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Use of Oligodeoxynucleotide Signature Probes for Identification of Physiological Groups of Methylotrophic Bacteria

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Oligodeoxynucleotide sequences that uniquely complemented 16S rRNAs of each group of methylotrophs were synthesized and used as hybridization probes for the identification of methylotrophic bacteria possessing the serine and ribulose monophosphate (RuMP) pathways for formaldehyde fixation. The specificity of the probes was determined by hybridizing radiolabeled probes with slot-blotted RNAs of methylotrophs and other eubacteria followed by autoradiography. The washing temperature was determined experimentally to be 50 and 52°C for 9-α (serine pathway) and 10-γ (RuMP pathway) probes, respectively. RNAs isolated from serine pathway methylotrophs bound to probe 9-α, and RNAs from RuMP pathway methylotrophs bound to probe 10-γ. Nonmethylotrophic eubacterial RNAs did not bind to either probe. The probes were also labeled with fluorescent dyes. Cells fixed to microscope slides were hybridized with these probes, washed, and examined in a fluorescence microscope equipped with appropriate filter sets. Cells of methylotrophic bacteria possessing the serine or RuMP pathway specifically bind probes designed for each group. Samples with a mixture of cells of type I and II methanotrophs were detected and differentiated with single probes or mixed probes labeled with different fluorescent dyes, which enabled the detection of both types of cells in the same microscopic field.

Methylotrophs are those bacteria that use methane, methanol, methylamines, halomethanes, or other reduced one-carbon compounds as energy sources and assimilate formaldehyde as the major carbon source. Methanotrophs, methane-utilizing methylotrophs, are a diverse group of microorganisms which play an important role in the global carbon cycle (14, 36) and are classified as type I, type X, and type II methanotrophs according to their carbon assimilation pathway, intracytoplasmic membrane arrangement, the presence of a complete tricarboxylic acid cycle, and the chain length of membrane phospholipid fatty acids (3, 36). Those methylotrophs that do not grow on methane lack intracytoplasmic membranes and a few other features of methanotrophs. They are grouped according to the pathways used for formaldehyde assimilation (3; M. Lidstrom, *in* A. Baloues, H. G. Truper, M. Dworkin, W. Harder, and K. Schleifer, ed., *The Procaryotes*, in press).

Methanotrophs have been shown to insert one oxygen from dioxygen into many alkenes, alkanes, aromatic hydrocarbons, and polycyclic aromatic compounds (6, 15). These reactions are catalyzed by methane monooxygenase (MMO), which has a broad substrate specificity (15, 31). Some methanotrophs were able to degrade low-molecular-weight halogenated hydrocarbons such as trichloroethylene (33; H. C. Tsien, G. A. Brusseau, L. P. Wackett, and R. S. Hanson, *in Proceedings of the IGT 2nd International Symposium on Gas, Oil, Coal, and Environmental Biotechnology*, in press). Trichloroethylene and other low-molecular-weight halogenated hydrocarbons are industrial wastes which pollute the environment and contaminate groundwater (27). Some of them are toxic chemicals that are on the high priority list of toxic environmental pollutants of the Environmental Protection Agency (21, 32). Methanotrophs are, therefore, important in biotechnology for their ability to oxidize many organic molecules (6).

Methylotrophic bacteria are found in a variety of ecosystems, such as freshwater lakes, ponds, marshes, marine sediments, and soils (12; R. S. Hanson, A. I. Netrusov, and K. Tsuji, *in* A. Baloues, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer, ed., *The Procaryotes*, in press). Ecological studies of methylotrophic bacteria have been limited by available methodology and the diversity of these microorganisms (1, 10, 12, 13, 28). Fluorescent-antibody techniques, among others, have been used extensively in microbial ecology (4). Fluorescent-antibody techniques have been used in studies of the distribution of some methane-utilizing bacteria in both freshwater and marine environments (1, 10, 26). Due to their high specificity, however, these techniques have limitations. To prepare specific antibodies, it is necessary to isolate the target microorganism from its natural environment and to cultivate it in pure culture prior to the immunization of the animal. Some methylotrophic microorganisms are inherently difficult to isolate in pure culture. Enrichment and plating techniques preferentially select some microorganisms according to the method and the culture medium used (Hanson et al., in press). It is possible that some methylotrophs have not been isolated. Therefore, fluorescent-antibody techniques may be inadequate for studies of the ecology of these microorganisms. A technique is needed that does not require the isolation of target organisms and that is capable of identifying single cells in environmental samples.

