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Minireview

MAR-FISH—An Ecophysiological Approach to Link Phylogenetic Affiliation and *In Situ* Metabolic Activity of Microorganisms at a Single-Cell Resolution

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A major goal of microbial ecology is to study the abundance, localization, and activities of microorganisms *in situ* in order to understand ecophysiological roles that the microorganisms play in complex natural ecosystems. In fact, in typical microbial habitats such as biofilms, sediments, and microbial aggregates, resources and physicochemical conditions are dynamically changing with time and across even a very tiny distance because of metabolic activities and substrate transport limitation. To directly correlate microbial identity (16S rRNA-based phylogeny) to the specific metabolic function of individual cells within such complex and heterogeneous microbial habitats, several new molecular-based techniques have been developed in the last decade. These techniques exploit *in situ* simultaneous phylogenetic identification and metabolic capabilities of even uncultured microorganisms without the need to isolate them in culture. Microautoradiography is a powerful but "rather old" tool, with which the *in situ* uptake of specific radiolabeled substrates by individual cells can be determined. Fluorescence *in situ* hybridization (FISH) is a new molecular-based technique that allows the *in situ* phylogenetic identitification of individual cells. However, FISH cannot provide sufficient information on metabolic capabilities, because phylogeny and phenotype are rarely congruent. Recently, microautoradiography and FISH have been successfully combined to further improve the complementary strengths of the two methods. Microautoradiography combined with FISH (MAR-FISH) can be used to simultaneously examine the phylogenetic identity and the relative or actual specific activity of microorganisms within a complex microbial community at a single-cell level. This article overviews the principle, experimental protocol and application of the MAR-FISH technique, as well as current developments of other analytical techniques for *in situ* microbial functions (metabolic activities) from a single-cell level to community levels.

Key words: microautoradiography combined with fluorescence *in situ* hybridization (MAR-FISH), microsensors, microbial community, *in situ* activity

Introduction

Understanding the structure and function of complex microbial communities is a central theme in microbial ecology. However, traditional cultivation-dependent methods are inadequate to fulfill this task because most members of microbial communities in natural and engineered systems cannot be cultured. It is now widely recognized that only a small fraction, possibly up to 1–10%, of the naturally occurring microorganisms in natural ecosystems have been isolated and characterized so far, even though a great diversity

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of microorganisms are present[7,86]. In recent years, new molecular biological techniques that are virtually based on the 16S rRNA gene sequence (the so-called full cycle rRNA approach) have been applied to analyze microbial community structure. This approach includes the cloning and sequencing of PCR-amplified rRNA genes from samples of interest, subsequent design of specific probes and eventually their *in situ* detection by fluorescence *in situ* hybridization (FISH)[7,60]. These techniques permit characterization of the communities without the need to cultivate the microorganisms. Thus, the cultivation-independent 16S rRNA approach allows one to obtain more complete phylogenetic information on molecular biodiversity of microbial communities and on the existence of heretofore unknown microorganisms. Especially, the FISH technique is relatively easy to perform and allows for *in situ* detection, localization, and quantification of single microbial cells or cell clusters in complex heterogeneous microbial communities such as biofilms, aggregates and sediments. Several recent reviews provide technical details, applications, and limitations of FISH and the rRNA approach[3,6,29,81,82]. However, 16S rRNA-based phylogenetic identification generally does not provide information on the physiology of the detected microorganisms. 16S rRNA is highly conserved and therefore has low resolution, somewhere near the species level at best. Knowing only what broad groups are present, it is usually difficult to say much for sure about specific function. There are now many major groups of Bacteria and Archaea known only from molecular sequences, and their eco-physiological role in the environment is uncertain until these microorganisms can be cultivated[33]. Even when a microorganism can be cultivated, eco-physiological properties determined in the laboratory may not necessarily reflect the *in situ* activities and physiology of their counterparts in the environment. As a consequence, our understanding of the ecological function of microbial populations in the environment is generally limited.

Microsensors

For analysis of microbial structure and function (activity) of such complex microbial communities, classical microbiological techniques like isolation and physiological characterization have limitations. Therefore, appropriate methods with sufficiently high spatial resolution are needed for (1) *in situ* identification, localization, and quantification of microbial populations, (2) the determination of the physicochemical microenvironment, and (3) the measurement of their *in situ* activity[5]. Combination of FISH and microsensor technology became a powerful and reliable tool during the last two decades. The spatial resolution of microsensors is about two times the tip diameter of the sensors as long as analyte consumption by the sensor is negligible and the sensor is small enough to cause minimum disturbance[73]. The tip diameter of microsensors applied to biofilms and aggregates is about 10 μm, indicating a spatial resolution of about 20 μm. This resolution is good enough to characterize the concentration gradients across the biofilms, microbial mats and sediments and to calculate the net rates (areal and volumetric) of production and consumption at a certain depth or of a whole microbial community. During the last decade, microelectrode measurement was nicely combined with FISH to relate microbial community structure and function of sulfate-reducing bacteria (SRB)[35,57,65,71] and nitrifying bacteria[14,56,58,59,72,74–76] in biofilms. The combination of these two methods allows relating *in situ* microbial activity directly to the occurrence of specific microorganisms within complex microbial communities. Microelectrodes, however, only measure net chemical profiles, and the spatial resolution is also above a single-cell level. To address the question of the higher abundance and activity of SRB in oxic zones of biofilms for example[57], the resolution of microelectrode measurements is not high enough. In addition, when the resources used by an uncultured microorganism are unknown or the abundance of the targeted microorganism is low in complex and heterogeneous habitats, the chemical profiles and fluxes are not correlated with the abundance of specific bacterial populations. Therefore, an analytical method at a single-cell level that allows us to more directly correlate 16S rRNA-based phylogenetic affiliation and specific metabolic activity of individual cells is desirable.

