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Combined Microautoradiography–16S rRNA Probe Technique for Determination of Radioisotope Uptake by Specific Microbial Cell Types In Situ

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We propose a novel method for studying the function of specific microbial groups in situ. Since natural microbial communities are dynamic both in composition and in activities, we argue that the microbial “black box” should not be regarded as homogeneous. Our technique breaks down this black box with group-specific fluorescent 16S rRNA probes that simultaneously determine 3H-substrate uptake by each of the subgroups present via microautoradiography (MAR). Total direct counting, fluorescent in situ hybridization, and MAR are combined on a single slide to determine (i) the percentages of different subgroups in a community, (ii) the percentage of total cells in a community that take up a radioactively labeled substance, and (iii) the distribution of uptake within each subgroup. The method was verified with pure cultures. In addition, in situ uptake by members of the α subdivision of the class Proteobacteria (α-Proteobacteria) and of the Cytophaga-Flavobacterium group obtained off the California coast and labeled with fluorescent oligonucleotide probes for these subgroups showed that not only do these organisms account for a large portion of the picoplankton community in the sample examined (~60% of the universal probe-labeled cells and ~50% of the total direct counts), but they also are significant in the uptake of dissolved amino acids in situ. Nearly 90% of the total cells and 80% of the cells belonging to the α-Proteobacteria and Cytophaga-Flavobacterium groups were detectable as active organisms in amino acid uptake tests. We suggest a name for our triple-labeling technique, substrate-tracking autoradiographic fluorescent in situ hybridization (STARFISH), which should aid in the “dissection” of microbial communities by type and function.

In situ characterization of the marine picoplankton “black box” has been restricted mostly to determinations of community composition by a number of molecular techniques, including fluorescent in situ hybridization (FISH) (2, 3, 13, 16, 18, 33). In FISH fluorescently labeled oligonucleotide rRNA probes are used to identify and enumerate microorganisms in situ (13, 16). Probe sequences can target organisms at various taxonomic levels. Some probes are universal and target all organisms (18, 32), while others are for the domains Archaea (17, 30, 35) and Bacteria (16, 18); other probes target intermediate levels, such as the α subdivision of the class Proteobacteria (α-Proteobacteria), β-Proteobacteria, and γ-Proteobacteria (29), as well as the Cytophaga-Flavobacterium group (28); and still other probes are specific for species and subspecies (37).

In addition to composition, the marine bacterioplankton community can be characterized by its ecological role in the uptake of dissolved organic matter (DOM) via autoradiography (6, 15, 24, 38). Workers have reported that the bacterioplankton may be responsible for transferring up to 50 to 60% of the local primary production from the DOM pool into higher trophic levels through the microbial loop (7, 14, 15, 20). Standard measurements of in situ nutrient uptake, however, do not discriminate between different prokaryotic groups. Since analysis of 16S rRNA sequences shows that the marine picoplankton community is dynamic and is composed of various diverse organisms (3, 36), while studies of nutrient uptake by pure cultures show that the type and rate of nutrient uptake vary among different strains (22), we can conclude that in situ substrate uptake is dependent on the local picoplankton community composition. Hence, in order to better understand and model DOM uptake in the ocean, microbiologists need to “dissect” the bacterioplankton black box into smaller compartments on the basis of taxonomy and function instead of looking at it as a single homogeneous group.

In situ identification of the different prokaryotic groups in mixed communities, however, is limited by the lack of morphological features (9); this is the prime reason why FISH has become such an important tool in microbial ecology (3). Also, isolation of marine prokaryotes in pure cultures has been demonstrated to be difficult; less than 1% of the total natural community grows under laboratory conditions (39).

Here we suggest a new method in which FISH and microautoradiography are combined to determine in situ nutrient uptake by members of specific picoplankton groups on a single slide. The cells are triple-labeled with a general stain, a fluorescent oligonucleotide probe, and a tritiated substrate. With the same microscopic field it is possible to determine not only the percentage of a specific prokaryotic phylogenetic group in a mixed sample but also the distribution of nutrient uptake within each subgroup. Results obtained with both mixed cultures and natural marine samples are presented below.