DNA-based hybridization probes have been used to detect and identify microorganisms in a few microbial communities (16, 22). rRNA is an abundant constituent of all living cells, and the sequence of rRNA is highly conserved. Studies of the sequence homology of 16S rRNA have been used to describe evolutionary relationships among living organisms, as well as to define a new kingdom, *Archaebacteriae* (9, 38). The 16S rRNAs are especially suitable for phylogenetic studies because of their size, which is approximately 1,500

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TABLE 1. Methylotrophs used in this study

Bacteria	Growth temp (°C)	Carbon source[a]	Growth medium (reference)	Source[b]
Methylobacillus glycogenes	30	Methanol	37	ATCC
Methylococcus capsulatus Bath	37	Methane	7	R. Whittenbury
Methylococcus luteus	30	Methane	5	P. Green
Methylomonas albus BG8	30	Methane	5	R. Whittenbury
Methylomonas gracilis	37	Methane	29 (VCR medium)	P. Green
Methylomonas methanica	30	Methanol	5	R. Whittenbury
Methylomonas methanolica	30	Methanol	37	ATCC
Methylomonas methylovora	25	Methane	37	ATCC
Methylophilus methylotrophus AS1	30	Methanol	37	D. Stirling
Methylobacterium extorquens	25	Methanol	37	NCIMB
Methylobacterium extorquens AM1	30	Methanol	19	P. Goodwin
Methylobacterium organophilum XX	30	Methanol	19	This laboratory
Methylobacterium sp. strain DM4	30	Methanol	18	T. Leisinger
Methylobacterium sp. strain M27	25	Methanol	37	NCIMB
Methylocystis parvus OBBP	30	Methane	5	R. Whittenbury
Methylocystis pyriformis #14	30	Methane	5	Y. Trotsenko
Methylosporovibrio methanica 81Z	30	Methane	5	S.-J. Zhao
Methylosinus trichosporium OB3b	30	Methane	5	R. Whittenbury
Methylosinus sporium #27	30	Methane	5	Y. Trotsenko
Methylosinus sp. strain B	30	Methane	5	This laboratory
Unidentified methanotroph (SH-1)	30	Methane	5	D. Grbic-Galic
104	30	Methane	24	This laboratory
NP-1	30	Methane	24	This laboratory

[a] 0.5% methanol and 25% methane were used.
[b] ATCC, American Type Culture Collection; NCIMB, National Collection of Industrial and Marine Bacteria.

nucleotides in length, and abundance in bacterial cells (23). Oligodeoxynucleotide probes complementary to 16S rRNA have been used successfully in quantifying target species in their natural habitats (30). Radiolabeled kingdom-specific probes as well as species- and group-specific probes have been used for the identification of single microbial cells (11), bacterial species (25), and estimation of the abundance of 16S rRNAs from specific bacteria in ecological studies of the rumen (30). This technique requires the use of radiolabeled probes and autoradiography, a somewhat tedious process which requires the use and the disposal of radiochemicals. Due to the abundance of 16S rRNAs per cell, fluorescently labeled, kingdom-specific probes have been used successfully for the detection of single cells (8). Recently, Amann et al. (2), using this technique, studied the distribution of 14 strains of *Fibrobacter* spp.

Based on the analysis of 16S rRNA sequence and the study of phylogenetic relationships, ribulose monophosphate (RuMP) pathway methylotrophs were grouped in the β and γ subdivisions and serine pathway methylotrophs were grouped in the α subdivision of the purple eubacteria (*Proteobacteria*) (34). Two group-specific oligodeoxynucleotide probes, which were specific to the α- and β/γ-subdivision methylotrophs, were designed and synthesized. The sequences of these probes are complementary to the 16S rRNAs of their respective group of methylotrophs. In this study, we describe the use of these probes, which were fluorescently labeled, for the identification of serine and RuMP pathway methylotrophs. They were used in in situ hybridization with formaldehyde-fixed cells and viewed by epifluorescence microscopy. The radioactively labeled probes were hybridized with slot-blotted RNAs from a variety of eubacteria to determine the scope and specificity of the probes.

MATERIALS AND METHODS

Microorganisms and growth conditions. All methylotrophs used and their sources and conditions are listed in Table 1. Most cells used in this study were in either mid- or late-exponential growth phase. Occasionally, cells in stationary growth phase were also used. Depending on the methylotroph used, either methane or methanol was used as growth substrate. *Pseudomonas putida* F1 and *P. fluorescens* were grown in mineral salts medium as described by Wackett and Gibson (35). *Bacillus megaterium* and *Bacillus subtilis* were grown in nutrient broth (Difco Laboratories, Detroit, Mich.) at pH 7. *Bacillus* sp. strain A1 was grown in nutrient broth at pH 5.5. *Escherichia coli* was grown in LB broth.