Combination of microautoradiography and FISH

Microautoradiography is a powerful tool, with which the *in situ* uptake of specific radioisotopes by individual cells can be determined. This method has been used to study the *in situ* metabolic activity of microbes in many ecological studies for a long time[15,27,31,39,44,61]. Measurement of *in situ* nutrient uptake by heterotrophic bacteria via autoradiography was first suggested in the mid 1960s by Brock and Brock[15] to determine the nutritional requirements of microorganisms and to estimate cell growth rate and production. However, they noticed that autoradiography alone was limited in assigning function to specific groups of microorganisms due to the lack of distinct morphological features among these microorganisms. In previous studies, either the

organisms of interest were identifiable by their morphological features[16,27] or the entire microbial community was studied as a group. These communities were considered to be homogeneous both in terms of composition and function in the uptake of substrates of interest[23,39,54,83]. The major limitation is obviously the inability to link the substrate uptake by individual cells to their phylogenetic identities[28]. Recently, microautoradiography and FISH have been successfully combined to further improve the complementary strengths of the two approaches. In this combination, the activity or function of interest can be demonstrated by microautoradiography (the accumulation of a suitable isotope, e.g., ^{3}H, ^{14}C, ^{35}S, or ^{33}P, inside or adjacent to the cells), and then the phylogenetic identity of microorganisms can be determined with FISH. This analysis will show which phylogenetic types and/or groups actively uptake a specific radiolabeled substrate during the time of incubation. Many different names were given for this approach with slight modifications, e.g., MAR-FISH (microautoradiography-fluorescence *in situ* hybridization)[42], STAR-FISH (substrate tracking autoradiography-fluorescence *in situ* hybridization)[62] and MICRO-FISH (microautoradiography-fluorescence *in situ* hybridization)[18]. All the methods differ only in detail; the idea and principle are identical, and we shall use the term "MAR-FISH" in this review. We discuss the combined application of MAR and FISH as a new tool for cultivation independent analysis of the microbial community structure and its function(s). Applying this technique, the phylogenetic identity and the specific activity of the microorganisms can be simultaneously examined *in situ* within a complex microbial community at a single-cell level.

Description of methodology

The experimental procedure of MAR-FISH is summarized from principle to application[42,52]. The typical MAR-FISH procedure is composed of (i) incubation with radiolabeled substrates, (ii) fixation and handling, (iii) staining (FISH, DAPI, or gram-staining), (iv) microautoradiographic procedure (MAR), and (v) microscopic observation (Fig. 1). Several important methodological options have been previously described elsewhere[52] concerning the application of MAR-FISH to complex microbial communities. The following are several additional possible improvements and important options described in recent publications. Lee *et al.*[42] discussed the order of MAR and FISH. In conclusion, FISH should be conducted before MAR. Detachment of biomass and autoradiographic film was frequently observed if FISH was performed after the developing procedure (MAR). The cellular signal intensities obtained by FISH were significantly higher if the FISH procedure was performed prior to the developing protocol (MAR).

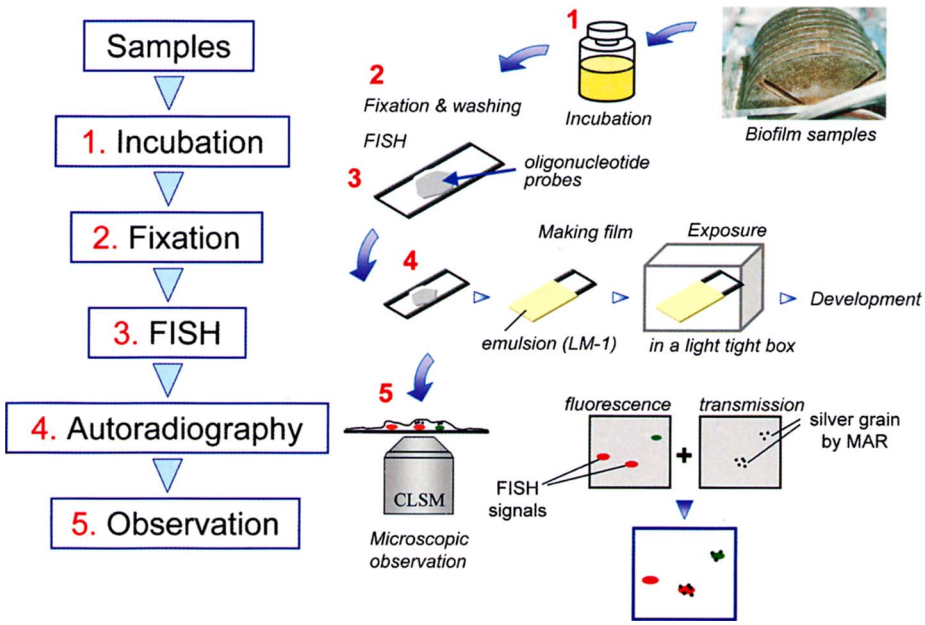

Fig. 1. Flow scheme of overall procedure for microautoradiography combined with FISH analysis.

(i) Incubation

It is very important to determine the following experimental parameters in advance; the right amount of biomass, the amount of radioactivity added ("hot substrate"), the background concentration level of "cold" substrate, the presence of other electron donors or acceptors (e.g., oxygen or nitrite), and the length of incubation. A typical sample volume is a few mL with a biomass concentration of 1–2 g L^{-1}. For this biomass concentration, a typical radioactivity added would be 1–25 μCi mL^{-1} (or 37–925 kBq mL^{-1})[9]. Optimum incubation conditions must be determined in advance, which totally depends on the microorganisms targeted and radiolabeled substrates used. For anaerobic incubation, it is critical to completely remove trace amounts of oxygen, which may cause a false substrate uptake within the initial few minutes. For examples, preincubation (1–3 h) is often carried out for anaerobic experiments of SRB to remove traces of oxygen[34] and to release orthophosphate in polyphosphate-accumulating organism (PAO) cells completely[48]. It is also important to include proper negative controls to evaluate non-specific uptake or absorption of radiolabeled substrates onto cell surfaces. The samples must be incubated with radiolabeled substrates, but microbial activity should be stopped by the addition of specific inhibitors. For example, molybdate (MoO_4) and bromoethanesulfonic acid (BES) can be used as a specific inhibitor for sulfate-reduction[34] and for methane fermentation[48], respectively. Furthermore, arylethiourea (ATU) is a good specific inhibitor for nitrifying activity. A pasteurized sample (70°C, 10 min) is also used in parallel to check adsorption phenomenon and chemography. It is also recommended to run the incubations in duplicate or triplicate.

