Combination of 16S rRNA-Targeted Oligonucleotide Probes with Flow Cytometry for Analyzing Mixed Microbial Populations

RUDOLF I. AMANN,[1]† BRIAN J. BINDER,[2] ROBERT J. OLSON,[3] SALLIE W. CHISHOLM,[2]
RICHARD DEVEREUX,[1] AND DAVID A. STAHL[1]*

*Departments of Veterinary Pathobiology and Microbiology, University of Illinois, Urbana, Illinois 61801[1];
Ralph M. Parsons Laboratory 48-425, Massachusetts Institute of Technology, Cambridge,
Massachusetts 02139[2]; and Biology Department, Woods Hole Oceanographic
Institution, Woods Hole, Massachusetts 02543[3]*

Fluorescent oligonucleotide hybridization probes were used to label bacterial cells for analysis by flow cytometry. The probes, complementary to short sequence elements within the 16S rRNA common to phylogenetically coherent assemblages of microorganisms, were labeled with tetramethylrhodamine and hybridized to suspensions of fixed cells. Flow cytometry was used to resolve individual target and nontarget bacteria (1 to 5 μm) via probe-conferred fluorescence. Target cells were quantified in an excess of nontarget cells. The intensity of fluorescence was increased additively by the combined use of two or three fluorescent probes complementary to different regions of the same 16S rRNA.

Assessments of diversity, abundance, and activity of water column microorganisms are fundamental to studies in aquatic microbiology. Yet, most past measurements have been compromised by methodology, principally the requirement for pure-culture isolation and the use of subjective criteria for classification. Recent studies in molecular evolution and molecular phylogeny have provided new perspectives and new tools for studies in environmental microbiology. Most notably, the comparative sequencing of homologous biopolymers has served to reveal phylogenetic relationships among microorganisms. Extensive sequencing of the ribosomal RNAs (5S and 16S- and 23S-like rRNAs) has been particularly informative. Sequence divergence among these individual rRNAs has defined the outline of a natural classification of microorganisms (28). These data can also be used to design hybridization probes for determinative studies in microbiology.

The 16S-like rRNA has been the common target for determinative hybridization probes (1, 25). By using selected regions within the larger rRNA molecules (16S- and 23S-like rRNAs) as hybridization targets for synthetic oligonucleotides, probe specificity can generally be freely adjusted. Microbial species or subspecies can be distinguished by oligonucleotides complementary to the most variable regions of the molecule (25). By targeting regions of increasing conservation, probes can be made to encompass specific genera or higher taxons (e.g., the three kingdoms: archaebacteria, eucaryotes, and eubacteria) (29). Finally, some regions of the rRNAs have remained essentially unchanged in all sequenced species; these can be used as targets for universal probes. Universal probes have been used to measure total rRNA abundance in the environment and to assess differences in cellular rRNA content (1, 25).

A single *Escherichia coli* cell has between 10^4 and 10^5 ribosomes and, consequently, as many copies of 5S and 16S- and 23S-like rRNAs (15). Thus, the rRNA is a naturally amplified target for hybridization probes. Giovannoni and

co-workers first demonstrated that because of the high copy number, individual cells can be identified by using singly radiolabeled oligonucleotides (10). More recently, fluorescent dye-labeled probes have been used for the direct microscopic identification of single cells; the term "phylogenetic stain" was coined to describe the unique character of these probes (1, 8). Although fluorescent antibodies have been applied to the identification of single cells in the environment, their specificity is generally restricted to the species or subspecies level (3, 5, 16, 26) and their development requires previous isolation of the target organism. In contrast, the potential specificity of rRNA-targeted fluorescent probes spans the entire phylogenetic spectrum. Furthermore, based on the extensive 16S rRNA sequence data base, probes can be designed for organisms which have not yet been brought into culture (19).

Flow cytometry is a well-established method for measuring selected physical and chemical characteristics of individual cells. Multiple parameters (e.g., forward and 90° light scatter and fluorescence emission at wavelengths of interest) can be determined individually for a large number of cells in a short time (up to several thousand cells per second). In the last 5 years flow cytometers have been applied to ecological studies, especially to measure the distribution and abundance of marine picoplankton (6, 14, 18, 20, 23).

Currently, most applications of flow cytometry to environmental samples make use of various morphological and physiological characteristics of the cells (e.g., size and pigment content of photosynthetic organisms) (20). These criteria generally are not sufficient for identification at the genus or species level. Staining with DNA-specific fluorochromes offers information about numbers of bacterial cells but not about their identity. The combined use of dyes that bind preferentially to G·C or A·T base pairs has been used to distinguish organisms of different G+C content (C. A. Sanders, D. M. Yajko, W. Hyun, R. G. Langlois, P. S. Nassos, M. J. Fulwyler, and W. K. Hadley, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, R-1, p. 280) but offers limited insight into community composition: organisms with identical percents G+C are not necessarily related phylogenetically. Although flow cytometric detection of rRNA in fixed eucary-