Synthesis and labeling of oligodeoxynucleotide probes. Probes used in this study and their sequences and position on the *E. coli* 16S rRNA sequence are listed in Table 2. Oligodeoxynucleotides were synthesized on a DNA Synthesizer (Applied Biosystems, Foster City, Calif.). Aminolinker (Aminolink 1; Applied Biosystems), an aminoethyl group, was introduced at the last step during the synthesis of oligodeoxynucleotide probes. Coupling of oligodeoxynucleotides with fluorescent dyes was carried out by mixing 100 μg of oligodeoxynucleotide with 400 μg of fluorescent dye, fluorescein isothiocyanate or X-rhodamine isothiocyanate, in 250 μl of 200 mM sodium carbonate buffer, pH 9, at room temperature in the dark for 16 h with constant stirring, as described by DeLong et al. (8). After conjugation, the mixture was passed through a Sephadex G-50 column and the fluorescently labeled oligodeoxynucleotides were purified by high-pressure liquid chromatography, using an RP 304 column (Bio-Rad Laboratories, Richmond, Calif.). Fluorescein- or X-rhodamine-labeled probes were used at a concentration of 1.7 ng/μl in hybridization mixtures. The hybridization mixture had the following composition: 0.75 M NaCl, 5 mM EDTA, 0.1 M Tris (pH 7.8), 10% dextran, 0.2%

TABLE 2. Sequences of oligodeoxynucleotide probes used in this study and their location on 16S rRNA molecules

Probe description	Nucleotide sequence	Location[a]	No. of nucleotides	T_d (°C)[b]
9-α[c]	5′-CCCTGAGTTATTCCGAAC-3′	142–159	18	50
10-γ[d]	5′-GGTCCGAAGATCCCCCGCTT-3′	197–216	20	52
Universal[e,f]	5′-GWATTACCGCGGCKGCTG-3′	519–536	18	ND
Eubacterial[e]	5′-ACCGCTTGTGCGGGCCC-3′	927–942	17	ND
Control[e,f]	5′-GTGCCAGCMGCCGCGG-3′	NA	16	ND

[a] Position numbers refer to the *E. coli* 16S rRNA sequence. NA, Not applicable.
[b] T_d, Washing temperature at which 50% of bound probe was removed. Values were experimentally determined. ND, Not determined.
[c] Serine pathway methylotroph signature probe.
[d] RuMP pathway methylotroph signature probe.
[e] Synthesized according to sequences given by Giovannoni et al. (11).
[f] W, A, or T; K, G, or T; M, A, or C.

bovine serum albumin, and 0.01% poly(A). Oligodeoxynucleotides were also radiolabeled at the 5′ end with 5′-[γ-^{32}P] ATP and T_4 polynucleotide kinase to a specific activity of approximately 10^8 cpm/μg. The reaction mix consisted of kinase buffer (50 mM Tris hydrochloride [pH 7.8], 10 mM $MgCl_2$), 15 mM dithiothreitol, 0.33 μM ATP, 10 to 20 pmol of 5′ ends, 20 μCi of [γ-^{32}P]ATP, and 20 U of T_4 polynucleotide kinase. After incubating for 15 min at 37°C, an additional 7.2 mM $MgCl_2$ and 20 μCi of [γ-^{32}P]ATP was added. Incubation was resumed for another 15 min and then stopped with 40 mM EDTA. Next, tRNA was added as a carrier and the nucleic acid was precipitated twice with ammonium acetate and ethanol (20) to remove unincorporated nucleotides. The precipitate was dissolved in distilled water, and 1 μl was counted to determine probe activity.

Fixation, treatment, and in situ hybridization of cells. Two 30-μl cell suspensions were smeared on microscope slides predipped into a gelatin solution (0.1% gelatin, 0.01% chromium potassium sulfate at approximately 70°C) and air dried. Both prefixed and unfixed cell suspensions of 0.1 A_{600} were used for smearing. Exponential-phase cultures fixed with 3.7% formaldehyde at room temperature for 1 h and washed by centrifugation three times with culture media were used as prefixed cells. After smearing, slides were fixed in 3.7% formaldehyde in 90% methanol for 10 min and treated, after a brief rinse with distilled water, with 50 mM sodium borohydride for 30 min in the dark with occasional agitation. Slides were rinsed with distilled water and air dried.

For in situ hybridization, 10 μl of hybridization solution containing probe (1.7 ng/μl) was added to the smear, and slides were covered immediately with a glass cover slip and incubated at 37°C overnight in a sealed and moisture-saturated container. Duplicate smears on the same slide were hybridized with two different probes, usually one with a 9-α probe and the other with a 10-γ probe, or with another combination. After incubation, cover slips were removed by immersing slides in ice-cold 5× SET (1× SET is 750 mM NaCl, 100 mM Tris hydrochloride [pH 7.8], 5 mM EDTA), and the slides were washed three times in 0.2× SET at 37°C for 10 min each time. Slides were then air dried in the dark and were ready for microscopic examination. Slides were stored at 4°C in the dark until viewing without losing fluorescence intensity.

Epifluorescent microscopy. Samples were mounted in a synthetic mountant (0.01 M phosphate buffer, 0.15 M NaCl, 0.1% [wt/vol] *p*-phenylenediamine [Sigma Chemical Co., St. Louis, Mo.], 90% [vol/vol] glycerol, pH 8.0) to reduce the fading of fluorescence during microscopy (17). Slides were viewed with a Neofluor ×100 objective on a Zeiss microscope equipped with epifluorescent optics, a mercury lamp, and filter sets 487709 and 487715 for fluorescein and X-rhodamine, respectively. Kodak Ektachrome 400 daylight film was used for photography.