Selection of radiolabeled substrates

In order to obtain the single-cell level resolution in microautoradiography, adequate radiolabeled substrate(s) or suitable isotopes must be selected for the microbial group(s) targeted. The best spatial resolution is obtained with weak beta-emitters such as ^{3}H and ^{14}C because the radioactivity does not travel far from its source, permitting better localization of the source. For ^{3}H and ^{14}C, the resolution is 0.5–2 μm[70]. Although ^{3}H-labeled substrates have a good resolution, the sensitivity is lower than for ^{14}C. ^{33}P has also been used for targeting PAOs[41]. Since MAR-FISH using ^{35}S has not been reported to date, it could be useful to apply to the study of sulfur-oxidizing bacterial groups.

(ii) Fixation and handling (cryosectioning and homogenization)

Samples were usually fixed in 4% paraformaldehyde (PFA) for 3 h at 4°C. After fixation, the samples must be washed three times by repeated centrifugation (at 14,000×*g* for 10 min) and the addition of washing buffer to remove excess radioactive substrate. Samples are then spotted on gelatin (0.1% gelatin and 0.01% chromium potassium sulfate)-coated cover glasses (24×60 mm) for air-drying. The sample can be stored (frozen or cold and dry) until use.

When tritium-labeled substrates are used, the leakage problem (excretion of $^{3}H_2O$) of tritium-labeled compounds from cells will occur if the fixation is not enough. The leakage significantly decreases the number of silver grains on the bacterial cells. The fixation protocol has been recently improved to overcome this leakage problem[47]. It indicates that the fixation for 3 h in 4% PFA is long enough to minimize the leakage. Repeated freezing and thawing of the sample from freezer to room temperature also causes the leakage. Therefore, immediately after fixation in PFA solution for 3 h, the cells should be immobilized on gelatin-coated cover glass and then stored at −20°C until use[47]. The amount of radioactive substrate incorporated in the samples is easily quantified by liquid scintillation counting. When ^{3}H-labeled substrate is used, the liquid scintillation counting may underestimate the actual amount of the uptake since a fraction of the radioactive label is excreted as $^{3}H_2O$ during metabolic processes. In the case of studying phosphorus (^{33}Pi) uptake, an additional washing step at pH 2 should be performed after normal fixation and washing in order to remove chemical phosphate precipitation, which causes false MAR-positive signals in the pasteurized negative control[42].

Cryosectioning

Fixed samples (e.g., biofilms and microbial aggregates) are embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN) overnight to infiltrate the OCT compound into the samples and subsequently frozen at −20°C. The frozen samples are cut into 5 to 10-μm-thick sections with a cryostat (Reichert-Jung Cryocut 1800, Leica) at −20°C. Each sectioned specimen should be placed on a gelatin- or poly-L-lysine-coated cover glass and air-dried over night. The specimen is finally dehydrated by successive passage through 50, 80, and 98% ethanol washes (for 3 min each) and air-dried. This cryosectioning step is needed to obtain a single-cell resolution for samples with high biomass density and activity like biofilms. Figure 2 shows a hypothetical il-

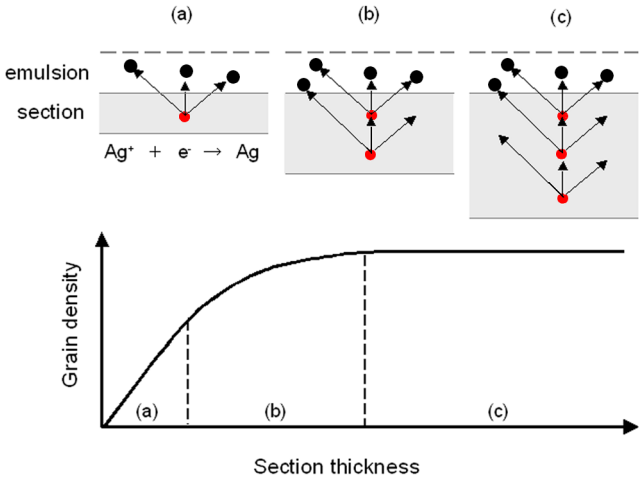

Fig. 2. Top figure: vertical sections of a specimen coated with emulsion, showing the effect of increasing section thickness on grain density (a)–(c). With increasing the section thickness, not all of the particles emitted can reach the emulsion so there is no longer a linear proportionality between section thickness and grain density (b–c). Bottom figure: graphic illustration of the relationship between section thickness and grain density (modified from Baker[13]).

lustration of the effect of increasing section thickness on accessibility of particles emitted to the emulsion layer. When the section thickness is thin, the number of grains formed in a given area of emulsion is proportional to the section thickness (Fig. 2 (a)). With an increase in the section thickness, not all of the particles emitted can reach the emulsion, so there is no longer a linear proportionality between section thickness and grain density (Fig. 2 (b) (c)). In general, the thinner section (ca. 5 to 10 μm-thick depending on cell density and activity) gives the better spatial resolution of MAR[42,48].

Homogenization

It is sometimes required to homogenize the samples to quantitatively count the MAR-positive cell numbers[34,42]. The fixed and washed samples are homogenized with a mini cordless grinder (Funakoshi, Tokyo, Japan) for a few minutes. The homogenized samples can be spotted on cover glasses and prepared for the following FISH as described above.