* Corresponding author.
† Present address: Lehrstuhl für Mikrobiologie, Technische Universität München, 8000 Munich 2, Federal Republic of Germany.

```
eubacterial                                        eukaryotic

probe        3'-TGAGGATGCCCTCCGTCG-5'              probe         3'-CCTCCCGTTCAGACCA-5'
target       5'-ACUCCUACGGGAGGCAGC-3'              target        5'-GGAGGGCAAGUCUGGU-3'
E.coli*      UCCAGACUCCUACGGGAGGCAGCAGUG           E.coli*       -AAGCACCGGCUAACUCC-GUGCCA
D.gigas*     UCCAGACUCCUACGGGAGGCAGCARUG           D.gigas*      -AAGCACCGGCUAACUCC-GUGCCA
D.hydrogen*  UCCAGACUCCUACGGGAGGCAGCAGUG           D.hydrogen*   -AAGCGCCGGCNAACUCN-GUGCCA
Dsv.desulf   UCCAGACUCCUACGGGAGGCAGCAGUG           Dsv.desulf    -AAGCACCGGCUAACUCC-GNGCCA
Dssar.var    UCCAGACUCCUACGGGAGGCAGCAGUG           Dssar.var     -AAGCACCGGCUAACUCC-GUGCCA
Myx.xant     UCCAGACUCCUACGGGAGGCAGCAGUG           Myx.xant      -AAGCACCGGCUAACUCU-GUGCCA
Agr.tume     CCCAAACUCCUACGGGAGGCAGCAGUG           Agr.tume      -AAGCCCCGGCUAACUUC-GUGCCA
Flav.fer     GCNNGACUCCUACGGGAGGCAGCAGUA           Flav.fer      -AAGCACCGGCUAACUCC-GUGCCA
B.subtil     CCCAGACUCCUACGGGAGGCAGCAGUA           B.subtil      -AAGCCACGGCUAACUAC-GUGCCA
Mc.van       CCCCAGGCCCUACGGGGCGCAGCAGGC           Mc.van        -AAGGGCUGGGCAAGUUCGGUGCCA
D.discoideum CUACCACUUCUACGGAAGGCAGCAGGC           D.discoideum  CAAUUGNAGGGCAAGUCUGGUGCCA


SRB                                                Desulfobacter

probe        3'-GGACTGCGTCGCTGCGGC-5'              probe         3'-AAACCCCTACTCARACGCMT-5'
target       5'-CCUGACGCAGCGACGCCG-3'              target        5'-UUUGGGGAUGAGUYUGCGKA-3'
E.coli*      GCAAGCCUGAUGCAGCCAUGCCGCGUG           E.coli*       ----GCCAUCGGAUGUGCCCAGAUGGGAU
D.gigas*     GAAAGCCUgACGCAGCGACGCCGCGUG           D.gigas*      ----ACAAUGAGAUGAGUCCGCGUCUCAU
D.hydrogen*  GCAACCCUGACGCAGCAACGCCGCGUG           D.hydrogen*   ----GUUUGGGGAUGAGUCUGCGGACCAU
Dsv.desulf   GAAAGCCUGACGCAGCGACGCCGCGUG           Dsv.desulf    ----ACGUAAGGAUGAGUCCGCGUCCCAU
Dssar.var    GAAAGCCUGACGCAGCGACGCCGCGUG           Dssar.var     ----GUUUGAAGAUGGGCCCGCGUACCAU
Myx.xant     GAAAGCCUGACGCAGCAACGCCGCGUG           Myx.xant      ----ACAUUCAGAUGAGUCCGCGGCCCAU
Agr.tume     GCAAGCCUGAUCCAGCGACGCCGCGUG           Agr.tume      ----GGGGUAUGAUGAGCCCGCGUUGGAU
Flav.fer     GAAAGUCUGAACCAGCCAUGCCGNGUG           Flav.fer      ----GCUAGAAGACGGGUGUGCGGCUGAU
B.subtil     GAAAGUCUGACGGAGCAACGCCGCGUG           B.subtil      ----ACUUACAGAUGGACCCGCGGCCGCAU
Mc.van       GAAAGUGCGACGGGGGGACCCCAAGUG           Mc.van        -----CCCGAGGAUAGGACUGCGCUCGAU
D.discoideum -AUACGGGGAAGUAGUGACAAUAAAU-           D.discoideum  -----AAGUCUACUGUGUCACUGCCCUAU
```

FIG. 1. 16S rRNA sequence alignments showing target regions of probes. An asterisk (*) marks the sequences of the three species used in this study. The 16S rRNAs compared in these alignments are of the following organisms: E. coli*, *E. coli* (4); D. gigas*, *Desulfovibrio gigas* (9); D. hydrogen*, *Desulfobacter hydrogenophilus* (9); Dsv. desulf, *Desulfovibrio desulfuricans* (21); Dssar. var, *Desulfosarcina variabilis* (9); Myx. xant, *Myxococcus xanthus* (21); Agr. tume, *Agrobacterium tumefaciens* (30); Flav. fer, *Flavobacterium ferrugineum* (27); B. subtil, *Bacillus subtilis* (11); Mc. van, *Methanococcus vannielii* (13); and D. discoideum, *Dictyostelium discoideum* (17). Mismatched pairings and deletions are shaded.

otic cells has been demonstrated by using biotin-labeled transcription products as probes (2), long probes encompass conserved tracts of sequence and therefore lack the specificity offered by short oligonucleotide probes. Here we document the use of flow cytometry to quantify defined mixtures of bacteria hybridized with 16S-like rRNA-targeted fluorescent oligonucleotide probes. Although the limits of cell size and ribosome content have not yet been evaluated, this approach could extend the use of flow cytometry to direct detection and identification of virtually any microorganism in the aquatic environment.

