JOURNAL OF BACTERIOLOGY, Feb. 1990, p. 762–770
0021-9193/90/020762-09$02.00/0

Vol. 172, No. 2

Fluorescent-Oligonucleotide Probing of Whole Cells for Determinative, Phylogenetic, and Environmental Studies in Microbiology

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Received 29 August 1989/Accepted 8 November 1989

Fluorescent-dye-conjugated oligonucleotides were used to classify 14 *Fibrobacter* strains by fluorescence microscopy. On the basis of partial 16S rRNA sequences of six *Fibrobacter* strains, four hybridization probes were designed to discriminate between the species *Fibrobacter succinogenes* and *Fibrobacter intestinalis* and to identify *F. succinogenes* subsp. *succinogenes*. After in situ hybridization to whole cells of the six sequenced strains, epifluorescence microscopy confirmed probe specificity. The four probes were then used to make presumptive species and subspecies assignments of eight additional *Fibrobacter* strains not previously characterized by comparative sequencing. These assignments were confirmed by comparative sequencing of the 16S rRNA target regions from the additional organisms. Single-mismatch discrimination between certain probe and nontarget sequences was demonstrated, and fluorescent intensity was shown to be enhanced by hybridization to multiple probes of the same specificity. The direct detection of *F. intestinalis* in mouse cecum samples demonstrated the application of this technique to the characterization of complex natural samples.

Comparative sequencing and molecular systematics are rapidly changing the character of studies in determinative and environmental microbiology. For the first time, it is possible to define natural relationships within a comparative molecular framework. The most encompassing of molecular descriptions of microbial diversity has been provided by comparative sequencing of rRNAs. Of available sequence collections of homologous biopolymers, this is the largest and most far reaching. Sequence divergence among the different species (5S, 16S-like, and 23S-like rRNAs) has served to define the primary lines of evolutionary descent and provided a framework for a natural classification of microorganisms (26, 27). For historical and technical reasons, the largest available data sets of complete sequences are for the 5S and 16S-like rRNAs. Although both have proved valuable for determinative, phylogenetic, and environmental studies, the greater information content of the larger rRNA species makes them the preferred reference sets.

The general approach to determinative and environmental studies has been to target discrete regions of the rRNAs or their genes for hybridization to group- and species-specific oligonucleotide probes. Since the rRNAs differ along their lengths in relative sequence conservation, the targeting of regions of greater or lesser conservation offers exquisite control of probe specificity. For example, kingdom-, genus-, and species-specific oligonucleotide probes have been applied to determinative and environmental studies, and the universally occurring regions of sequences have been used to identify rRNA genes in recombinant libraries of environmental DNA (17, 25).

Most past studies have started with total nucleic acid extracted from pure cultures or the environment for analysis. However, the demonstration that fixed whole cells are permeable to short-oligonucleotide probes extended these studies to single-cell identification (8). The first demonstration of this approach used radioactively labeled probes in combination with autoradiography. Fluorescent-dye-conjugated probes would extend this technique to the direct observation and identification of single cells by fluorescence microscopy. We here detail the use of phylogenetically defined, fluorescent-oligonucleotide probes for determinative and autecological studies of ruminal and intestinal microbiota. These studies complement and extend similar studies recently published by DeLong et al. (7).

MATERIALS AND METHODS

Organisms. Strains of *Fibrobacter succinogenes* subsp. *succinogenes* were thankfully obtained from M. P. Bryant, University of Illinois at Urbana-Champaign (S85), and B. A. Dehority, Ohio State University, Wooster (A3c); strains of *F. succinogenes* subsp. *elongata* were isolated by L. Montgomery, University of Illinois ($HM2^T$), and R. E. Hungate, University of California, Davis (REH 9-1). *Fibrobacter intestinalis* NR9 and DR7 were obtained from L. Montgomery. *Fibrobacter* strains GC5, JG1, LH1, MB4, and MM4 were isolated by L. Montgomery. Isolates BL2 and B1 were provided by C. S. Stewart, Rowett Research Institute, Bucksburn, United Kingdom; C1A was provided by V. H. Varel, U.S. Department of Agriculture Meat Animal Research Center, Clay Center, Neb. Table 1 summarizes sources of isolation and corresponding references.

Culture technique. Cells were grown at 37°C in a complex medium derived from the rich PMC broth described by Montgomery and Macy (16). Cellobiose (0.4%) was substituted for the pebble-milled cellulose. Growth was monitored spectrophotometrically (optical density at 550 nm) (Apparatus from Beckman Instruments, Inc., Irvine, Calif.), and the cells were harvested in mid-log phase to optimize the rRNA content.

Nucleic acid extraction and dot blot hybridization. Nucleic acid was isolated from ca. 200 mg of cells by hot phenol extraction followed by ethanol precipitation as previously described (18). RNA was denatured by addition of 3 volumes

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TABLE 1. Source of *Fibrobacter* isolates

Strain	Source	ATCC no.	Reference
A3c	Bovine rumen		6
B1	Bovine rumen		22
BL2	Bovine rumen		22
C1a	Porcine cecum		23
DR7	Porcine cecum	43855	16
GC5	Bovine cecum		—[a]
HM2	Ovine rumen	43856^T	16
JG1	Ovine rumen		—
LH1	Ovine rumen		—
MM4	Ovine rumen		—
MB4	Ovine rumen		—
NR9	Rat cecum	43854^T	16
REH9-1	Bovine rumen	43857^T	16
S85	Bovine rumen	19169^T	4

[a]—, L. Montgomery, M. S. Zino, and H. R. Mansfield, manuscript in preparation.

of 2% glutaraldehyde immediately before dilution to 0.5 μg/ml in water made to 1 μg of poly(A) (Sigma Chemical Co., St. Louis, Mo.) per ml. Samples were applied in a total volume of 100 μl to a nylon membrane (0.45-μm Magnagraph; Micron Separation Inc.) by using a dot blot device (Schleicher & Schuell, Inc., Keene, N.H.) under slight vacuum. Membranes were air dried and baked for approximately 30 min at 80°C before hybridization.

