography.

Hybridization probes can be used to evaluate the relative amounts of particular organisms, or groups of organisms, that are present in mixed populations. One approach to this is measuring the amount of a group-specific probe that is bound to bulk rRNA or to whole cells in mixed populations. Since the rRNA content of cells is proportional to growth rate over a wide range of growth rates (8), the amount of a universal probe hybridized per unit of biomass could provide an estimate of the metabolic activity of a population. Phylogenetic group-specific probes would provide information on the relative composition of the population and could be used to follow population dynamics or distributions along clines. We have found it convenient to probe bulk preparations of glutaraldehyde-fixed cells stored in 50% ethanol. Such samples are readily prepared in the field and retain the ability to hybridize probes after months of storage at −70°C. However, significant uncertainties must be considered if the binding of probes to bulk preparations is to be used for the quantitative analysis of natural samples. The amount of a probe that is specifically bound may be influenced by many variables, including the permeability of fixed cells and the accessibility of the rRNAs in fixed preparations.

A second approach to quantifying natural microbial populations is the use of microautoradiography to detect in situ hybridization. This approach provides a means of microscopically counting the constituents of microbial populations (2). Since numbers of labeled cells, not the amount of probe bound, are determined, this method is relatively insensitive to variations in the efficiency of probe binding by different cell types. Furthermore, for some purposes (for example, locating symbionts within host tissues or characterizing morphologically conspicuous bacteria), in situ hybridizations offer a means of achieving a detailed phylogenetic identification of organisms without the requirement for isolation or cultivation. We have stored fixed, desiccated slides at −70°C for 1 year without significant loss of the ability to bind hybridization probes. Thus, this method can be applied readily to field studies.

The use of nucleic acid hybridization probes for the detection, quantitation, and identification of microorganisms has expanded rapidly in recent years. Such methods are clearly applicable to the study of uncultivatable microorganisms from natural microbial populations, either through the use of phylogenetic group-specific probes, such as those described here, or by the construction of organism-specific probes from sequences of rRNA genes cloned from natural microbial populations. The large number of rRNA molecules in each cell renders them particularly suitable as hybridization targets. We believe that hybridization techniques based on rRNA sequences will contribute substantially to studies of microbial ecosystems.

ACKNOWLEDGMENTS

We are grateful to Mitchel Sogin for providing us with the eucaryotic 18S rRNA sequencing primer that we used as a eucaryotic probe.

This work was supported by Public Health Service grant GM34527 from the National Institutes of Health to N.R.P., National Science Foundation grant BSR-8600170 to S.J.G., and Office of Naval Research grant N14-86-K-0268 to G.J.O.

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