(iii) FISH

The *in situ* hybridization technique as described by Amann *et al.*[8] and Amann[2] can be used for MAR-FISH. Since the details of the FISH procedure can be found elsewhere[7], we do not cover them in this review. It should be noted that the signal intensity of the hybridized cells tends to be reduced after MAR development. The application of Cy3- and/or Cy5-labeled probes is, therefore, recommended since these fluorescent dyes show higher signal intensity than FITC[52]. Alexa-labeled probes may show more stable signals, which is independent of pH variation. In addition to FISH, other staining techniques such as DAPI and gram-staining can be performed at this stage.

(iv) Autoradiographic procedure (MAR)

Emulsion

After FISH, the cover glass with dried sample should be coated with the sensitive liquid film emulsion (LM1; Amersham Pharmacia Biotech) by a standardized procedure[9,42]. It is apparent that increasing emulsion thickness increases grain density where beta-particle range is greater than emulsion thickness. In the case of ^{3}H, a thickness of 3–4 μm is sufficient to absorb all beta-particles entering the emulsion layer[13]. In the case of ^{14}C (the long path of beta-particles), a thicker (10–100 μm) and uniform emulsion layer is required to absorb all particles and accurately determine grain density. It should be noted that increasing the thickness of the emulsion too much would negate the advantage for quantification and increased efficiency of ^{14}C microautoradiography. To optimize the efficiency of ^{14}C microautoradiography, an emulsion of high sensitivity should be used.

Exposure

Exposure time is another important factor affecting the resolution of MAR and consequently the interpretation of results. In general, prolongation of exposure will increase the grain density of a microautoradiograph. The exposure times depend on the radiolabeled substrate used and the amount of incorporation, and are usually 2 to 6 days. Longer exposure time often makes it difficult to distinguish MAR positive cells from background radiation or radiation emitted by larger adjacent colonies[21]. Therefore, correct exposure times should be determined in advance by trial and error.

Development

The number of silver grains on the surface of the cells is linearly developed as a function of development time and then saturated within a few minutes, whereas the background rises significantly after between 5 and 10 minutes. To obtain a higher contrast (signal-to-background (noise) ratio), the optimal length of development time should, therefore, be somewhere between 2 and 5 min[17].

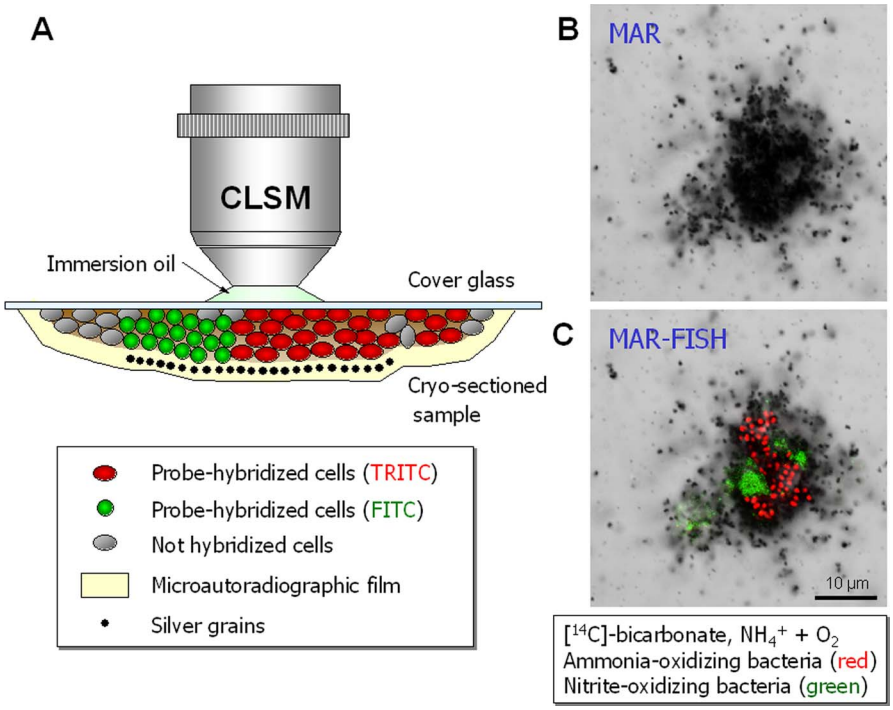

Fig. 3. A: Schematic presentation of confocal laser scanning microscopic (CLSM) observation (modified from Lee *et al.*[42]). A sample is viewed through a cover glass located above the fixed sample with the microautoradiographic film. B: Microautoradiographic image of a thin section (5 µm) of autotrophic nitrifying biofilm incubated with [^{14}C]bicarbonate and NH_4^+ as a sole electron donor under oxic conditions for 4 h. C: A combined MAR image (B) and FISH image of the microscopic field in panel B. *In situ* hybridization was performed with TRITC (red)-labeled probe Nso190 (specific for ammonia-oxidizing bacteria; AOB) and FITC (green)-labeled Ntspa1026 (specific for *Nitrospira*-like nitrite-oxidizing bacteria; NOB). This *Nitrospira*-like bacterium is uncultured NOB and a dominant species of NOB in wastewater treatment plants. Both AOB and NOB took up [^{14}C]bicarbonate even though only NH_4^+ was supplied as an electron donor, indicating that AOB oxidized NH_4^+ to NO_2^-, which was subsequently utilized by NOB sitting adjacent to AOB. This evidence clearly demonstrated that efficient transfer of NO_2^- occurred between AOB and NOB.