Synthetic DNA oligonucleotide probes were 5′ end labeled with ^{32}P by using polynucleotide kinase and 5′-[γ-^{32}P] ATP to a specific activity of 10^8 to 10^9 cpm/μg.

Baked membranes were prewetted in water and sealed in plastic bags. Approximately 100 μl of hybridization buffer [0.9 M NaCl, 50 mM sodium phosphate (pH 7.0), 5 mM EDTA, 10× Denhardt solution (14), 0.1% sodium dodecyl sulfate (SDS), 0.5 mg poly(A) per ml] was added per cm^2 of membrane. Membranes were incubated (prehybridized) at 40°C for several hours before addition of 2×10^5 cpm of probe per sample dot. Incubation was continued for 12 to 16 h, and filters were washed in 1× SSC (0.15 M sodium chloride, 0.015 M sodium citrate [pH 7.0])–1% SDS for 30 min at the specified wash temperature. Membranes were dried and exposed to preflashed film (Kodak XRP; Eastman Kodak Co., Rochester, N.Y.) with intensifying screens (Cronex Lightning-Plus; E.I. du Pont de Nemours & Co., Inc., Wilmington, Del.) at −85°C. Bound probe was quantitated by scintillation counting or densitometry after autoradiography.

Cell fixation. Cells were fixed by adding 3 volumes of 4% paraformaldehyde in 200 mM sodium phosphate buffer (pH 7.2) to 1 volume of bacterial suspension. For rat cecum samples, approximately 100 mg of cecum contents was suspended in 2 ml of sodium phosphate buffer (pH 7.0) and fixed by adding 3 volumes of fixative. The mixture was gently vortexed and incubated at room temperature for at least 3 h. Fixed cells were stored at 4°C for up to 8 weeks. A slight decrease of hybridization signal with time suggested that the available rRNA content decreased with prolonged storage.

T_d study with whole cells. Approximately 10^8 fixed cells were suspended in 500 μl of hybridization solution and incubated at 40°C for 30 min. After addition of 4×10^7 cpm of 5′-^{32}P-end-labeled oligonucleotide, incubation was continued for 3 h. Cells were pelleted by centrifugation (6,000 rpm for 1 min; Eppendorf microfuge), and the hybridization solution was decanted. Cells were suspended in 500 μl of 1× SSC–0.1% SDS and washed twice at 40°C for 15 min each. After resuspension in 500 μl of 1× SSC–0.1% SDS, 50-μl samples in 1.5-ml Eppendorf tubes were incubated for 30 min at selected temperatures between 38 and 62°C. Incubation was stopped by adding 1 ml of 1× SSC–0.1% SDS (20°C), pelleting the cells as above, and discarding the supernatant. The cell pellet was washed once with 1 ml of 20°C 1× SSC–0.1% SDS and counted in the tube after addition of 300 μl of Ecoscint scintillation solution (National Diagnostics, Manville, N.J.).

Preparation of cell smears. Glass slides with a heavy Teflon coating to form six separate hybridization wells per slide were purchased from Cel-Line Associates, Inc. (Newfield, N.J.). The slides were pretreated by soaking for 1 h in ethanolic KOH (10% potassium hydroxide) and rinsing in double-distilled water. After air drying, the cleaned slides were dipped in a gelatin solution (0.1% gelatin, 0.01% chromium potassium sulfate [70°C]) and dried in a vertical position.

The fixed cell suspension was mixed with 0.1 volume of 1% Nonidet P-40 (Sigma) and centrifuged for 1 min at 6,000 rpm (Eppendorf microfuge). The supernatant was removed, and the cells were resuspended in 0.1% Nonidet P-40 to a final concentration of 10^9 cells per ml. Samples of 3 μl were spread on each well, allowed to air dry, and dehydrated by serial immersion of the slide in 50, 80, and 100% ethanol (3 min each). Slides were stored at room temperature indefinitely.

Fluorescent-dye labeling of oligonucleotides. Oligonucleotides between 15 and 25 nucleotides in length were synthesized on a DNA synthesizer (Applied Biosystems, Foster City, Calif.). In the last coupling cycle, an aminoethylphosphate linker (Aminolink 1; Applied Biosystems) was attached to the 5′ end of the oligonucleotide (10). Free ammonium ions were removed by lyophilization, and the oligonucleotide was used for the coupling reaction without further purification. A 100-μg sample of 5′-aminoethyl oligodeoxynucleotide and 200 μg of tetramethylrhodamine isothiocyanate (10-mg/ml solution in dimethyl formamide; Research Organics, Cleveland, Ohio) were combined with 250 μl of 200 mM sodium carbonate buffer (pH 9.0). After incubation at 37°C for 8 to 16 h in the dark, unreacted dye was separated from the oligonucleotide by passing the reaction mixture twice through a G25 spin column (5′-3′ Inc., West Chester, Pa.). The volume was reduced to 40 μl by lyophilization, and unreacted oligonucleotide was separated from the oligonucleotide-dye conjugate by electrophoresis on a 15% nondenaturing polyacrylamide gel (300 V, 15 V/cm, 3 to 4 h [14]). The gel was removed from the apparatus and placed on a thin-layer plate impregnated with a UV indicator (Analtech Inc., Newark, Del.). Nucleic acid was visualized by shadowing the plate by illumination with a short-wave UV lamp (254 nm). The higher-molecular-weight dye-conjugated oligonucleotide and residual dye were visualized by long-wave UV illumination (302 nm). The conjugate was usually well resolved (retarded) from the unreacted oligonucleotide. This region of the gel was excised and crushed, and the labeled probe was passively eluted in TE buffer (10 mM Tris hydrochloride [pH 7.2], 1 mM EDTA). A Nensorb 20 column (du Pont) was used for final purification (11). After lyophilization, the fluorescent oligonucleotide was dissolved in TE buffer to a final concentration of 50 ng/μl and stored in equal samples at −20°C.