MATERIALS AND METHODS

The sequence of events in our triple-labeling technique involved collection of samples, transfer of cells onto emulsion-gelatin-coated slides, emulsion exposure and development, whole-cell hybridization, and epifluorescence counting. All solutions, including the culture media and phosphate-buffered saline (PBS), were filtered with 0.2-μm-pore-size Nuclepore filters, autoclaved, and checked for the presence of prokaryotes by DAPI (4’6-diamidino-2-phenylindole) staining before they were used throughout the entire procedure. Formalin was filtered with 0.02-μm-pore-size Whatman Anodisc filters before it was used.

Culture preparation. The following organisms were selected based on their uptake of glucose and morphological characteristics for easy differentiation in

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nutrient uptake tests and during identification by epifluorescence microscopy and on photomicrographic: Escherichia coli, a short bacillus which can take up glucose; and Monasella carthalthus, a coccolid (whose cells usually occur in pairs) that does not take up glucose (22). The two main purposes of the culture experiment were to test the protocol under highly controlled conditions and to test whether 16S RNA, the target molecule for the oligonucleotide probes, could endure the conditions in the photographic solutions.

Replicates of both cultures were grown separately in Luria-Bertani enriched medium at 37°C to the mid-exponential growth phase, as determined with a Klett colorimeter (Klett-Summerson, New York, N.Y.) at an optical density of 0.25 nm. Cells were concentrated on centrifugation at 2,300 × g at 17°C for 5 min and were washed three times in 1× PBS; this was followed by two additional centrifugation steps, and each time the cells were resuspended in M9 minimal medium (27) lacking glucose.

**Natural sample preparation.** The following natural samples were collected in 250-ml acid-washed dark bottles from two sites in southern California: (i) Catalina Island surface water, which was collected on 16 January 1998 at 33°26.71’ N and 118°29.07’ W; and (ii) water collected in the San Pedro Channel 5 m below the surface on 31 May 1998 at 33°33’ N and 114°24’ W. Sample preparation. Tritiated D-glucose (Dupont) was added at a concentration of 10 nM to two replicate cultures growing in minimal medium. Two killed cultures: a formalin-fixed culture containing 6.74 ng/ml and a formalin-fixed culture containing 50 ng/ml of Cy3-labeled oligonucleotide labeled probes. The emulsion-gelatin-coated slides were left to dry in total darkness for 30 min before they were placed in a light-proof box which was kept in total darkness, as well as for the natural subsamples, by withdrawing 2-ml aliquots from each tube. Two of the subsamples that were killed by treatment with 10% formalin were placed in a light-proof box which was protected from light. The slides were left to dry in total darkness for 30 min before they were placed in a light-proof box which was protected from light. The slides were left to dry in total darkness for 30 min before they were placed in a light-proof box which was protected from light. The slides were left to dry in total darkness for 30 min before they were placed in a light-proof box which was protected from light.

**Slide preparation.** In a darkroom, a photographic emulsion (type NTB2; Eastman Kodak, Rochester, N.Y.) was mixed with 0.5% formalin solution (1× formalin) for 1 h served as controls. All four subsamples were supplemented with a 5 mM pH-amino acid mixture (Amer sham) and were incubated at in situ seawater temperatures.

Nutrient uptake was monitored over time for all of the live and killed cultures, as well as for the natural subsamples, by washing 2-ml aliquots from each tube. Two of the subsamples that were killed by treatment with 10% formalin for 1 h served as controls. All four subsamples were supplemented with a 5 mM pH-amino acid mixture (Amer sham) and were incubated at in situ seawater temperatures.

Sample treatment. Tritiated D-glucose (Dupont) was added at a concentration of 10 nM to two replicate cultures growing in minimal medium. Two killed controls for each culture were prepared by treatment with 10% formalin for 1 h prior to addition of D-glucose. Cultures were incubated on a rotary shaking platform at 250 rpm at 37°C.

Natural samples were divided into four 40-ml subsamples in sterile conical tubes. Two of the subsamples that were killed by treatment with 10% formalin for 1 h served as controls. All four subsamples were supplemented with a 5 mM pH-amino acid mixture (Amer sham) and were incubated at in situ seawater temperatures.