(v) Microscopic observation

Stained bacteria can also be examined for radioactive labeling by a combination of phase-contrast and epifluorescence microscopy. The key to the high-resolution analysis is to optimize the number of developed grains per radioactively-labeled bacterium, which can be achieved by balancing the combination of isotope used, incubation conditions, exposure time and development time. This can be done only by trial and error. One crucial device to make MAR-FISH more successful is the use of cover glasses instead of slide glasses, on which the samples are immobilized. At this step, FISH and MAR images can therefore be observed through the cover glasses from the backside by either inverse or normal microscopy. The use of inverse microscopy is the better way to avoid detachment or disruption of the autoradiographic film by movement of the objective[42]. In the case of normal microscopy, the cover glass is turned down and then placed on the slide glass, which allows the same view as in the case of inverse microscopy without putting pressure on the cover glass (Fig. 3A). A confocal laser microscope is highly recommended to use for acquiring sharp FISH images and efficiently combining them with MAR images (Figs. 3B and 3C).

Drawbacks

The substrates added are foreign to the microbial communities and may not represent the natural food source of the microorganisms of interest. Furthermore, the number of potential substrates present in samples (e.g., wastewater and sediments) is so large that a comprehensive assessment might be difficult. Therefore, it may be helpful to reduce the domain substrates by determining what types of substrates can be utilized by a population of interest and under what conditions (e.g., oxic and anoxic conditions) in advance. Substrate cross-feeding is of particular importance at a prolonged substrate incubation time. This effect could be ex-

ploited to investigate the transfer of carbon through microbial communities but can cause problems in differentiating between primary substrate consumers and microorganisms that live on the secretions or lysis products of those primary consumers.

The sensitivity and reproducibility of the MAR-FISH, in general, depend on several factors. First, the concentration of bacterial target cells in the system and their mean cellular ribosome content determine the detection limit of MAR-FISH. Second, it must be considered that the incorporation rates of radiolabeled carbon into biomass might vary significantly depending on the type of microorganisms and the activity of the same species. Third, the ratio of hot and cold substrates and the specific activity of the radiolabeled substrate selected significantly influence the sensitivity. For successful MAR-FISH analysis, all these experimental factors must be carefully determined in advance basically by trial and error, and the experiments must be conducted in duplicate or triplicate. Thus, MAR-FISH is rather time consuming and tedious, and the radiolabeled compounds are also expensive. In addition, legislation of the use of isotopes in some countries restricts application of MAR (e.g., use of certain isotopes).

Due to the low resolution of MAR, sample homogenization or sectioning is sometimes required if single bacterial cells within biofilms or thick sludge flocs are analyzed by MAR. In contrast to fluorescently stained cells, the 3-D distribution of silver grains in the emulsion layer (e.g., above a sample) cannot be accurately analyzed even by confocal laser scanning microscopy (Figs. 3A and 3B).

Literature review

MAR-FISH is an elegant approach to evaluate substrate uptakes by different phylogenetic groups in complex microbial communities. To date, this technique has been successfully applied in a limited, but rapidly increasing, number of studies on microbial communities in natural and engineered environments as summarized in Table 1.

Filamentous bacteria causing bulking in activated sludge

"Bulking" due to excessive growth of filamentous bacteria in activated sludge treatment plants is a general and serious problem in many treatment plants. A better understanding of factors controlling the growth of various filamentous bacteria is necessary to efficiently control the bulking problems. This requires phylogenetic identification and detailed knowledge about the ecophysiology of the different filamentous bacteria, which are indistinguishable on the basis of morphological observations alone. In addition, these filamentous bacteria are notoriously difficult to maintain in laboratory cultures. For these reasons, MAR-FISH has been first applied to various industrial or municipal activated sludge systems to investigate the *in situ* substrate uptake patterns of the predominant filamentous bacteria especially *Thiothrix* spp.[47,49,51,84] and *Microthrix parvicella*[10,53], which were responsible for bulking and foaming problems. These papers described how microautoradiography (MAR) and FISH could be used to characterize and enumerate functionally important groups of microorganisms in activated sludge. The principles for the methods, some important methodological aspects and limitations of the procedures were also briefly discussed[52]. The studies clearly demonstrated that strain differences with regard to substrate utilization were likely to occur among bacteria within the same genera and designated types which are indistinguishable on the basis of morphological observations alone and by the 16S rDNA probes used in the study.

It is well known that *Thiothrix* spp. are phylogenetically related to *Leucothrix* and the filamentous sulfur-oxidizing bacterium Eikelbooms Type 021N[87,90]. The morphology is also very similar for *Thiothrix* spp. and Type 021N, hence a proper identification can only be performed by using FISH based on the 16S rRNA sequence[30]. Eikelbooms Type 021N bacteria have been recently reclassified as *Thiothrix* and divided into three distinct groups on the basis of their genotypic and phenotypic characteristics[12,38]. Both types can be present simultaneously[49,87]. Identification and enumeration using FISH with species-specific 16S and 23S rRNA probes revealed that 5–10% of the bacteria in the activated sludge were *Thiothrix* spp.[49]. Physiological characteristics of *Thiothrix* were also quantitatively determined by counting the number of silver grains formed on the top of the filaments[49]. MAR-FISH results indicated the *Thiothrix* filaments were very versatile and showed incorporation of [^{14}C]-acetate and/or [^{14}C]-bicarbonate under heterotrophic, mixotrophic and chemolithoautotrophic conditions. The key properties that *Thiothrix* spp. might be employing to outcompete other microorganisms in activated sludge are probably related to the mixotrophic growth potential with strong stimulation of acetate uptake by thiosulphate, as well as stimulation of bicarbonate incorporation by acetate in the presence of thiosulphate or intracellular elemental sulfur. This phenomenon has not been described in pure culture study. The occurrence of mixotrophic growth of *Thiothrix* spp. was a significant factor giving *Thiothrix* spp. a compet-

Table 1. Examples of previous studies on the *in situ* substrate uptake patterns of microorganisms inhabiting various microbial ecosystems