The following six probes were used (all numberings correspond to the complementary positions in the *Escherichia coli* 16S rRNA): 1400 (ACGGGCGGTGTGT[G/A]C), a uni-

versal oligonucleotide complementary to virtually all 16S-like rRNAs (positions 1392 to 1406) (17); succinogenes (TG CCCCTGAACTATCCAAGA), a species-specific probe for *F. succinogenes* (positions 650 to 669); intestinalis 1 (CCGC ATCGATGAATCTTTCGT; positions 207 to 226) (21) and intestinalis 2 (GCCCCGCTGCCCATTGTACCGCCC; positions 1242 to 1265), which are species-specific probes for *F. intestinalis*; ssp. succinogenes (CCATACCGATAAATCTC TAGT), an *F. succinogenes* subsp. *succinogenes*-specific probe (21); and Arch915, an archaebacterium-specific oligodeoxynucleotide (D. Stahl, unpublished data) used as a control for nonspecific binding.

Whole-cell hybridization with fluorescently labeled probes. A moisture chamber equilibrated with 1 M sodium chloride (approximately isotonic to the hybridization solution) was used for the whole-cell hybridization. A 9-μl sample of hybridization mixture [0.9 M NaCl, 50 mM sodium phosphate (pH 7.0), 5 mM EDTA, 0.1% SDS, 0.5 mg of poly(A) per ml, 10× Denhardt solution] was applied to each of the six wells. After a 30-min incubation at the hybridization temperature, 50 ng of fluorescent oligodeoxynucleotide was added and incubation continued for 2 to 5 h. The hybridization mixture was then removed by flushing the slide with several milliliters of wash solution (0.9 M NaCl, 50 mM sodium phosphate [pH 7.0], 0.1% SDS) at the hybridization temperature and immersing the slide for 15 min in wash solution at specified temperatures (37 to 56°C), followed by thorough rinsing with distilled water. The slides were air dried in the dark and stored at room temperature in the dark or viewed immediately.

Epifluorescence microscopy and photomicrography. The samples were mounted in distilled water and viewed under oil immersion with a UV Fluorite Flatfield 100× objective on an Olympus BH2 microscope (Olympus Optical Co., Tokyo, Japan) fitted with a high-pressure mercury bulb and blue and green filter sets (no. BL 0892; Olympus). Black-and-white photomicrographs were made with Kodak Tri-X Pan 400 film. Exposure times were 0.5 s for phase-contrast micrographs and 30 s for epifluorescence micrographs.

Sequencing of the 16S rRNAs. Nucleotide sequences were determined by the dideoxynucleotide method, using reverse transcriptase and the 16S rRNA as template as previously described (12). The primer sequences and their complementary regions within the *E. coli* 16S rRNA were (i) CTACGGG (G/A)(G/C)GCAGCAG, positions 342 through 357; (ii) TCT ACGCATTTCACC, positions 690 through 704; and (iii) AC GGGCGGTGTGT(G/A)C, positions 1392 through 1406.

RESULTS AND DISCUSSION

Previous studies. Members of the genus *Fibrobacter* are anaerobic, gram-negative, nonmotile, nonsporeforming rods, cocci, or lemon-shaped cells that characteristically are cellulolytic and produce primarily succinic and acetic acids. On the basis of these and limited additional physiological criteria, they were, until recently, placed in the genus *Bacteroides* as *B. succinogenes* (Hungate). However, comparative sequencing of 16S rRNA revealed no close relationship between these strains and other members of the genus *Bacteroides* (Fig. 1) (15). In fact, the aerobic flavobacteria and the gliding cytophaga are more closely related, by this criterion, to the type strain of *Bacteroides fragilis* (19, 24) than are strains of *Fibrobacter*. This served as the basis for the formation of a new genus, and "*B. succinogenes*" was transferred to *Fibrobacter* gen. nov. as *Fibrobacter succinogenes* comb nov. In addition, two subspecies were defined

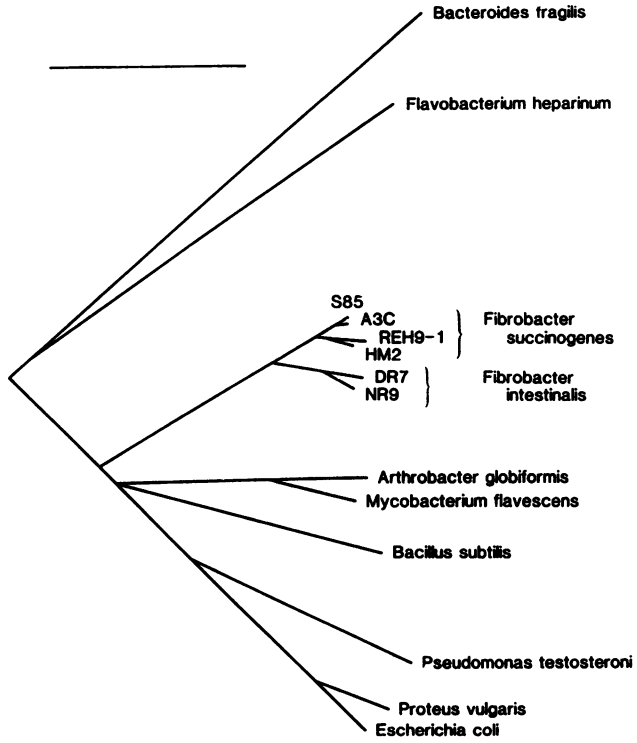


FIG. 1. Phylogenetic tree based on 16S rRNA sequence comparisons of *Fibrobacter* spp. and representatives of several eubacterial phyla. The global topology was deduced from an evolutionary distance matrix by using nearly complete sequences (ca. 1,400 nucleotides each) of *F. succinogenes* $S85^T$ and representatives of other phyla. The branching order among the *Fibrobacter* strains and the final branch length for the entire tree were based on the 834 nucleotides of sequence available for all *Fibrobacter* strains. The position of the root of the tree was suggested by a separate analysis that included an archaebacterial outgroup organism (data not shown). The phyla represented are bacteroides-flavobacteria (*Bacteroides fragilis* and *Flavobacterium heparinum* [24]), gram-positive bacteria (*Bacillus subtilis* [9], *Mycobacterium flavescens* ATCC 14474^T [21a], and *Arthrobacter globiformis* [C. R. Woese, unpublished data], and the purple bacteria (*E. coli* [3], *Proteus vulgaris* [5], and *Pseudomonas testosteroni* [28]). The bar represents a 0.1-nucleotide change per position, i.e., 0.1 evolutionary distance unit. Reproduced with permission from reference 15.

within the species: subspecies *succinogenes* strains are thick rods, often pleomorphic, coccoid, or lemon shaped, whereas cells of subspecies *elongata* are slender rods.