MATERIALS AND METHODS

Design of oligodeoxynucleotide probes. A data base of about 250 complete and partial 16S rRNA sequences was used to identify probe targets of appropriate specificity. The following oligonucleotides were used (all numberings list the corresponding positions in the *E. coli* 16S rRNA): (i) eubacterial (5'-GCTGCCTCCCGTAGGAGT-3'), specific for all eubacteria (positions 338 to 355); (ii) sulfate-reducing bacteria (SRB) (5'-CGGCGTCGCTGCGTCAGG-3'), inclusive for most species of the δ-group of purple bacteria (positions 385 to 402); (iii) desulfobacter [5'-T(C/A)CGCA(G/A)ACTCAT CCCCAAA-3'], specific for the genus *Desulfobacter* (positions 220 to 239); (iv) eucaryotic (5'-ACCAGACTTGCCC TCC-3'), specific for eucaryotes (positions 502 to 516). Figure 1 shows sequence alignments of the 16S rRNA of several microbial species in the target region for these probes.

Source and growth of strains. Three species of eubacteria of known 16S rRNA sequence were used: *E. coli* TB1 (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), *Desulfovibrio gigas* (ATCC 19364), and *Desulfobacter hydrogenophilus* (DSM 3380). Growth temperatures and media for the different organisms were: *E. coli* at 37°C in aerobic TY medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl

[pH 7.2]), *D. gigas* at 30°C in anaerobic freshwater-lactate medium (7), and *D. hydrogenophilus* in anaerobic saltwater-acetate medium (7). The cells were grown to the late logarithmic phase and harvested by centrifugation in an Eppendorf microcentrifuge (12,000 rpm, 2 min, 4°C). The growth medium was decanted, and the cells were suspended by thorough vortexing in cold phosphate-buffered saline (PBS) solution (130 mM sodium chloride, 10 mM sodium phosphate buffer [pH 7.2]).

Cell fixation and storage. The cells were fixed with fresh (not older than 24 h), cold paraformaldehyde solution (4% in PBS). One volume of cell suspension was mixed with 3 volumes of fixative, and the mixture was incubated at 4°C for 16 h. The cells were pelleted by centrifugation (Eppendorf microcentrifuge; 12,000 rpm, 2 min, 4°C), washed with PBS solution to remove residual fixative, pelleted again, and resuspended at a concentration of 10^8 to 10^9 cells per ml in PBS solution. One volume of fixed cell suspension was added to 1 volume of cold absolute ethanol (10), and the mixture was stored at −20°C for up to 2 weeks without apparent influence on the hybridization.

Direct counting of cells. The concentration of cells in the fixed suspensions was determined by direct counting of 4',6'-diamidino-2-phenylindole-stained cells on membrane filters (12, 22). Samples of the fixed-cell suspension were diluted to 1 ml in 0.2-μm-pore-size-filtered water and stained for 10 min in a filter tower by addition of 10 μl of a 1-mg/ml stock solution of 4',6'-diamidino-2-phenylindole. Cells were concentrated onto a 0.2-μm-pore-size membrane filter (25-mm diameter; Poretics Corp., Livermore, Calif.) by applying a slight vacuum. After washing with 1 ml of 0.2-μm-pore-size-filtered water, the filters were mounted in oil and observed immediately by epifluorescence microscopy.