RNA extraction, membrane blotting, and hybridization. rRNAs were isolated by using a modification of the low-pH–hot-phenol extraction method of Stahl et al. (30). Equal volumes of phenol (pH 5.1), cell suspension, and glass beads plus 0.5% sodium dodecyl sulfate (SDS) were agitated vigorously in a bead beater (Biospec Products, Bartlesville, Okla.) and incubated for 10 min at 60°C. Following an additional bead beating, the aqueous phase was removed after separation by centrifugation. The phenol extraction was repeated, followed by two extractions with 4:1 phenol-chloroform (pH 5.1) and two chloroform extractions. RNA was precipitated by adding 1/10 volume of 3 M sodium acetate and 2 volumes of absolute ethanol. The precipitate was washed three times with 80% ethanol and solubilized in distilled water. RNA concentrations were determined by measuring the A_{260}.

RNAs were immobilized on a nylon membrane with a slot-blotting device (Schleicher & Schuell Inc., Keene, N.H.). After air drying, the nylon membranes were baked at 80°C for 30 min to 1 h. The baked membranes were sealed in plastic bags with 100 μl of prehybridization buffer {0.9 M NaCl, 50 mM sodium phosphate [pH 7.0], 50 mM EDTA [pH

TABLE 3. Computer-assisted design of oligodeoxynucleotide probes targeted for 16S rRNA of methylotrophs

Bacteria	Formaldehyde assimilation pathway utilized	No. of mismatches	
		Probe 9-α	Probe 10-γ
Methylococcus capsulatus Bath	RuMP	4	0
Methylomonas methanica	RuMP	4	0
Methylophilus methylotrophus AS1	RuMP	5	0
Methylobacterium extorquens AM1	Serine	0	6
Methylobacterium organophilum XX	Serine	0	7
Methylobacterium sp. strain DM4	Serine	0	4
Methylocystis parvus OBBP	Serine	0	6
Methylosporovibrio methanica 81Z	Serine	0	6
Methylosinus trichosporium OB3b	Serine	0	6
Methylosinus sp. strain B	Serine	0	7
E. coli	NA[a]	7	3
R. rubrum	NA	4	6
A. tumefaciens	NA	4	5
P. testosteroni	NA	7	3

[a] NA, Not applicable.