Strains of *F. succinogenes* form a dominant part of the fiber-digesting ruminal microbiota and are routinely isolated from the rumen contents of cattle and sheep. In contrast, other strains of the genus are more frequently isolated from the intestines and ceca of nonruminant animals. These were found to represent an assemblage phenotypically similar to, but phylogenetically distinct from (ca. 92% 16S rRNA sequence similarity), strains of *F. succinogenes*, and a second species (*F. intestinalis*) was created to encompass them. The type species and available strains were isolated from the ceca of nonruminant animals (Table 1) (15).

The comparative sequence analysis is also directly applicable to environmental studies, and this data set was used to design oligonucleotide probes complementary to regions of 16S rRNA sequence unique to *Fibrobacter* spp. for studies of their natural distribution and abundance (21). The present study used these and additional phylogenetically designed

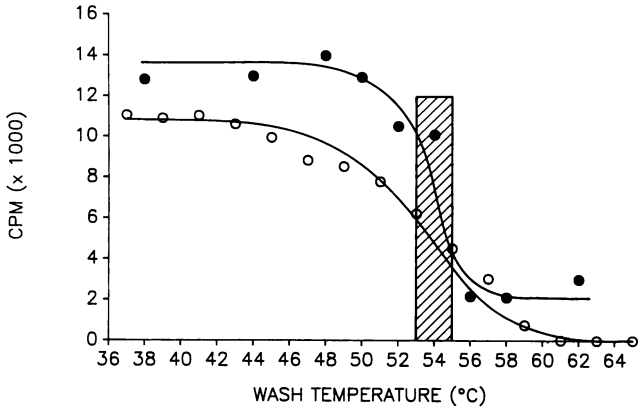


FIG. 2. Comparison of the melting curves derived from whole-cell (●) and dot blot (○) hybridization for the duplex between the intestinalis 1 oligonucleotide probe and *F. intestinalis* NR9 16S rRNA. The shadowed area marks the region corresponding to the estimated T_d values.

oligonucleotide probes conjugated to a fluorescent dye (tetramethyl-rhodamine) for the microscopic identification of species and subspecies of *Fibrobacter* in pure culture or directly in the environment.

Temperature-dependent dissociation of probes from whole cells in contrast to membrane-bound RNA. The T_d (temperature of dissociation) of an oligonucleotide from its complementary sequence (target) is the most important characteristic of the specific duplex structure. This value is usually established for the duplex formed between membrane-bound target nucleic acid and labeled probe. Thus, a comparison of whole-cell and membrane dissociation profiles was necessary to establish their comparability. A comparison of experimental T_d determinations for the intestinalis 1 probe hybridized to fixed cells (*F. intestinalis* NR9) and membrane-bound rRNA is shown in Fig. 2. Although the melting transition appears steeper for whole cells, the T_d values were not appreciably different, in both cases ranging between 53 and 55°C. Some nonspecific binding of probe to fixed cells is indicated by the elevated base line in the whole-cell experiment. Similar results were obtained for the ssp. succinogenes probe (data not shown). We assume that the dissociation temperature of the oligonucleotide-rRNA duplex from whole cells can be estimated from membrane studies with purified components.

Use of multiple probes to enhance specific single-cell fluorescence. For initial studies, a set of three oligodeoxynucleotides was designed to discriminate between the species *F. succinogenes* and *F. intestinalis* and to identify the subspecies *succinogenes*. Hybridization of *F. intestinalis* (strain NR9) cells with the *F. intestinalis*-specific (intestinalis 1) and universal probes resulted in a relatively weak fluorescent signal. This we attributed to the low rRNA content of these cells grown under the specified conditions. We therefore designed, synthesized, and fluorescently labeled a second *F. intestinalis*-specific oligonucleotide (intestinalis 2) that addressed a different unique target site within the 16S rRNA. Hybridization with these probes in combination approximately doubled the signal intensity (results not shown) and correspondingly reduced the exposure time for photographic documentation. Therefore, multiple probes can be used to alleviate problems of low rRNA content or reduced target availability in the detection of specific microorganisms.