Synthesis and purification of fluorescent oligodeoxynucleotides. The oligodeoxynucleotides were synthesized with one primary amino group at the 5' end (Aminolink 2; Applied
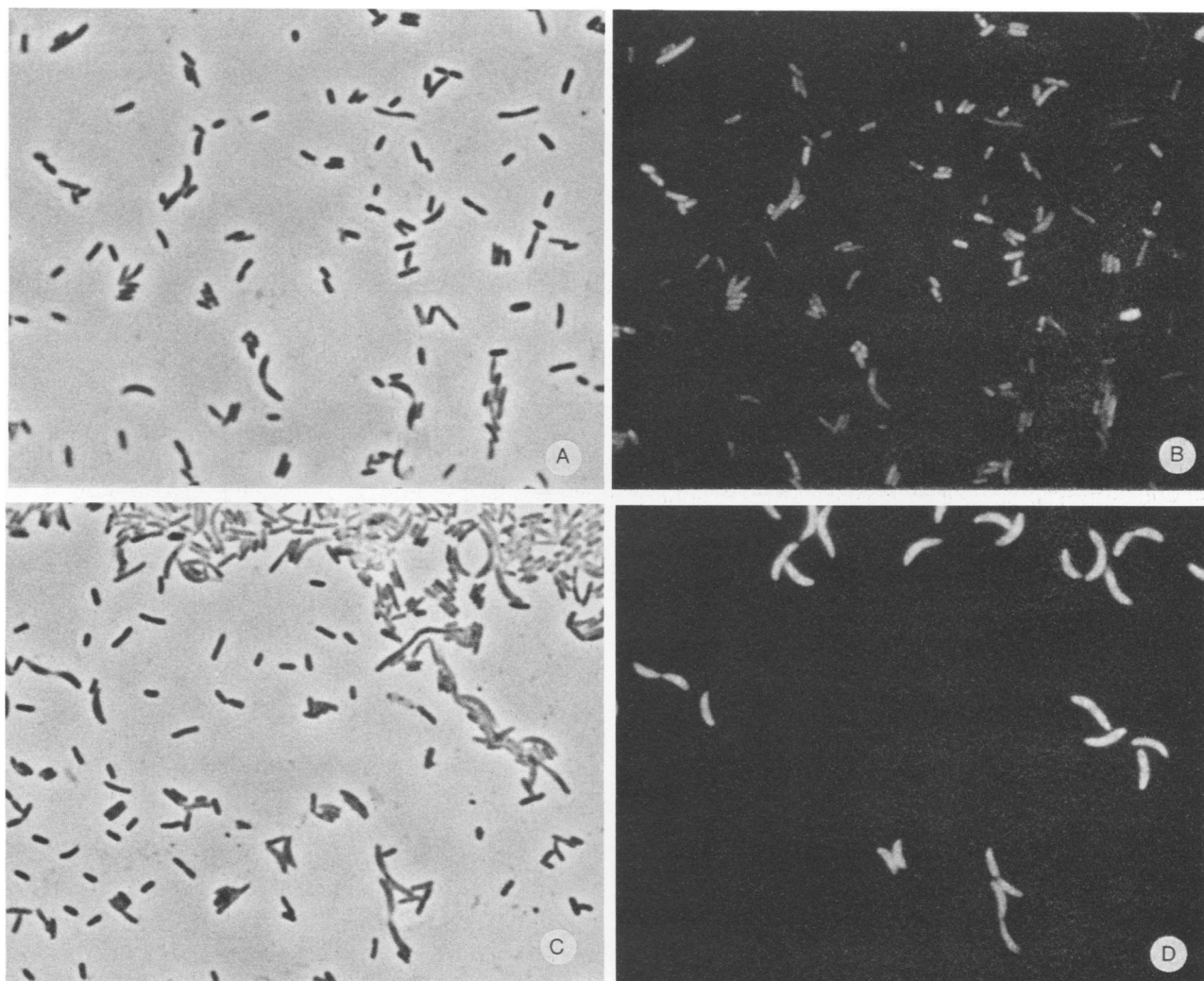
FIG. 2. Whole-cell hybridization with tetramethylrhodamine-labeled probes. All hybridizations were performed at 45°C for 1 h followed by a 30-min washing step at 48°C. Phase-contrast (left) and epifluorescence (right) photomicrographs are shown for each field. (A and B) *D. gigas* (vibrios) and *E. coli* (rods) hybridized with the eubacterial probe. (C and D) *D. gigas* and *E. coli* hybridized with the SRB probe.

Biosystems, Foster City, Calif.). Tetramethylrhodamine iso-thiocyanate (Research Organics, Cleveland, Ohio) was co-valently bound to the amino group as earlier described (1). The dye-oligonucleotide conjugate (1:1) was purified from unreacted components (1) and stored at −20°C in double-distilled water at a concentration of 50 ng/μl.

Hybridization of fixed cells with fluorescent probes. The purity and quality (intact morphology and high rRNA content) of the fixed cells as well as the quality of the probe were evaluated by hybridization of cells bound to gelatine-coated slides. Small portions (1 to 3 μl) of the cell suspension in ethanol-PBS were spread on gelatin-coated slides and allowed to air dry. After dehydration in 50, 80, and 100% ethanol (2 min each), the cells were hybridized at 45°C with probes as previously described (1). Following hybridization, the slides were washed for 30 min at 48°C in 0.9 M sodium chloride–0.1% sodium dodecyl sulfate–20 mM Tris hydrochloride (pH 7.2). The cells were observed with an Olympus BH2 microscope (Olympus Optical Co., Tokyo, Japan) fitted for epifluorescence with a high-pressure mercury bulb and

filter set no. BL 0892 and documented by photography as previously described (1).

Hybridization for flow cytometric analysis was modified as follows: 1 to 7 μl of fixed-cell suspension was transferred to a prewarmed (45°C) 1.6-ml Eppendorf tube and mixed with 50 μl of prewarmed hybridization buffer (0.9 M sodium chloride, 0.1% sodium dodecyl sulfate, 100 μg of polyade-nylic acid per ml, 20 mM Tris hydrochloride [pH 7.2]). After adding 200 ng of tetramethylrhodamine-labeled oligodeoxy-nucleotide probe, the tube was immediately transferred to a 45°C incubator. After 1 h, the hybridization was stopped by adding 1 ml of 0.2-μm-pore-size-filtered double-distilled water and the resulting suspension was immediately used.

Flow cytometric analysis. Analyses were performed on a flow cytometer (model Epics-V; Coulter Electronics, Inc., Hialeah, Fla.) equipped with a Biosense flow cell and a 6-W argon ion laser. The laser was tuned for 515-nm emission (500 mW) and focused with a 38-mm spherical quartz lens (spot size, approximately 20-μm diameter). Fluorescence was measured as light passing a 585-nm band pass interfer-

ence filter (45 nm at full band width at half maximum transmission) and a 530-nm-long pass absorbance filter. Signals were amplified with 3 decade log amps and collected at a rate of approximately 10^3 signals per s; histograms shown represent a total of 30,000 to 60,000 events each. Fluorescence data were normalized to calibration beads (0.5-μm diameter "polychromatic" beads; Polysciences, Inc., Warrington, Pa.), which were added in known concentration to each sample but gated out of the histograms presented here. To compare the fluorescence of individual cells, we used mode values, as these are less biased by cell clumping than are means. The modes reported here have been linearized by using a calibration factor for the log amp derived by the method of Schmid et al. (24).