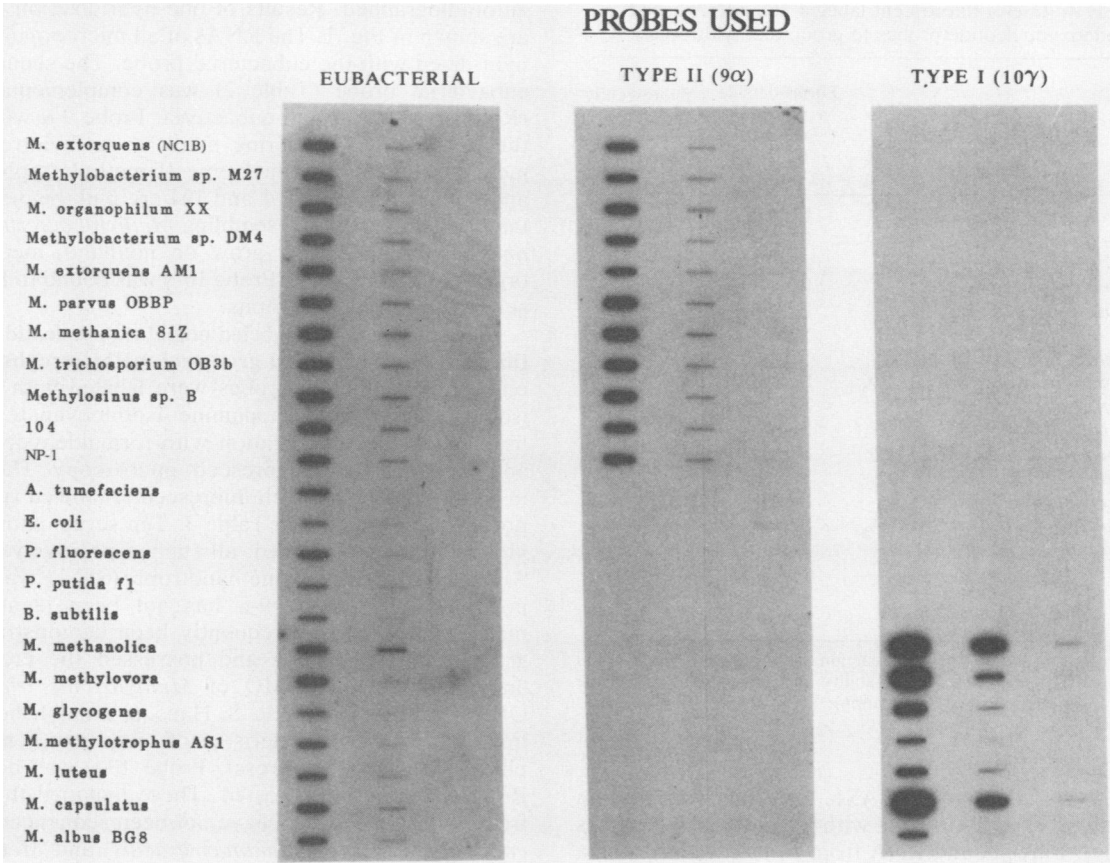


FIG. 1. Eubacterial RNA survey to determine probe specificity. Amounts of 100, 10, and 1 ng of total RNA from 23 different eubacteria were spotted onto triplicate nylon membranes. After baking, the triplicate filters were hybridized with the eubacterial, 9-α, or 10-γ probe. The eubacterial probe bound to all RNAs on the membrane, but the 9-α probe specifically bound only to RNA from serine pathway methylotrophs while the 10-γ probe specifically bound only to RNA of RuMP pathway methylotrophs. Although all of the five control organisms except *B. subtilis* are proteobacteria, their RNAs did not hybridize with either signature probe.

7.2], 0.5% SDS, 10× Denhardt solution [20], 0.5 mg of poly(A) per ml} per cm^2 of membrane. The membranes were incubated for a minimum of 2 h at 40, 45, or 47°C for the eubacterial, 9-α, and 10-γ probes, respectively. The solution was removed from each bag and replaced with 50 μl of hybridization buffer (0.9 M sodium phosphate [pH 7.2], 1.0% SDS, 1% bovine serum albumin, 1 mM EDTA [pH 8.0]) per cm^2 plus the appropriate probe at approximately 1×10^6 to 2×10^6 cpm/ml of buffer. Incubation was continued for another 18 to 20 h. The filters were then washed once for 30 min at room temperature in 40 mM sodium phosphate (pH 7.2) with 1 mM EDTA, 0.5% SDS, and 0.5% bovine serum albumin (wash 1 solution), followed by two 30-min washings in 40 mM sodium phosphate (pH 7.2) with 1% SDS and 1 mM EDTA (wash 2 solution) at 37, 50, or 52°C for the eubacterial, 9-α, and 10-γ probes, respectively. The washed membranes were exposed to film (Kodak X-Omat AR) with an intensifier screen (DuPont Cronex Lightning-Plus) at −70°C for 12 to 24 h.

Determination of T_d values. Triplicate filters with 2 μg of bound RNA were prepared and hybridized with appropriate probes. Filters were washed with wash 1 solution two times at room temperature for 30 min and then washed for 10 min with wash 2 solution at increasing 2°C increments up to 70°C. Samples of the washing fluid were collected at each temperature and counted.

RESULTS

Construction of signature probes. While studying phylogenetic relationships among methylotrophs (34), we created a data base containing 16S rRNA sequences of several methylotrophs. By comparing 16S rRNA sequences of six serine pathway and three RuMP pathway methylotrophs and several purple eubacteria, and by using a pattern-matching computational algorithm, two unique oligodeoxynucleotide probes, 9-α and 10-γ, with sequences complementary to the sequences of 16S rRNAs of methylotrophic bacteria were designed. These probes were targeted to serine pathway and RuMP pathway methylotrophs, respectively. The sequences of these probes and the number of nucleotide residues and their positions on 16S rRNA sequences are listed in Table 2. The number of nucleotide mismatches of probes 9-α and 10-γ to all existing methylotroph 16S rRNA sequences and 16S rRNA sequences of four reference microorganisms are listed in Table 3. The probe 9-α sequence had zero mismatch to all serine pathway methylotrophs sequenced, and the probe 10-γ sequence matched perfectly with RuMP methylotrophs and *Methylococcus capsulatus* Bath, a type X methanotroph. Probes 9-α and 10-γ were synthesized and used in the following experiments.

Determination of T_d values. RNAs, isolated from two methylotrophs, *Methylosinus trichosporium* OB3b and

TABLE 4. Use of fluorescent labeled 16S rRNA-based oligodeoxynucleotide probes to group methylotrophs

Bacteria	Formaldehyde assimilation pathway utilized	Binding of fluorescently labeled probes	
		Probe 9-α	Probe 10-γ
Methylococcus capsulatus Bath	RuMP	−	+
Methylococcus luteus	RuMP	−	+
Methylophilus methylotrophus AS1	RuMP	−	+
Methylomonas albus BG8	RuMP	−	+
Methylomonas gracilis	RuMP	−	+
Methylobacterium extorquens AM1	Serine	+	−
Methylobacterium sp. strain DM4	Serine	+	−
Methylobacterium organophilum XX	Serine	+	−
Methylocystis parvus OBBP	Serine	+	−
Methylocystis pyriformis #14	Serine	+	−
Methylosporovibrio methanica 81Z	Serine	+	−
Methylosinus trichosporium OB3b	Serine	+	−
Methylosinus sporium #27	Serine	+	−
Methylosinus sp. strain B	Serine	+	−
Unidentified methanotroph (SH-1)	Serine[a]	+	−
P. putida F1	NA[b]	−	−
B. megaterium	NA	−	−
Bacillus sp. strain A1	NA	−	−

[a] Based on the formation of cross-reaction with anti-*M. trichosporium* OB3b-soluble MMO antibodies and the ability to degrade trichloroethylene (Tsien and Hanson, unpublished observation).
[b] NA, Not applicable.