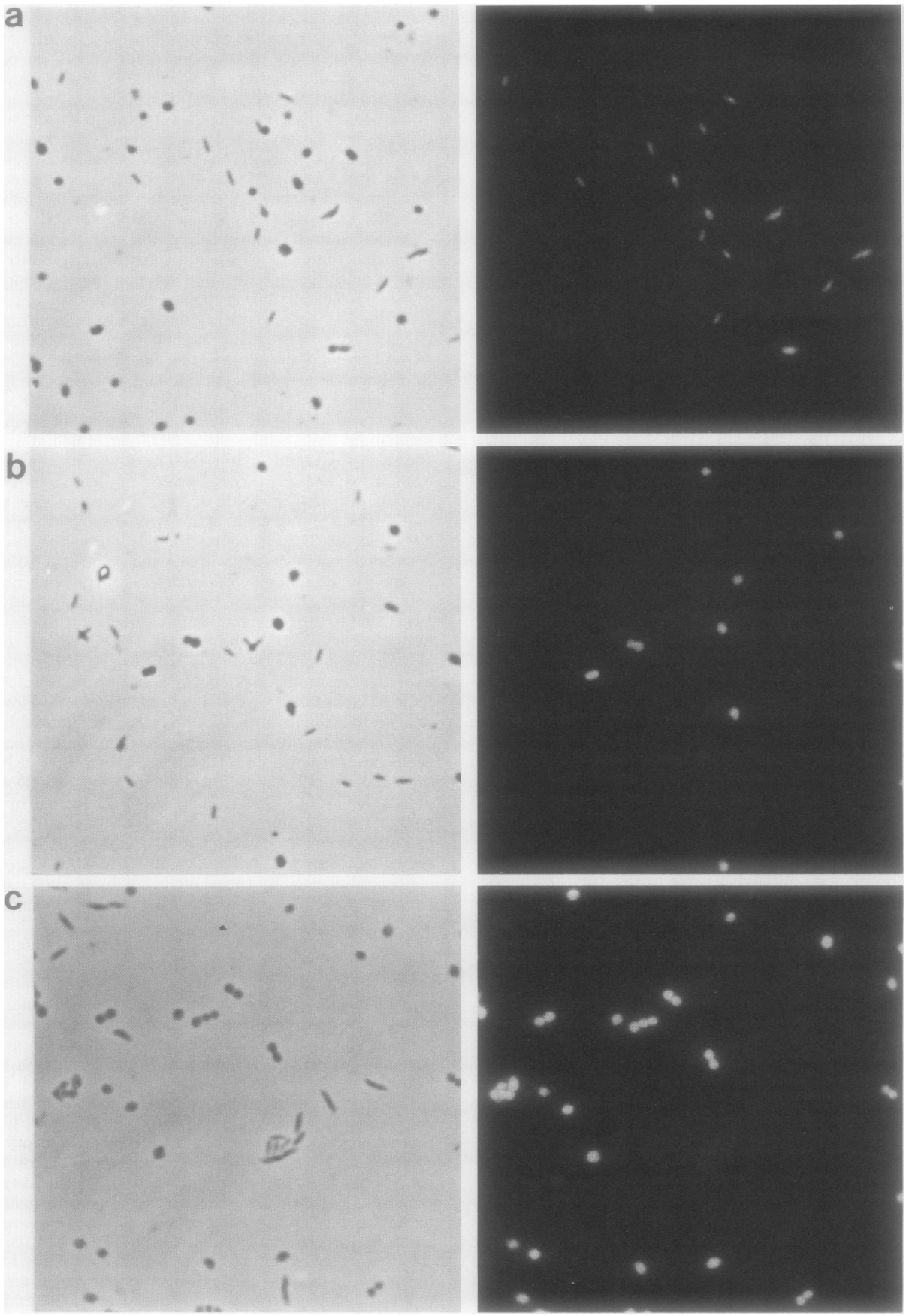
Mismatch discrimination. The resolution of closely related target sequences, and therefore closely related organisms, is central to determinative applications. We therefore examined the specificity of the fluorescent probes on pairs of morphologically distinct *Fibrobacter* strains containing known mismatches to the various probes (see Fig. 4 and Table 3) (15). For unambiguous interpretation of each hybridization, a positive and a negative control were applied to two of the six wells on each slide. Autofluorescence was routinely checked and found to be very low for all fixed cells. The universal probe 1400 served to estimate the content of 16S rRNA and act as a positive control.

An archaebacterium-specific probe was used to evaluate nonspecific binding. This can result from evaporative concentration of the hybridization solution (e.g., a leak in the moisture chamber during the hybridization) or apparently from binding to secondary, low-affinity sites within the rRNAs. Thus, nonspecific binding varies according to the rRNA content of the cell, and this must be evaluated in order to distinguish between a specific and a nonspecific fluorescent signal.

Representative photomicrographs for a study in which all incubations and washing steps were performed at 37°C are shown in Fig. 3a to d. In a mixture of *F. intestinalis* NR9 (thin rods) and *F. succinogenes* S85 (lemon to coccoid shape), the combined use of the species-specific probes intestinalis 1 and intestinalis 2 detected only *F. intestinalis* NR9 (Fig. 3a). In the remaining panels, the subspecies probe (ssp. succinogenes) was used to evaluate mismatch discrimination among different *Fibrobacter* strains. This probe is complementary to the S85 strain. Mismatches (within the target site) to the remaining strains varied from one for HM2 to three for REH9-1 and six for NR9 (Fig. 4). At this hybridization temperature (37°C), strain NR9 showed no hybridization to the ssp. succinogenes probe (Fig. 3b) and REH9-1 showed a very low amount (Fig. 3c). However, strain HM2 (single mismatch) was strongly fluorescent (Fig. 3d). Consistent with this result, the T_d of the ssp. succinogenes probe from strain HM2 16S rRNA was only 3°C lower than that observed for the complementary S85 rRNA (45 versus 48°C in 1× SSC–1% SDS). Nevertheless, by using stringent hybridization and washing conditions (56°C), we could discriminate between S85 and HM2 cells by fluorescence microscopy (Fig. 3e). Correcting for the different salt concentration used for the whole-cell hybridization (20), this temperature is 2.8°C below the T_d of the ssp. succinogenes-S85 rRNA duplex.

Although hybridization at high stringency resulted in an overall reduction of fluorescence intensity, it was nevertheless possible to distinguish between strains that differed by a single mismatch. However, the ability to discriminate complementary from single-mismatch hybrids varied markedly between different probe and target sequences; in general, single-mismatch discrimination must be empirically established.