RESULTS AND DISCUSSION

Flow cytometric detection of specific probe binding to single cells. Pure cell suspensions of *D. gigas* and *E. coli* were initially hybridized with the SRB probe and analyzed by flow cytometry. This probe is complementary to the 16S rRNA of *D. gigas* but has three mismatches to the corresponding region in *E. coli*. These mismatches destabilize the heterologous hybrid (45°C hybridization) enough for clear microscopic discrimination between *D. gigas* and *E. coli* (Fig. 2). The resulting two-parameter histograms of forward light scatter and fluorescence showed well-defined differences between the complementary (*D. gigas*) and noncomplementary (*E. coli*) cells (Fig. 3A and B). The mode relative intensities of fluorescence were 60 for *D. gigas* and 2.9 for *E. coli* (Table 1). Subsequently both cell suspensions were hybridized with the eubacterial probe, which is complementary to both organisms (Fig. 3C and D). As expected, the fluorescence of *D. gigas* labeled with the eubacterial probe was the same as that with the SRB probe (Fig. 3C; Table 1). *E. coli* hybridized with this probe was about threefold less fluorescent than *D. gigas* (Fig. 3D; Table 1) but still about an order of magnitude more fluorescent than the same cells hybridized with the noncomplementary SRB probe.

In order to assess nonspecific probe binding, both organisms were hybridized with the eucaryotic probe (Fig. 3E and F). This probe has seven mismatches and one deletion with the 16S rRNA of both *D. gigas* and *E. coli* (Fig. 1). The *E. coli* fluorescence with the eucaryotic probe was very low and, as expected, comparable to that of hybridization with the SRB probe (Table 1). Although *D. gigas* fluorescence with the eucaryotic probe was significantly less than with either the SRB or eubacterial probe, it was more fluorescent than *E. coli* hybridized with the same probe. This was evidenced by a population clearly separated from the noise (Fig. 3E; Table 1). Since this positive signal persisted in the presence of a 50-fold excess of unlabeled eucaryotic probe, it apparently does not represent oligonucleotide hybridization to alternative sites on the rRNA. It originates in part from autofluorescence of the fixed *D. gigas* cells. Autofluorescence of *D. gigas* is significantly higher than that of *E. coli* (Table 1). Also, nonspecific binding of the dye-oligonucleotide conjugate to other cellular constituents could contribute to fluorescence; unlabeled oligonucleotide is not a competitor.

Detection and enumeration of specific cells in mixtures. Defined mixtures of *D. gigas* and *E. coli* hybridized with the SRB probe were used to determine the utility of this technique for enumerating specific bacteria against a background of nontarget cells. Cell concentrations of the fixed-cell suspensions were first determined by direct counting of
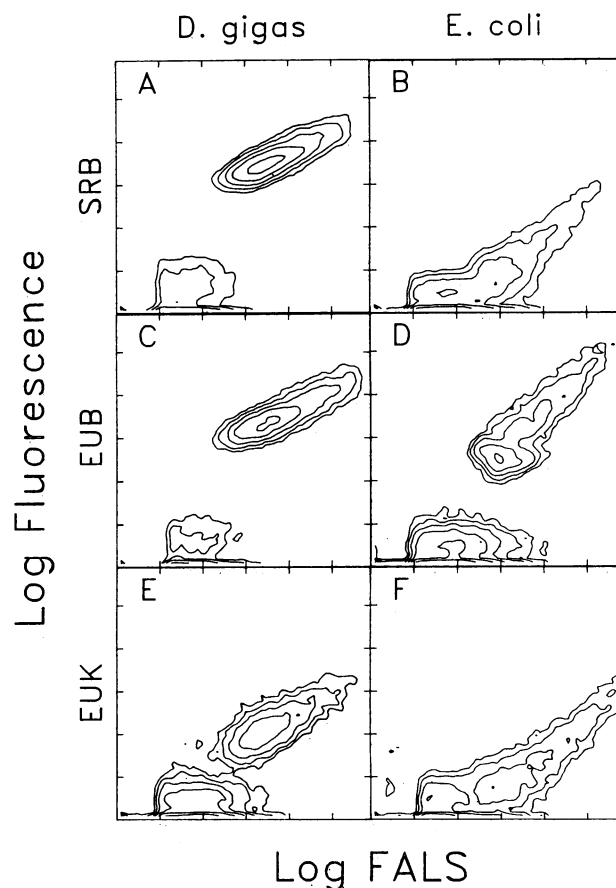

FIG. 3. Flow cytometric analysis of *D. gigas* and *E. coli* populations hybridized with three different probes: SRB (A and B), eubacterial (C and D), and eucaryotic (E and F). Data are presented as two-parameter histograms: x axis, log forward angle light scatter per cell (relative units); y axis, log fluorescence per cell (normalized to standard beads); and z axis (elevation on these contour plots), relative cell number. Contour levels correspond to 0.05, 0.1, 0.2, 0.4, and 0.8% of the total cell number. The x and y axes cover 3 decade log amps each.