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briefly rinsed with distilled water at 43°C, and finally immersed three times in 0.2% SET at 43°C for 10 min each time. After air drying, the slides were mounted in a glycerol-10% SET (50:50, vol/vol) solution and kept at −20°C for at least 1 h before they were observed by fluorescence and transmitted light microscopy.

**Autoradiographic and fluorescent-cell counts.** For each microscopic field, the following four types of counts were obtained: (i) total DAPI cell counts with UV excitation (Olympus type U-MWU UV filter; excitation wavelength, 330 to 385 nm; emission wavelength, >420 nm); (ii) probe fluorescence cell counts, which included autofluorescent and probe-labeled cell counts with green light excitation (Chroma type tetramethylrhodamine isothiocyanate [TRITC] U-M41002 filter; excitation wavelength, 535 ± 50 nm; emission wavelength, 565 nm); (iii) microautoradiography counts (cells labeled with one of the tritiated nutrients) under transmitted light; and (iv) counts for cells labeled with a fluorescent probe and simultaneously labeled autoradiographically (overlap between green excitation and transmitted light). The last three types of counts were computed as percentages of the total DAPI cell counts.

All counts were obtained within 2 days of hybridization by using an Olympus Bmax epifluorescence microscope equipped with a UPlanApo objective lens (magnification, ×100), a type HBO 100 Hg vapor lamp, and the filters described above. The images were captured and intensified by using a microchannel plate image intensifier (model COHU intensified charge-coupled device camera), and the background was reduced by image averaging with a model DSP-2000 image processor (Dage-MTI, Inc., Michigan City, Ind.). The images were visualized with a Sony Trinitron color video monitor (model PVM-153MD). This video system could display cells with fluorescence considerably less than the fluorescence directly detectable by eye.

**Comparing counts with FISH counts.** In order to determine whether the treatment of cells during the triple-labeling technique had any effect on the natural picoplankton community composition (from selective transfer of cells to or selective loss of cells from the emulsion-coated slides), the fluorescent probe counts obtained from the triple-labeling experiments were compared to standard FISH counts obtained for the San Pedro Channel sample collected on 31 May 1998. The standard FISH protocol used has been described in detail previously (32), and no chloramphenicol treatment was performed before the cells were preserved.

**Photomicrography.** Culture samples were photographed with the Olympus Bmax microscope described above by using Fujifilm Provia 1600 ISO film. The exposure times were 5 s with UV excitation (DAPI-stained cells), about 1 min with green excitation (probe-labeled and autofluorescent cells), and 3 s with transmitted light (autoradiographically labeled cells). The oligonucleotide probe fluorescence from some cells in the natural sample was too dim (compared to that of the intensified video system) to be properly recorded on the photographic film.

**RESULTS**

**Cultures.** The results of the culture experiment were as expected. From the nutrient uptake curves, we confirmed that the *E. coli* strain used was capable of taking up glucose but that the *M. catarrhalis* strain was not. When these strains of *E. coli* (short bacilli in Fig. 1) and *M. catarrhalis* (cocci in Fig. 1) were used in the triple-labeling experiment, the autoradiography counts showed an all-or-nothing type of results. While 100% of the live *E. coli* cells were labeled with [3H]glucose (compare the short bacilli in Fig. 1D with the autoradiographic marks at the same positions in Fig. 1E), the *M. catarrhalis* cells remained unlabeled (compare the cocci in Fig. 1D with the autoradiographic marks at the same positions in Fig. 1E). None of the killed cells in the control samples took up [3H]glucose (Fig. 1A and 1B).

Less than 2% of cells left an autoradiographic record on the emulsion (Fig. 1E) without a corresponding attached cell (Fig. 1D). The silver grain marks left by labeled cells were distinct in shape and size. It is also possible that cells were washed off the emulsion during in situ hybridization, but no tests were done to confirm this.