Use of fluorescent probes for determinative studies. For determinative studies, hybridizations with all probes were routinely performed at 45°C. This temperature offered a compromise between specificity (sufficient to clearly distinguish more than one mismatch) and signal intensity. For each determination, the species-specific (succinogenes and the intestinalis 1+2 mixture), subspecies-specific (ssp. succinogenes), kingdom-specific (Arch915), and universal (1400) probes were directly compared in adjacent hybridization wells. In this way, the entire battery of probes was used to group eight uncharacterized *Fibrobacter* isolates with the sequenced strains they most closely resembled in fluores-


a
b
c

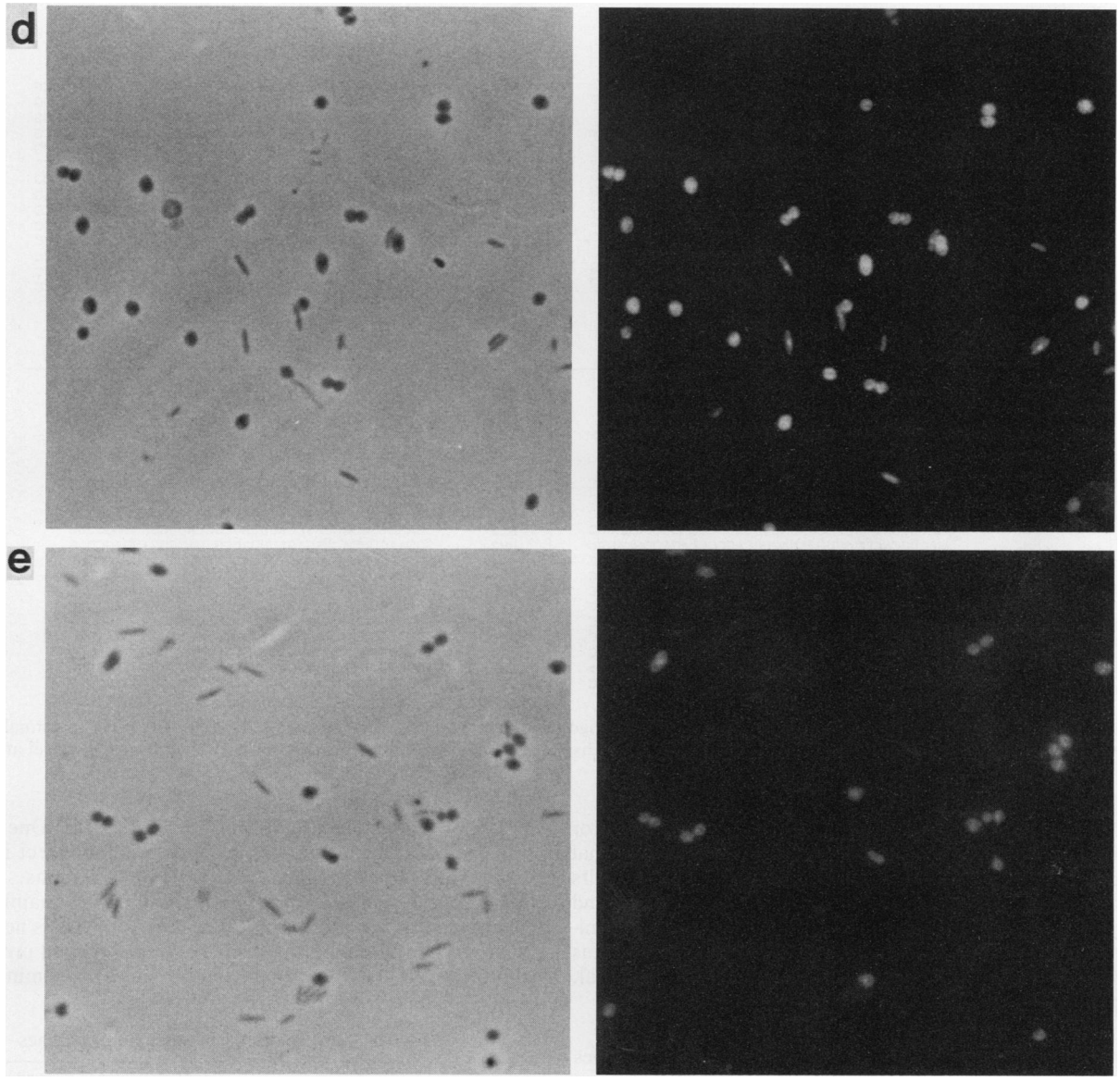


FIG. 3. Whole-cell hybridization with tetramethyl-rhodamine-labeled probes. All hybridizations and wash steps except for the study shown in panel e were performed at 37°C. Phase-contrast (left) and epifluorescence (right) photomicrographs are displayed for each field. (a) *F. succinogenes* S85 (cocci) and *F. intestinalis* NR9 (thin rods) hybridized with the intestinalis 1+2 probe; (b) *F. succinogenes* S85 and *F. intestinalis* NR9 hybridized with the ssp. succinogenes probe; (c) *F. succinogenes* S85 and *F. succinogenes* subsp. *elongata* REH9-1 (thick rods) hybridized with the ssp. succinogenes probe; (d) *F. succinogenes* subsp. *succinogenes* S85 and *F. succinogenes* subsp. *elongata* HM2 (thin rods) hybridized with the ssp. succinogenes probe; (e) as for panel d but hybridized and washed at 56°C.

cence intensity (Table 2). Importantly, the hybridization results from the determinative study were consistent; strains that hybridized to the succinogenes probe did not hybridize to the intestinalis 1+2 probe.

To evaluate the accuracy of the determinative study, we sequenced the target regions for each of the four specific probes from the additional *Fibrobacter* isolates (Fig. 4). The sequence data reflect the hybridization data (numbers of mismatches are listed in Table 3). By both criteria (fluorescent probing and comparative sequencing), we grouped isolates B1 and BL2 with *F. succinogenes* subsp. *succinogenes*. Strains MM4 and MB4 closely resembled the *F. succinogenes* subsp. *elongata* strain HM2 in mismatch pattern, and GC5 apparently belongs to the same subspecies. Strain C1a was complementary to both *F. intestinalis*-specific probes.