Methylophilus methylotrophus AS1, were blotted on nylon membrane filters and hybridized with ^{32}P-end-labeled probes 9-α and 10-γ. Filter-bound RNA from *E. coli* was used as a control. T_ds, the temperature at which 50% of the bound nucleotides were removed, were found to be 50 and 52°C, respectively, for probes 9-α and 10-γ. These temperatures were used in stringent washes in critical experiments. They were also used to determine the temperatures for prehybridization and hybridization, which were set at 45 and 47°C, i.e., 5°C below T_d values for probes 9-α and 10-γ, respectively.

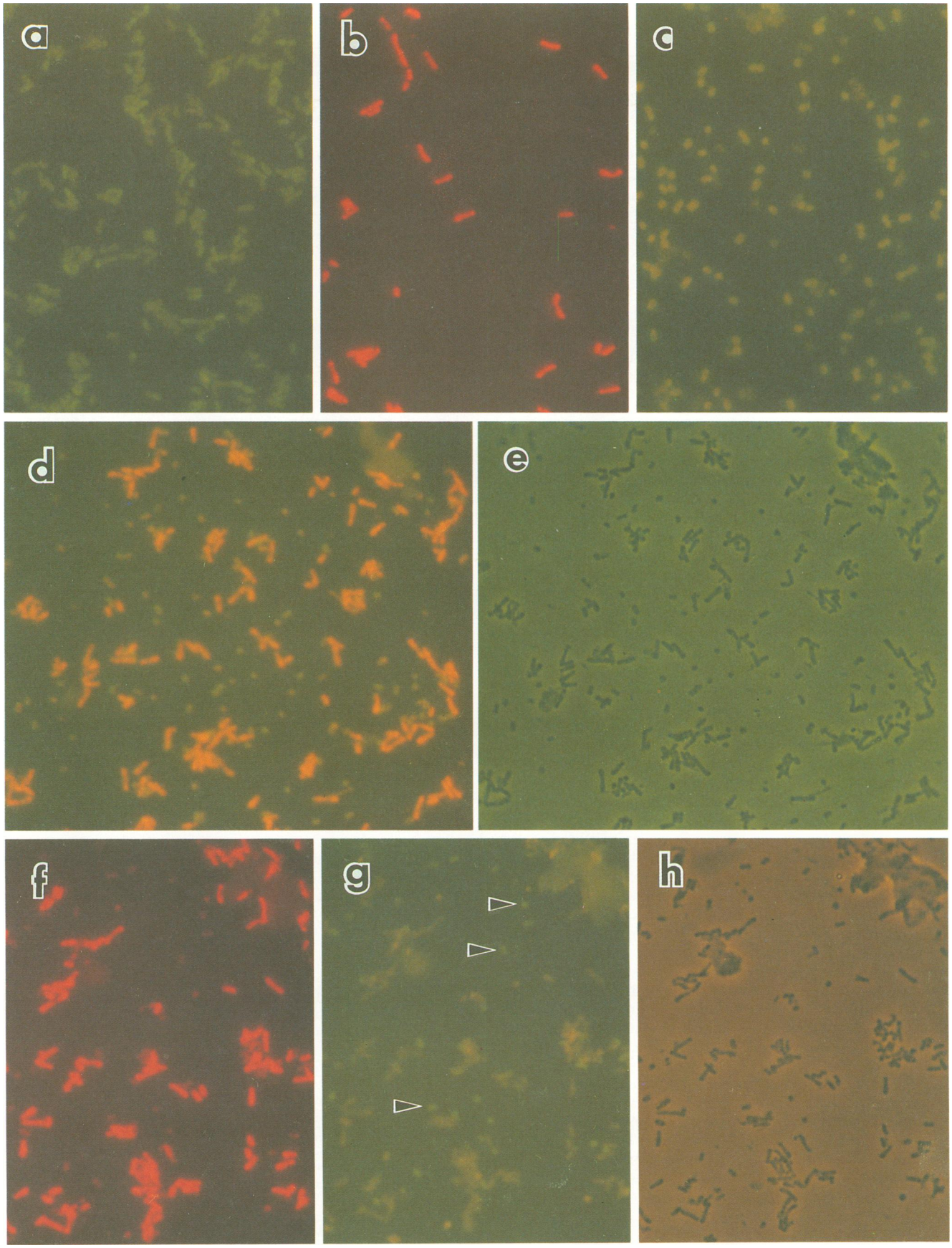
Hybridization of probes to membrane-bound RNA. Twenty-three isolated RNAs, including 18 methylotrophs and 5 nonmethylotrophic microorganisms, were slot blotted on a nylon membrane. The amount of RNAs applied to slots in each row were 100, 10, and 1 ng, respectively. Prehybridization and hybridization were carried out at temperatures specified for 9-α, 10-γ, and eubacterial probes. After hybridization, the filters were washed at room temperature followed by two washes at 50, 52, or 37°C for 9-α, 10-γ, and eubacterial probes, respectively. The membranes were then autoradiographed. Results of one hybridization experiment are shown in Fig. 1. The RNAs of all microorganisms tested hybridized with the eubacterial probe. The sequence of the eubacterial probe (Table 2) was complementary to 16S rRNA sequences of all eubacteria. Probe 9-α was bound to the RNAs of all 11 serine pathway methylotrophs tested including two unidentified type II methylotrophs, "NP-1" and "104." Strains NP-1 and 104 are pink-pigmented facultative methylotrophs resembling *Methylobacterium organophilum* strain XX that grow on methane, methanol, and organic substrates (24). Probe 10-γ was bound to RNAs of all seven RuMP methylotrophs.