4',6'-diamidino-2-phenylindole-stained cells on membrane filters. Cells were mock hybridized by incubating in hybridization buffer for 1 h before diluting with 1 ml of 0.2-μm-pore-size-filtered water and counting. Pairs of dividing cells and cell clusters contribute to a single event in the flow cytometric analysis and were therefore counted as one. The *D. gigas* cell preparation had a high number of dividing cells (ca. 50%), and the *E. coli* preparation consisted of about one-third cell clusters containing up to 10 cells. Fixed *D. gigas* (1.2×10^8/ml) and *E. coli* (7.0×10^8/ml) cell suspensions were mixed to give final concentrations of *D. gigas* of 50, 20, 3, and 0.8% of the total cell number. The mixtures were then hybridized with the SRB or eubacterial probe and analyzed on the flow cytometer. Hybridization with the eubacterial probe provided a total cell count.

Target cells comprising as few as 3% of the total were clearly visible as a well-separated population (Fig. 4). In order to enumerate the cells of interest, a bitmap that surrounded the area in which homologous hybridized cells appear on the two parameter plot was generated (Fig. 4). The results of the flow cytometric analysis (Table 2) closely

TABLE 1. Mode fluorescence values of fixed-cell preparations after hybridization with tetramethylrhodamine-labeled oligodeoxynucleotide probes

Probe	Mode fluorescence value	
	D. gigas	E. coli
SRB	60.0	2.9
Eubacterial	60.0	21.0
Eucaryotic	15.0	3.1
None	2.1	<1.1[a]

[a] Minimum log amp signal.

matched the expected values (the 0.8% value is slightly elevated) and indicate that flow cytometry in combination with fluorescent oligodeoxynucleotide probes can identify and enumerate specific microorganisms over a wide range of concentrations.

Increasing the signal intensity with multiple probes. A unique oligodeoxynucleotide probe labeled with one molecule of fluorescent dye can be sufficient to detect and distinguish rapidly growing cells. However, organisms in the
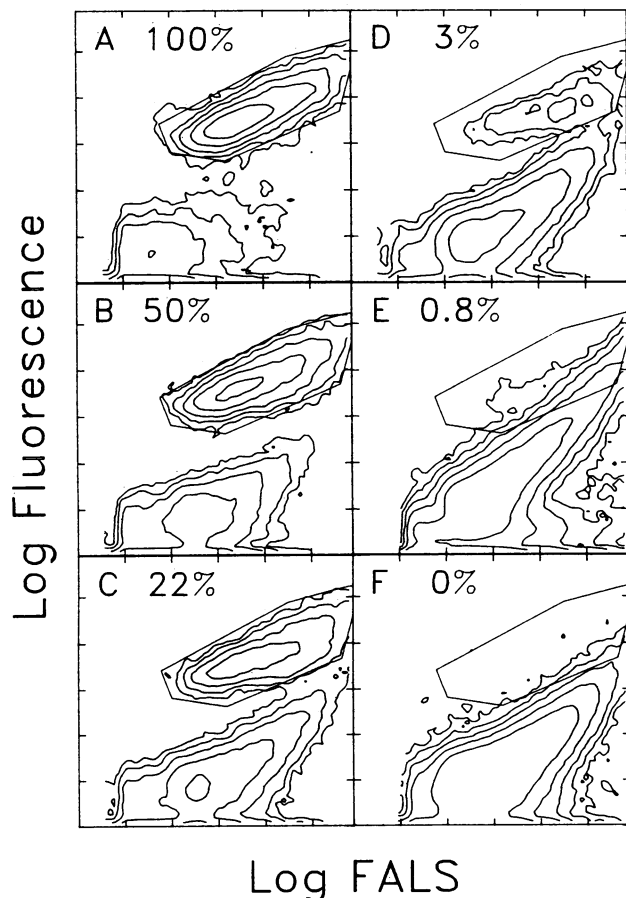

FIG. 4. Flow cytometric detection of D. gigas against an increasing background of E. coli. The two cell types were mixed and hybridized with the SRB probe; percentages of D. gigas were as follows: 100% (A), 50% (B), 20% (C), 3% (D), 0.8% (E), and 0.0% (F). Data presentation is as described in Fig. 3, except that the contour levels correspond to 1, 3, 9, 27, and 81 cells in all panels. The bitmap defining the D. gigas population was drawn based on panel A and used unaltered on the other histograms.

TABLE 2. Comparison of flow cytometry-measured D. gigas cell densities in the microscopically quantified mixtures of D. gigas and E. coli shown in Fig. 4[a]

Detection of D. gigas against increasing E. coli background					
Expected			Measured		
D. gigas/ml	Total/ml	%	D. gigas/ml[b]	Total/ml[c]	%
6.0×10^5	6.0×10^5	100.0	7.7×10^5	8.9×10^5	87.0
6.0×10^5	1.2×10^6	50.0	4.5×10^5	9.4×10^5	48.0
4.0×10^5	1.9×10^6	22.0	3.8×10^5	1.5×10^6	25.0
1.0×10^5	3.1×10^6	3.2	1.2×10^5	2.8×10^6	4.3
2.2×10^4	2.9×10^6	0.8	6.1×10^4	1.7×10^6	3.5
0.0	5.0×10^6	0.0	6.0×10^3	2.4×10^6	0.3

[a] Pure stocks of each cell type were enumerated microscopically and mixed in different proportions to yield the cell densities indicated.
[b] D. gigas counts were derived from the bitmaps shown in Fig. 4A to F.
[c] Total counts were derived from light-scattering data.

environment often do not grow at their optimal rate. DeLong and co-workers demonstrated that signal intensity is directly related to the physiological status of the cells (8). Cells growing at a slower rate have fewer 16S rRNA target sites and a reduced fluorescent signal. Furthermore, the relative concentrations of rRNA and the probe availability of rRNA target regions can vary for different species. For environmental studies, it therefore may be necessary to increase the number of dye molecules delivered to each target nucleic acid molecule.