Isolates LH1 and JG1 reacted identically to the battery of probes and therefore are closely related. However, they did not fit well into the existing determinative framework, demonstrating no hybridization with the succinogenes probe and only a weak hybridization (signal intensity approximately one-fifth of that of the target organism) to the intestinalis 1+2 probe mixture. Taking into account that the rRNA content is not unusually low (as assessed by hybridization with the 1400 probe), we anticipated that these strains would have one mismatch with one or both components of the *F. intestinalis*-specific probe mixture. This was verified by 16S rRNA sequencing. For definitive placement of isolates LH1

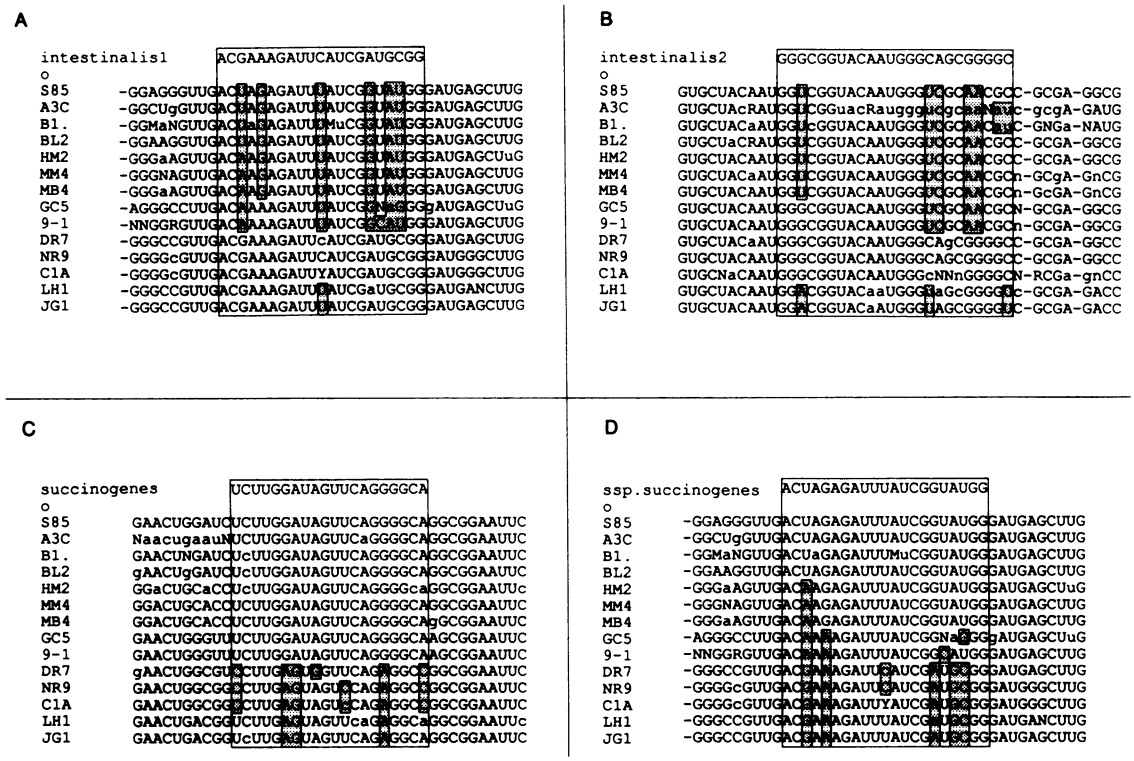


FIG. 4. Sequence alignment of 16S rRNAs in the vicinity of oligonucleotide target regions (boxed): (A) intestinalis 1; (B) intestinalis 2; (C) succinogenes; and (D) ssp. succinogenes. The target sequence is displayed in the upper row; mismatches between probe and target are boxed and shadowed.

and JG1 with one of the two known *Fibrobacter* species (or possibly with a third, yet to be defined species), additional comparative analysis is required. Nevertheless, these results clearly demonstrate the utility of a battery of species- and subspecies-specific oligonucleotide probes for rapid determinative studies in microbiology, particularly for organisms difficult to place by traditional morphological, biochemical, and physiological criteria.

TABLE 2. Results of the determinative use of fluorescent probes

Strain	Intensity of given fluorescent probe[a]			
	1400	Succino-genes	Intestinalis 1 + 2	ssp. succinogenes
F. succinogenes subsp. *succinogenes*				
S85	+	+	−	+
A3c	+	+	−	+
B1	+	+	−	+
BL2	+	+	−	+
F. succinogenes subsp. *elongata*				
HM2	+	+	−	w
REH9-1	+	+	−	−
MB4	+	+	−	w
MM4	+	+	−	w
GC5	+	+	−	−
F. intestinalis				
NR9	w	−	+	−
DR7	+	−	+	−
C1a	+	−	+	−
JG1	+	−	w	−
LH1	+	−	w	−

[a] +, Positive; w, weakly positive; −, negative.

Use of fluorescent probes in the environment. One aim in the development of fluorescent probes is the direct identification of microorganisms in complex ecosystems. Intense autofluorescence of ruminal samples (derived mainly from plant material but also from lower eucaryotes) was not much reduced by sodium borohydride reduction (7) and prevented direct observation of *Fibrobacter* species in ruminal con-

TABLE 3. Probe and target region mismatches

Strain	No. of mismatches with given fluorescent probe			
	Succino-genes	Intesti-nalis 1	Intesti-nalis 2	ssp. succino-genes
F. succinogenes subsp. *succinogenes*				
S85	0	6	5	0
A3c	0	6	7	0
B1	0	6	7	0
BL2	0	6	5	0
F. succinogenes subsp. *elongata*				
HM2	0	6	5	1
MM4	0	6	5	1
MB4	0	6	5	1
GC5	0	5	4	3
REH9-1	0	6	4	3
F. intestinalis				
DR7	6	0	0	6
NR9	6	0	0	6
C1a	6	0	0	5
LH1	3	1	3	5
JG1	3	1	3	5