When cells were excited under green light, none of the cells were visible with no probe (NP) or with the control probe...
FIG. 2. Fluorescent-oligonucleotide probe, 3H-amino acid mixture, and probe plus 3H-amino acid mixture counts as percentages of the total DAPI counts for the Catalina Island (30 km off the coast of Los Angeles) surface sample collected on 18 January 1998. All of the probes, including the control (CON), universal (UNI), and Bacteria domain (BAC) probes, were dually labeled with Cy3 dye. Autofluorescent cell counts were not subtracted from the probe counts. The error bars indicate standard errors. 3H-AA, 3H-labeled amino acids.

(CON), while all of the cells were visible with the universal probe (UNI) or with the Bacteria probe (BAC) (compare the probe-labeled cells in Fig. 1C and F with the corresponding DAPI-labeled cells in Fig. 1A and B, respectively).

Natural marine samples. Tritiated amino acid uptake curves for both of the marine samples revealed rapid uptake that reached the saturation level by 3 h. The final corrected levels of radioactivity (live-cell radioactivity minus killed-cell radioactivity) for these two samples were of the same order of magnitude, but the value for the Catalina Island sample (3.14 × 10^−2 dpm/cell or 2.8 × 10^−19 mol of 3H-amino acid/cell) was two times higher than the value for the San Pedro Channel sample (1.53 × 10^−2 dpm/cell or 1.4 × 10^−19 mol of 3H-amino acid/cell). The levels of radioactivity for the killed controls were nearly undetectable (5.6 × 10^−5 dpm/cell for the Catalina Island sample control and 8.0 × 10^−3 dpm/cell for the San Pedro Channel sample control).

Triple-labeling data confirmed that the killed controls did not take up the 3H-amino acid mixture when either the Catalina Island sample for (Fig. 2) or the San Pedro Channel sample (Fig. 3) was examined. The 3 to 4% live cells detected for either the NP counts (2.8%) or the CON probe counts (3.5%) with the Catalina Island sample were assumed to be autofluorescent counts that probably originated from chlorophyll and phycoerythrin pigments, and they were not detected autoradiographically (Fig. 2). Autofluorescent cells are probably autotrophic, and they did not seem to take up dissolved amino acids in this experiment. With the San Pedro Channel sample the NP or CON probe counts accounted for less than 6% of the live cells, and less than 1% of the cells were simultaneously labeled with probe and by autoradiography (Fig. 3). The differences between the killed and live subsample probe (and NP) counts were not significant for either the Catalina Island sample (P = 0.278, as determined by Student’s t test) or the San Pedro Channel sample (P = 0.128, as determined by Student’s t test).

The average overall percentage of cells labeled autoradiographically (average autoradiography counts as percentages of DAPI counts for all probe and NP preparations) for the live Catalina Island subsample was 83% (standard error, ± 3.62%) (autoradiography counts divided by DAPI counts) (Fig. 2). The percentage was slightly higher for the San Pedro Channel sample (Fig. 3), 91% (standard error, ± 0.6%). Counts for the Catalina Island sample when cells were simultaneously labeled autoradiographically and with fluorescent probe showed that nearly 80% (standard error, ± 4.0%) of all of the cells were labeled with the universal probe (UNI) and 3H-amino acids and 64% (standard error, ± 4.0%) of the cells were labeled with the Bacteria probe (BAC) and 3H-amino acids.

The following two Bacteria subgroup-specific probes were used for the San Pedro Channel sample instead of the BAC probe: the α-Proteobacteria probe (αPRT) (mean ± standard error, 33 ± 4%) and Cytophaga-Flavobacterium (CF) probe (mean ± standard error, 23 ± 3.5%). Labeled amino acid uptake with each of these group-specific probes (computed as the simultaneous probe and autoradiography counts divided by the probe count, including the autofluorescent cell count) showed that 77% of the cells in the San Pedro Channel sample were labeled with the αPRT probe and 78% of the cells labeled with the CF probe were also labeled autoradiographically with the amino acid mixture (Fig. 3).

A comparison between the triple-labeling technique and the FISH technique for the San Pedro Channel sample (Fig. 4) showed that there were no significant differences (P = 0.253, as determined by the Student t test) for the NP counts and for all four probe (CON, UNI, αPRT, and CF) counts.