Preliminary studies with oligonucleotides having more than one dye molecule attached (e.g., by tailing) demonstrated high levels of nonspecific binding (R. I. Amann, unpublished data). However, an alternative straightforward approach is to use multiple probes. We examined this by hybridizing D. hydrogenophilus with various combinations of the eubacterial, desulfobacter, and SRB probes. The eubacterial probe has no mismatches to the target organism. Although the desulfobacter probe contains two degeneracies (A/C and A/G) and is a mixture of four slightly different oligonucleotides, it demonstrated a hybridization intensity virtually identical to that of the eubacterial probe (Fig. 5; Table 3). The SRB probe has a single mismatch with the 16S rRNA of D. hydrogenophilus; its fluorescence intensity was correspondingly reduced but was still above that of the eucaryotic probe background (Fig. 5; Table 3). This is an example of the potential influence of a single mismatch on the stability of an oligonucleotide hybrid. The hybridization signal was approximately doubled by the combined use of the eubacterial and desulfobacter probes (Fig. 5; Table 3). Intensity was further increased by addition of the SRB probe to the hybridization mixture and demonstrated that the fluorescent signal was additively increased by combined probing. By using multiple probes of identical specificity, it should therefore be possible to detect even slow-growing microorganisms.

Future directions. This study demonstrated the potential use of fluorescent hybridization probes in combination with flow cytometry. In principal, the phylogenetic underpinnings of the analysis could offer the basis for integrated studies of natural systems. For example, the general approach might be used for hierarchical analyses of defined phylogenetic groups: determining the abundance of specific kingdoms in a first step, probing for various phyla in a second and for families or genera in a third, and so on. Having so defined the dominant resident microbiota, the easily automated flow cytometric detection could serve to track population changes.
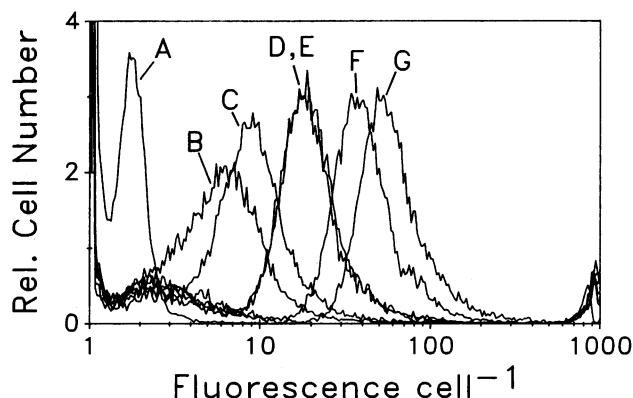
FIG. 5. Application of multiple probes to the same target cell. Suspensions of *D. hydrogenophilus* were hybridized with the following probes: no probe (A), eucaryotic (B), SRB (C), desulfobacter (D), eubacterial (E), desulfobacter and eubacterial (F), and desulfobacter, eubacterial, and SRB (G). Single-parameter histograms of relative cell number versus log fluorescence are shown. Histograms include any signal having forward and 90° light scatter greater than 0. Note that the SRB probe has a single mismatch to the target cells, the eubacterial probe has no mismatches, and the desulfobacter probe contains two degenerate positions (see the text).

Future developments must include increasing the fluorescent signal per ribosome. Although this was achieved here by use of multiple probes, other labeling strategies (e.g., indirect labeling) should be developed. The use of alternative fluorochromes must be evaluated. Labeling with rhodamine has certain disadvantages because some photosynthetic pigments are excited at approximately the same wavelength and because commonly used lasers (including the one used in this study) do not efficiently excite this fluorochrome. The relatively high levels of background fluorescence evidenced in this study likely will be reduced by improved hybridization protocols. To avoid clumping and cell loss, the current protocol does not include removal of unbound probe or a high-stringency washing step. Finally, precise relative abundance and activity measurements (as assessed by cellular ribosome abundance) should be possible by the combined use of general and specific probes labeled with different fluorochromes. In this configuration, the intensity of a specific hybridization signal could be normalized to the intensity of bound universal probe, thereby correcting for cell-to-cell differences in rRNA content or availability.

ACKNOWLEDGMENTS

TABLE 3. Mean fluorescence values of *D. hydrogenophilus* cells hybridized with tetramethylrhodamine-labeled probes

Probe	Mean fluorescence of *D. hydrogenophilus*
None	2.1
Eucaryotic	7.5
SRB	10.6
Eubacterial	23.7
Desulfobacter	24.0
Eubacterial plus desulfobacter	49.1
Eubacterial plus desulfobacter plus SRB	75.2