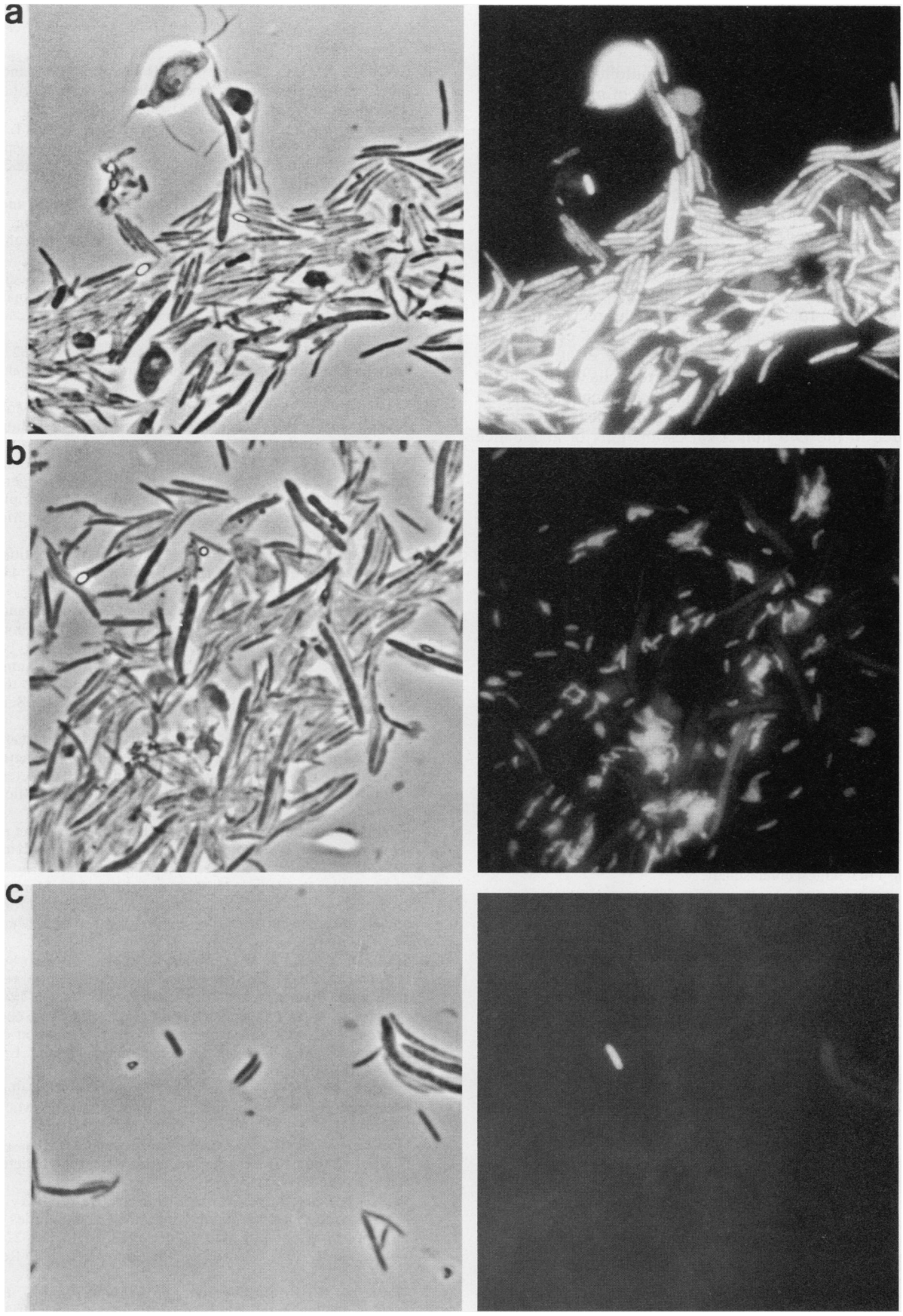


FIG. 5. Whole-cell hybridization of fixed cecum samples with tetramethyl-rhodamine-labeled probes. Phase-contrast (left) and epifluorescence (right) photomicrographs are displayed for each field. (a) Mouse cecum sample hybridized at 45°C with the 1400 probe; (b) mouse cecum sample seeded with *F. intestinalis* NR9 and hybridized with intestinalis 1+2 probe at 45°C; (c) mouse cecum sample hybridized with the intestinalis 1+2 probe at 45°C.

tents. However, substantially fewer autofluorescent particles were present in the cecum contents of mice, and direct detection was possible in this setting.

The rRNA content and target availability of cells in fixed cecal contents were estimated by hybridization with the 1400 probe (Fig. 5a). These estimations demonstrated that most cells in this environment are permeable to short-oligonucleotide probes and can be visualized by fluorescence microscopy. Cecum contents seeded with freshly grown *F. intestinalis* NR9 cells demonstrated specific detection of the target organism by the intestinalis 1+2 probe (Fig. 5b). In unseeded samples, long and short rods hybridized specifically to the intestinalis 1+2 probe (Fig. 5c). Although precise enumeration was not attempted, approximately 1 in 10^4 cells hybridized to the intestinalis 1+2 mixture. Macy et al. (13) estimated intestinal, cellulolytic *Fibrobacter* cells to account for up to 6% of total organisms as estimated by enumeration on nonselective medium. The difference of at least 1 order of magnitude in the two numerical estimations of abundance could reflect difficulties in cultivating all microorganisms. There are estimations that only 5 to 10% of all organisms in some ecosystems can be cultivated on nonselective media (1, 2). This has been a handicap in the evaluation of most natural systems.

In principal, the use of explicit molecular criteria will eliminate many of the uncertainties of cultural enumeration of natural ecosystems. The use of explicit molecular criteria within a phylogenetic framework, such as that provided by 16S rRNA-targeted probes, should markedly change the character of determinative and environmental studies in microbiology.

ACKNOWLEDGMENTS

We thank Larry Montgomery for supplying many of the strains used in this study and Berdena Flesher and Rebekah Key for technical assistance.

This research was supported by research agreements N00014-88-K-0093 to D.A.S. from the Office of Naval Research and CR815285-01-02 to D.A.S. from the U.S. Environmental Protection Agency. R.I.A. was supported by a postdoctoral grant from the Deutsche Forschungsgemeinschaft.

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