Target microorganisms and microbial community	Radio-labeled substrates used	Reference
·Filamentous bacteria related to sludge "Bulking"		
Filamentous bacteria in activated sludge	[^{14}C]acetate, [^{3}H]glucose, [^{14}C]ethanol, [^{3}H]glycine, [^{3}H]leucine, [^{3}H]oleic acid	Nielsen *et al.* (1998)
Microthrix parvicella	[^{14}C]acetate, [^{14}C]propionate, [^{14}C]butyrate, [^{3}H]glucose, [^{14}C]ethanol, [^{3}H]glycine, [^{3}H]leucine, [^{14}C]sodium dodecyl sulfate (SDS), [^{14}C]octadecanol, [^{3}H]palmitic acid, [^{3}H]oleic acid, [^{3}H]trioleic acid	Andersen and Nielsen (1998)
Microthrix parvicella in activated sludge	[^{14}C]oleic acid	Nielsen *et al.* (2002)
Microthrix parvicella in activated sludge	[^{14}C]oleic acid, [^{3}H]trioleic acid, [^{14}C]acetate	Andersen and Nielsen (2000)
Thiothrix spp. in activated sludge	[^{3}H]acetate, [^{3}H]butyrate, [^{14}C]bicarbonate,	Nielsen *et al.* (2000)
Type0041 and TM7 (uncharacterized filamentous bacteria)	[^{3}H]acetate, [^{3}H]glucose, [^{3}H]galactose, [^{3}H]manose, [^{3}H]glycine, [^{3}H]leucine, [^{14}C]oleic acid	Thomsen *et al.* (2002)
Thiothrix and single cells in activated sludge	[^{3}H]acetate	Nielsen *et al.* (2003)
Candidatus meganema perideroedes and *Thiothrix*	[^{14}C]acetate, [^{3}H]acetate, [^{3}H]glucose	Nielsen *et al.* (2003)
·Microbial communities in Wastewater treatments		
Pure culture microorganisms and activated sludge	[^{3}H]glucose, [^{14}C]acetate, [^{14}C]butyrate, [^{14}C]bicarbonate, ^{33}Pi	Lee *et al.* (1999)
Iron-reducing bacteria	[^{14}C]acetate, [^{3}H]acetate	Nielsen *et al.* (2002)
Nitrospira-like nitrite-oxidizing bacteria (NOB)	[^{14}C]bicarbonate, [^{3}H]acetate, [^{14}C]propionate, [^{14}C]butyrate, [^{14}C]pyruvate	Daims *et al.* (2001b)
Nitrifying autotrophic biofilm	[^{14}C]bicarbonate, [^{14}C]acetate, [^{14}C]NAG, [^{14}C]-labeled amino acid	Kindaichi *et al.* (2004)
Acetate-consuming bacteria in activated sludge	[^{3}H]acetate	Nielsen and Nielsen (2002)
SRB populations in wastewater biofilms	[^{14}C]acetate, [^{14}C]propionate, [^{14}C]butyrate, [^{14}C]bicarbonate, [^{14}C]formate	Ito *et al.* (2002a)
Phosphate-accumulation organisms (PAOs)	[^{33}P]phosphate	Lee *et al.* (2002)
·Marine samples		
Two seawater samples	[^{3}H]-labeled amino acids mixture, NAG, protein, and chitin	Cottrell and Kirchman (2000)
Marine picoplanktons	D-[^{3}H]glucose, [^{3}H]-labeled amino acid mixture	Ouverney and Fuhrman (1999)
Sediment containing *Achromatium* cells	[^{14}C]bicarbonate, [^{14}C]acetate	Gray *et al.* (2000)
Oligotrophic marine samples containing Archaea	[^{3}H]-labeled amino acids mixture	Ouverney and Fuhrman (2000)

itive and survival advantage over heterotrophs in complex activated sludge systems and led to bulking.

Microthrix parvicella is known as another filamentous microorganism responsible for bulking and foaming problems especially in activated sludge plants for nutrient removal such as nitrogen and phosphorous. However, the physiological capabilities of *M. parvicella* under *in situ* conditions are not well understood even though some studies of *M. parvicella* in pure cultures have been reported[79,80]. Therefore, MAR-FISH was used to investigate substrate utilization capabilities (especially long chain fatty acids (LCFA)) of *M. parvicella* under *in situ* conditions (e.g., oxic and anoxic conditions)[10,11]. The MAR-FISH results revealed that only LCFA including oleic acid, palmitic acid, and to some extent a lipid (trioleic acid) were assimilated under oxic conditions. This strongly indicated that the availability of LCFA, e.g., via the rate of hydrolysis of lipids, might be a controlling factor for the development of *M. parvicella* in activated sludge systems. Such improved knowledge of the physiology of *M. parvicella* under *in situ* conditions obviously could provide a better control strategy.

Other filamentous bacteria in activated sludge

Among the filamentous bacteria occasionally causing bulking problems in activated sludge treatment plants, three morphotypes are common, Eikelboom Type 0041, Type 1851 and Type 1701[77,85]. Very limited information is available about the phylogeny and physiology of these filamentous bacteria. MAR-FISH was therefore performed to investigate the identity and *in situ* physiology of the Type 0041-morphotype in two wastewater treatment plants[84]. The type 0041-morphotype is phylogenetically heterogeneous and probably comprises a number of distinct species, of which at least approximately 15% of the filaments belong to the TM7 phylum, a recently recognized major lineage in the bacterial domain[32]. This phylogenetic heterogeneity of Type 0041 again highlights the inadequacy of a morphology-based classification system. The *in situ* physiology of Type 0041 using MAR-FISH, however, revealed that the type 0041 filaments demonstrated several physiological similarities, such as the ability to consume glucose under oxic and anoxic conditions, and the inability to uptake acetate under oxic conditions. These are the first data on the *in situ* physiology of bacteria belonging to the almost entirely uncharacterized TM7 phylum and show that TM7 filamentous bacteria are physiologically versatile.