Use of fluorescently labeled oligodeoxynucleotides for identification of physiological groups of methylotrophs. Synthetic oligodeoxynucleotide probes were labeled with fluorescein isothiocyanate or X-rhodamine isothiocyanate and were used in in situ hybridization with formaldehyde-fixed cells and examined by epifluorescent microscopy. The results of in situ hybridization with fluorescently labeled type-specific probes are presented in Table 4. Ten serine pathway methylotrophs were examined; all stained positively with probe 9-α. One unidentified methanotroph (SH-1) which stained positively with probe 9-α has not been identified. This methanotroph has subsequently been demonstrated to degrade trichloroethylene and possessed the cross-reactive antigen of soluble MMO of *Methylosinus trichosporium* OB3b (H. C. Tsien and R. S. Hanson, unpublished observation). These are characteristics of some type II methanotrophs (Tsien et al., in press). Probe 10-γ was bound to all RuMP methylotrophs tested. These included three species from which 16S rRNAs have not been sequenced, *Methylococcus luteus*, *Methylomonas gracilis*, and *Methylomonas albus* BG8.

Examples of in situ hybridization with the fluor-labeled 9-α probe are shown in Fig. 2a and b for *Methylosinus trichosporium* OB3b bound to fluorescein- and X-rhodamine-labeled probes, respectively. X-rhodamine-bound cells appear as red rods, whereas fluorescein-bound cells are green in these photomicrographs. The binding of *Methylomonas albus* BG8 with fluorescein-labeled 10-γ is shown in Fig. 2c. All nonmethylotrophs tested did not bind any probes specific to methylotrophs (Table 4).

Universal and eubacterial probes described by Giovannoni et al. (11) were synthesized, fluor labeled, and used as positive controls to ensure that cells were permeable to probes. Oligodeoxynucleotide sequences of universal and eubacterial probes were complementary to the sequences of 16S rRNA of all living organisms and of all eubacteria, respectively. All eubacteria tested hybridized positively with these two probes. Yeast cells bound only to the universal

FIG. 2. Use of fluorescently labeled signature probes to identify methylotrophs. Formaldehyde-fixed cells of *Methylosinus trichosporium* OB3b, *Methylomonas albus* BG8, and the mixture of *Methylosinus trichosporium* OB3b and *Methylomonas gracilis* were hybridized with X-rhodamine- or fluorescein-labeled 9-α and fluorescein-labeled 10-γ probes which were designed for the determination of methylotrophic bacteria possessing the serine and RuMP pathways for formaldehyde fixation, respectively. (a and b) Cells of *Methylosinus trichosporium* OB3b hybridized with fluorescein- and X-rhodamine-labeled 9-α probe, respectively. (c) Cells of *Methylomonas albus* BG8 hybridized with fluorescein-labeled 10-γ probe. (d to h) Mixed cells of *Methylosinus trichosporium* OB3b and *Methylomonas gracilis* were hybridized with the mixed probes of X-rhodamine-labeled 9-α and fluorescein-labeled 10-γ probes. (d) Double-exposed photomicrograph, using two different filter sets for X-rhodamine and fluorescein fluorescence, respectively, shows X-rhodamine-stained cells (orange rods) and fluorescein-stained cells (green short rods). (f and g) Photomicrographs taken with respective filter sets for X-rhodamine and fluorescein show rod-shaped *Methylosinus trichosporium* OB3b cells (red) and short rod-shaped cells of *Methylomonas gracilis* (green). The shadowy images observed in panel g occur because the filter set used for fluorescein is unable to eliminate the red color completely from X-rhodamine-stained cells. Green short rods (arrowhead) are *Methylomonas gracilis*. (e and h) Respective phase-contrast photomicrographs for panels d, f, and g show rod-shaped *Methylosinus trichosporium* OB3b cells and short rod-shaped *Methylomonas gracilis* cells. Cells embedded in impurities as shown at upper right-hand corner in panels d through h were clearly revealed when stained with fluorescently labeled probes.


a
b
c
d
e
f
g
h

probe. An oligonucleotide with its sequence complementary to the sequence of the universal probe was used as a negative control (11).