DISCUSSION

Our results show that it is possible to obtain simultaneous in situ measurements for the composition of a bacterioplankton...
community and the capacity of the community to take up specific organic nutrients. Culture experiments in which E. coli and M. catarrhalis strains were mixed helped demonstrate the feasibility of the triple-labeling technique. Under these highly controlled conditions, culture experiments showed that it is possible to label cells with fluorescent probes even after the cells undergo the additional treatments of the triple-labeling technique. In addition, the culture experiments provided a simple example of one of the applications of the triple-labeling technique. Both E. coli and M. catarrhalis belong to the domain Bacteria, but only E. coli was capable of taking up glucose, supporting our previous statement that in mixed picoplankton communities nutrient uptake is dependent on the local community composition.

Furthermore, results obtained with the triple-labeling technique did not differ significantly from results obtained with FISH alone (P > 0.05) despite the lengthier procedures involved in the former protocol. Hence, any loss of cells from the emulsion-coated slides due to the additional rinse steps or during the development of the emulsion in the triple-labeling technique did not lead to a significant change in the apparent community composition.

Although the oligonucleotide probes used in our study targeted the entire domain Bacteria and two of its subgroups, the α-Proteobacteria and the Cytophaga-Flavobacterium group, other workers have used more group-specific probes (including probes specific at the species level) for in situ studies of mixed microbial communities (3–5, 42). In addition, the tritiated amino acid mixture used in our experiments served as an unspecialized label for easily labeling most or all heterotrophically active organisms. However, more specific substrates, such as simple sugars, polymers, nucleosides, phospholipids, or dissolved inorganic ions, could be used.

With the Catalina Island sample, the Bacteria probe tagged almost all of DAPI-labeled cells, and most of these cells took up detectable levels of amino acids, suggesting that members of most Bacteria subgroups contribute to the uptake of dissolved amino acids at this site. The results obtained with the more specific probes for the San Pedro Channel sample showed that cells labeled with the α-Proteobacteria probe (αPRT) and the Cytophaga-Flavobacterium probe (CF) combined accounted for almost 60% of all of the cells labeled with the UNI probe [(αPRT + CF)/UNI] at that site, assuming that there was no nontarget labeling that resulted in overlap between the two subgroup probes. Such results agree with previous findings which showed that most clones recovered in nearby waters belonged to members of these subgroups (12, 17). Furthermore, most of the cells of members of each of the subgroups (80% of each subgroup) were active in uptake of dissolved amino acids.

It is important to note that probe counts probably underestimate the total number of cells belonging to the probe target group for a given natural sample for two important reasons. First, some naturally occurring prokaryotes seem to have insufficient 16S rRNA for them to be detectable by whole-cell fluorescent hybridization (23–25, 32). These cells may grow too slowly (1, 13, 21), or they may be inactive (43). In contrast to the results of Zweifel and Hagström (43), who found that up to 70% of the cells in the Baltic Sea were inactive, the numbers of inactive cells in our samples ranged from 7 to 15% of the total cells based on probe counts. Furthermore, we showed previously (32) that treatment of natural samples with chloramphenicol at a concentration of 100 μg/ml for 1 h can increase the level of detection of prokaryotes with a universal fluorescent probe by 15% on average to about 95% of the total counts. Although we could not pretreat our samples with chlor-
a long time (9). In previous studies, however, either the organisms of interest (usually limited to a few species) were identifiable by their morphological features (11) or the entire prokaryotic community was studied as a group that was considered homogeneous both in terms of composition and in terms of function in the uptake of DOM (15, 24, 31, 40).

Perhaps due to the unspecialized ^3H-amino acid mixture and the general group probes used in our experiments, our results showed that the α-Proteobacteria and the Cytophaga-Flavobacterium subgroups had very similar percentages of cells (80%) involved in uptake of amino acids. Nevertheless, because the members of the α-Proteobacteria were 10% more abundant, their overall significance in DOM uptake may be greater than the significance of members of the Cytophaga-Flavobacterium group, assuming that the average amount of amino acid taken up per cell does not vary significantly between these two prokaryotic subgroups. With further investigation, STARFISH may help break down the bacterioplankton black box into smaller components and may provide a better understanding of the ecological role of various organisms in the uptake of DOM under natural conditions.

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