Phosphate-accumulating organisms (PAOs)

The microbiology of enhanced biological phosphorus removal (EBPR) in wastewater treatment plants is one of the great challenges in activated sludge microbiology, since the identification, population dynamics and interactions of the PAOs with other competing bacterial groups including glycogen-accumulating organisms (GAOs) are still largely unresolved. More than a decade ago, previous molecular studies demonstrated that *Acinetobacter*, the traditional model microorganism for EBPR, does not catalyse phosphorous removal in these plants[19,43,87]. Recently, the population size of PAOs in activated sludge was estimated to be approximately 4% of the total DAPI count on the basis of uptake ability of radiolabeled acetate under anoxic conditions[46]. The *in situ* physiology and identity of the PAOs in pilot and full-scale activated sludge plants was also investigated by MAR-FISH after incubations with radioactive $^{33}P_i$, [^{3}H]acetate, and D-[6-^{3}H]glucose, respectively[41]. The result revealed that a significant ^{33}Pi uptake was mainly observed for the *Rhodocyclus*-related bacteria within the beta-Proteobacteria and the gram-positive *Actinobacteria*. The *Rhodocyclus*-related bacteria also occurred in significant numbers in these EBPR plants. However, not all of the *Rhodocyclus*-related bacteria detectable with a specific probe accumulated polyphosphate during aerobic growth and showed uptake of [^{3}H]acetate and D-[6-^{3}H]glucose. This evidence suggests that either a part of them is inactive or that there are non-PAOs within this group. Furthermore, the results suggested that the uptake of P is probably mediated not only by the *Rhodocyclus*-related bacteria, but also by other bacterial division, i.e., yet unidentified bacteria groups.

Nitrifying bacteria community

Traditionally, *Nitrobacter* was considered to be the most important nitrite-oxidizing bacteria in wastewater treatment plants. Using the full cycle rRNA approach the occurrence of yet uncultured *Nitrospira*-like nitrite-oxidizing bacteria in nitrifying wastewater treatment plants (WWTPs) has been often demonstrated[20,21,24,36,40,56,58]. By using MAR-FISH, Daims and coworkers[21] investigated the ecophysiology of the uncultured *Nitrospira*-like nitrite-oxidizers in activated sludge and found that these bacteria are able to fix bicarbonate and to simultaneously take up pyruvate but not acetate, butyrate, and propionate. *Nitrospira*-like nitrite-oxidizers are probably *K*-strategists (with high substrate affinities and low maximum activity or growth rates) for oxygen and nitrite and thus outcompete *Nitrobacter* under substrate-limiting conditions in WWTPs[76] or in the deeper part of the biofilm where the O_2 concentration is low[56]. This hypothesis would also explain why *Nitrobacter* and *Nitrospira* co-exist in sequencing batch biofilm reactors with temporarily higher nitrite concentrations[21].

It is also important to note that physiologically inactive ammonia oxidizers are detected by FISH as these bacteria maintain high cellular ribosome contents under unfavorable conditions[88]. This is one of the difficult parts of the interpretation of FISH data. However, the number of physiologically active ammonia oxidizers can accurately be determined using MAR-FISH with ^{14}C-labeled bicarbonate as substrate[42].

Coexistence of heterotrophs in high abundance with nitrifiers has often been found even in autotrophic nitrifying biofilms cultured without the external organic carbon supply[56,58]. In such biofilm systems, the competitive interaction between heterotrophs and nitrifiers for dissolved oxygen and space is well known[55]. In the presence of organic carbon, nitrifiers are usually outcompeted by heterotrophs due to the slow growth rate and low growth yield. They also interact through the exchange of organic matter derived

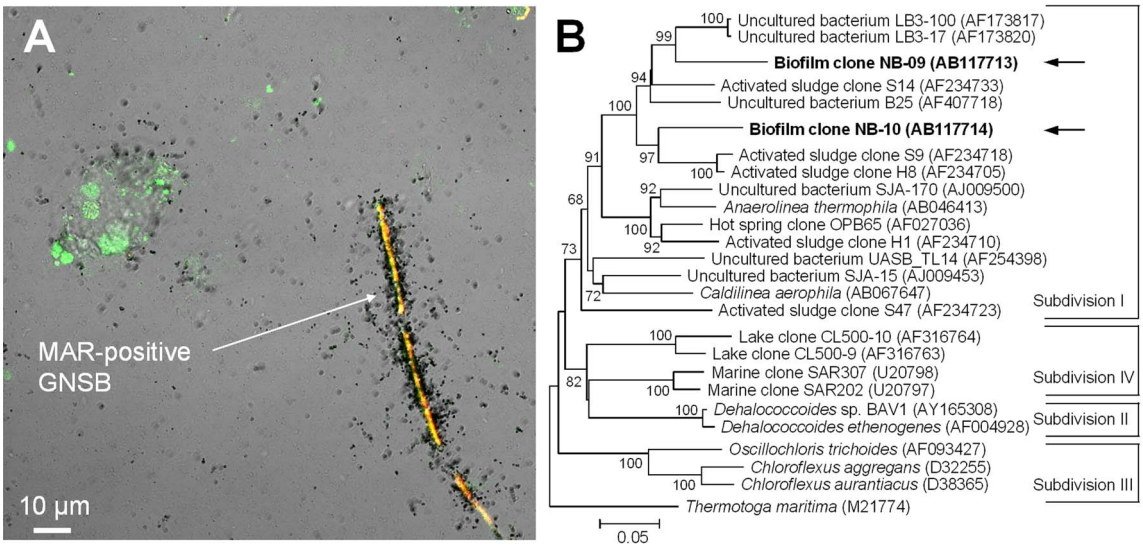

Fig. 4. Combined MAR-FISH images of homogenized autotrophic nitrifying biofilm samples. (A) Uptake of [^{14}C]*N*-acetyl-D-glucosamine by a member of the GNSB. *In situ* hybridization with FITC-labeled EUB338 probe[4] (green) and TRITC-labeled S-*-GNS-0667-a-A-18, GNSB-941, and CFX1223 probes (red) (Kindaichi *et al.*[40]). Yellow signals are yielded from binding both FITC- and TRITC-labeled probes to one cell. GNSB, Green non-sulfur bacteria. (B) Phylogenetic tree of Green non-sulfur bacteria (NGSB) showing the positions of the clones obtained from the autotrophic nitrifying biofilm as indicated by arrows. The tree was generated by using 1086-bp of the 16S rRNA genes and the neighbor-joining method. The scale bar represents 5% sequence divergence, and the values at the nodes represent bootstrap values (100 times resampling analysis). The *Thermotoga maritima* sequence served as the outgroup for rooting the tree.