When two type-specific probes were labeled with different fluorescent dyes, they could be used simultaneously in in situ hybridizations, using a single cell preparation. Cells of *Methylosinus trichosporium* OB3b and *Methylomonas gracilis* were mixed, fixed by formaldehyde, and smeared on microscopic slides. Preparations were hybridized with mixed probes of X-rhodamine-labeled 9-α and fluorescein-labeled 10-γ probes and viewed with an epifluorescence microscope, using two different filter sets. The results are shown in Fig. 2d to h. Figure 2d, a double-exposed photomicrograph, shows X-rhodamine-stained cells of *Methylosinus trichosporium* OB3b and fluorescein-stained cells of *Methylomonas gracilis*. The preparation could also be examined and photographed separately with each respective filter set. In this way, cells which bound different probes labeled with different fluorescent dyes could be identified as shown in Fig. 2f and g. Phase-contrast photomicrographs provided a view of all cells in the field (Fig. 2e and h).

DISCUSSION

In previous studies, we described the phylogenetic relationship among methylotrophic bacteria by analyzing 16S rRNA sequences (34). RuMP methylotrophs were grouped in the β/γ subdivisions of the purple eubacteria (*Proteobacteria*), whereas serine pathway methylotrophs were grouped in the α subdivision. The presence of unique oligonucleotide sequences which are common to all methylotrophs within a specific taxonomic group allowed us to design signature probes and to use these probes for the determination of methylotrophic bacteria without prior knowledge concerning these microorganisms.

Probe design was based on a data base with a limited number of sequences of methylotrophs. These probes, however, hybridized not only with those methylotrophs from which 16S rRNAs were sequenced, but also to those methylotrophs whose 16S rRNAs were not sequenced (Fig. 1; Table 4). They are, therefore, useful for grouping unidentified methylotrophs. An unidentified methanotroph (SH-1) which possessed some characteristics of type II methanotrophs stained positively with the 9-α probe (Table 4). The 16S rRNA of *Methylosinus* sp. strain B, an unidentified methanotroph which stained positively with the 9-α probe, has now been sequenced. The numbers of nucleotide mismatches between *Methylosinus* sp. strain B 16S rRNA and probes 9-α and 10-γ were 0 and 7, respectively (Table 3). Subsequent studies indicated that *Methylosinus* sp. strain B possessed the cross-reactive antigen of soluble MMO of *Methylosinus trichosporium* OB3b, degraded trichloroethylene, and had type II intracytoplasmic membrane (Tsien and Hanson, unpublished observation). The presence of hydroxypyruvate reductase and 18-carbon phospholipid fatty acids in this methanotroph was also confirmed (B. J. Bratina, D. C. White, and R. S. Hanson, unpublished observation). The sequence homology between *Methylosinus* sp. strain B and *Methylosinus trichosporium* OB3b is 92.8%; for that reason, the name *Methylosinus* sp. strain B was given this isolate. Type I methanotrophs contain 16-carbon phospholipid fatty acids and do not contain a soluble MMO that cross-reacts with antibodies prepared against the soluble MMO of the type II methanotroph *Methylosinus trichosporium* OB3b (36; Tsien et al., in press).

The specificity of probes has been confirmed and verified by hybridization of the probes to slot-blotted RNAs (Fig. 1). The 9-α probe hybridized positively with all serine pathway methylotrophs tested and the 10-γ probe hybridized to all RuMP pathway methylotrophs. As little as 1 ng of RNA is detectable. Occasional variation in radioactive labeling (Fig. 1, 10-γ probe-labeled RuMP methylotrophs) is probably due to the quality of the RNA preparations.

Although each actively growing cell contains 10^4 to 10^5 16S rRNA molecules, the intensity of fluorescence after hybridization with the fluorescent probes is, nevertheless, low when compared with immunofluorescent microscopy. For this reason, we prefer to hybridize duplicate preparations of an organism with two different probes side by side on the same slide to provide a comparison. In this way, we can differentiate positively stained cells from negatively stained cells without any difficulty as well as the fluorescence caused by autofluorescence. Autofluorescence seems to be a problem only at the wavelength region of fluorescein, around 500 nm. No autofluorescence was observed in the region of 600 nm where X-rhodamine fluorescence is maximum.

Some color variation in Fig. 2d caused the X-rhodamine-labeled *Methylosinus trichosporium* OB3b to appear as orange rods instead of red. The color modification of red rhodamine is caused by the double exposure during photography.

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