LITERATURE CITED

1. Amann, R. I., L. Krumholz, and D. A. Stahl. 1990. Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172:762–770.
2. Bauman, J. G. J., and P. Bentvelzen. 1988. Flow cytometric detection of ribosomal RNA in suspended cells by fluorescent in situ hybridization. Cytometry 9:517–524.
3. Bohlool, B. B., and E. L. Schmidt. 1980. The immunofluorescence approach in microbial ecology, p. 203–241. *In* M. Alexander (ed.), Advances in microbial ecology. John Wiley & Sons, Inc., New York.
4. Brosius, J., J. L. Palmer, J. P. Kennedy, and H. F. Noller. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA 75:4801–4805.
5. Campbell, L., E. J. Carpenter, and V. J. Iacono. 1983. Identification and enumeration of marine chroococcoid cyanobacteria by immunofluorescence. Appl. Environ. Microbiol. 46:553–559.
6. Chisholm, S. W., R. J. Olson, R. E. Zettler, R. Goericke, J. Waterbury, and N. Welschmeyer. 1988. A novel, free-living prochlorophyte occurs at high cell concentrations in the oceanic euphotic zone. Nature (London) 334:340–343.
7. Collins, M. D., and F. Widdel. 1986. Respiratory quinones of the sulfate-reducing and sulphur-reducing bacteria: a systematic investigation. Syst. Appl. Microbiol. 8:8–18.
8. DeLong, E. F., G. S. Wickham, and N. R. Pace. 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243:1360–1363.
9. Devereux, R., M. Delaney, F. Widdel, and D. A. Stahl. 1989. Natural relationships among sulfate-reducing eubacteria. J. Bacteriol. 171:6689–6695.
10. Giovannoni, S. J., E. F. DeLong, G. J. Olson, and N. R. Pace. 1988. Phylogenetic group-specific oligonucleotide probes for the identification of single microbial cells. J. Bacteriol. 170:720–726.
11. Green, C. J., G. C. Stewart, M. A. Hollis, B. S. Vold, and K. F. Bott. 1985. Nucleotide sequence of *Bacillus subtilis* ribosomal RNA operon, rrnB. Gene 37:261–266.
12. Hobbie, J. E., R. J. Daley, and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225–1228.
13. Jarsch, M., and A. Boeck. 1985. Sequence of the 16S ribosomal RNA gene from *Methanococcus vannielii*. Syst. Appl. Microbiol. 6:54–59.
14. Li, W. K. W., and M. Wood. 1988. Vertical distribution of North Atlantic ultraplankton: analysis of flow cytometry and epifluorescence microscopy. Deep-Sea Res. 35:1615–1638.
15. Maaloe, O., and N. O. Kjellgard. 1966. Control of macromolecular synthesis. W. A. Benjamin, Inc., New York.
16. Macario, A. J. L., E. Conway De Macario, U. Ney, S. M. Schoberth, and H. Sahm. 1989. Shifts in methanogenic subpopulations measured with antibody probes in a fixed-bed loop anaerobic bioreactor treating sulfite evaporator condensate. Appl. Environ. Microbiol. 55:1996–2001.
17. McCarroll, R., G. J. Olsen, Y. D. Stahl, C. R. Woese, and M. L. Sogin. 1983. Nucleotide sequence of the *Dictyostelium discoideum* small-subunit ribosomal ribonucleic acid inferred from the gene sequence: evolutionary implications. Biochemistry 22:5858–5868.
18. Neveux, J., D. Vaulot, C. Courties, and E. Fukai. 1983. Green photosynthetic bacteria associated with the deep chlorophyll maximum of the Sargasso Sea. C.R. Acad. Sci. Ser. III 308:9–14.
19. Olsen, G. J., D. J. Lane, S. J. Giovannoni, N. R. Pace, and D. A. Stahl. 1986. Microbial ecology and evolution: a ribosomal RNA approach. Annu. Rev. Microbiol. 40:337–365.

20. **Olson, R. J., S. W. Chisholm, E. R. Zettler, and E. V. Armbrust.** 1988. Analysis of *Synechococcus* pigment types in the sea using single and dual beam flow cytometry. Deep-Sea Res. **35**:425–440.

21. **Oyaizu, H., and C. R. Woese.** 1985. Phylogenetic relationships among sulfate respiring bacteria, myxobacteria and purple bacteria. Syst. Appl. Microbiol. **6**:257–263.

22. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25**:943–948.

23. **Robertson, B. R., and D. K. Button.** 1989. Characterizing aquatic bacteria according to population, cell size, and apparent DNA content by flow cytometry. Cytometry **10**:70–76.

24. **Schmid, I., P. Schmid, and J. V. Giorgi.** 1988. Conversion of logarithmic channel numbers into relative linear fluorescence intensity. Cytometry **9**:553–538.

25. **Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. **54**:1079–1084.

26. **Ward, B. B., and A. F. Carlucci.** 1985. Marine ammonia-oxidizing and nitrite-oxidizing bacteria: serological diversity determined by immunofluorescence in culture and in the environment. Appl. Environ. Microbiol. **50**:194–201.

27. **Weisburg, W. G., Y. Oyaizu, H. Oyaizu, and C. R. Woese.** 1985. Natural relationship between bacteroides and flavobacteria. J. Bacteriol. **164**:230–236.

28. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.

29. **Woese, C. R., E. Stackebrandt, T. J. Macke, and G. E. Fox.** 1985. A phylogenetic definition of the major eubacterial taxa. Syst. Appl. Microbiol. **6**:143–151.

30. **Yang, D. C., B. Kaine, and C. R. Woese.** 1985. The phylogeny of archaebacteria. Syst. Appl. Microbiol. **6**:251–256.