from nitrifying bacteria (e.g., biomass decay and production of soluble organic matter). Such ecophysiological interaction between nitrifiers and heterotrophic bacteria in a carbon-limited autotrophic nitrifying biofilm fed with only NH_4^+ as the energy source was investigated by a full cycle of 16S rRNA approach followed by MAR-FISH[40]. Despite low abundances in the biofilm community, members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) cluster and the green non-sulfur bacteria (GNSB) accounted for 64% and 27%, respectively, of the bacteria community that consumed *N*-acetyl-D-[1-^{14}C]glucosamine (NAG), considered a major structural component of bacterial cells (Fig. 4A). Based on 16S rRNA gene analysis, the NGSB detected by FISH with specific probes were affiliated with the GNSB subdivision I (Fig. 4B). The subdivision I contains the most diverse environmental clones among the four subdivisions; those clones were derived from various oxic and anoxic environments[33]. Their *in situ* metabolic functions are presently unknown. MAR-FISH revealed that the heterotrophic bacterial community was phylogenetically and metabolically diverse, and to some extent metabolically redundant, which assures the maximum utilization of organic matter produced by nitrifiers and consequently the stability of ecosystems as a biofilm.

Sulfate-reducing bacteria community

There are a few studies relating sulfate-reducing bacteria (SRB) community structure to their sulfate-reducing activity in wastewater biofilms, in which FISH was successfully combined with microelectrode measurements[35,57,65,71]. However, the spatial resolution of microelectrode measurements is not high enough to directly link phylogenetic information of individual SRB cells obtained by FISH to information about their *in situ* physiology (substrate uptake characteristics) at the single-cell level. For this reason, MAR-FISH was applied to simultaneously determine the phylogenetic affiliation and substrate uptake patterns of SRB inhabiting a sewer biofilm with oxygen, nitrate, or sulfate as electron acceptors[35]. The MAR-FISH analysis revealed that *Desulfobulbus* hybridized with probe 660 was a dominant SRB subgroup in this sewer biofilm and approximately 9 and 27% of them could take up [^{14}C]propionate with oxygen and nitrate, respectively, as an electron acceptor. This evidence might explain the high abundance of this species in the oxic surface zone of the biofilm. Furthermore, SRB were numerically important members of H_2-utilizing and $^{14}CO_2$-fixing microbial populations in this microaerophilic sewer biofilms, accounting for roughly 42% of total H_2-utilizing bacteria hybridized with probe EUB338. These results

provide further insight into the correlation between the 16S rRNA phylogenetic diversity and the *in situ* physiological diversity of SRB populations inhabiting sewer biofilms.

Sulfur-oxidizing bacterium (*Achromatium* spp.)

Achromatium oxaliferum is a large, morphologically conspicuous sulfur-oxidizing bacterium found principally in freshwater and brackish sediments. A recent study has shown that natural communities of this uncultured bacteria from the genus *Achromatium* comprised a number of phylogenetically, morphologically, and ecologically distinct subpopulations[26]. In addition, a solely microautoradiographic study of mixed natural populations of these bacteria indicated that not all cells assimilated [^{14}C]-bicarbonate and [^{14}C]-acetate, suggesting that the *Achromatium* community exhibited physiological as well as phylogenetic diversity[27]. These experimental results indicated that MAR-FISH provides the only reliable means for identifying and differentiating the coexisting *Achromatium* species present in natural environments and investigating their carbon metabolisms[28]. The results of the MAR-FISH study revealed that *Achromatium* spp. probably exhibit a range of physiologies, i.e., facultative chemolithoautotrophy, mixotrophy, and chemoorganoheterotrophy, similar to other large sulfur-oxidizing bacteria (e.g., *Beggiatoa* spp). This evidence can explain the consistent presence of a large population of *Achromatium* spp. within the natural environmental communities.

Denitrifying bacteria

Molecular studies of the community composition of denitrifying bacteria are difficult to perform since the denitrifying phenotype cannot be inferred from the phylogeny of microorganisms. However, the combination of FISH and microautoradiography allows identification of denitrifiers *in situ* by incubating with radiolabeled substrates (e.g., acetate) under anoxic conditions in the presence and absence of nitrite and nitrate. The use of MAR-FISH in combination with the full-cycle rRNA approach revealed that novel, uncultured *Beta-proteobacteria* related to the *Azoarcus-Thauera* complex are probably abundant denitrifiers in an industrial nitrifying and denitrifying wastewater treatment plant[37]. Further study is needed to identify truly important microorganisms responsible for denitrification in various WWTPs.

Degradation of xenobiotic compounds

MAR-FISH has been used for identification of microorganisms degrading xenobiotic contaminants such as *o*-nitrophenol and salicylate, both of which are environmentally important widespread aromatic compounds[91]. MAR-FISH allowed rapid and accurate identification of microorganisms responsible for degradation of [^{14}C] *o*-nitrophenol. In future, further studies should be performed for biodegradation of other contaminants such as petroleum hydrocarbons, pesticides, and dyes.

Marine picoplnkton

The application of MAR-FISH to marine bacterioplankton has demonstrated that nearly 90% of *alpha-Proteobacteria* and members of the *Cytophaga-Flavobacterium* group, which together accounted for 50–60% of the bacterial cells present, assimilated tritiated amino acids[62]. MAR-FISH has also been used to determine the relative contribution made to the utilization of marine dissolved organic matter (DOM) by different prokaryotic groups[18]. The results showed that no phylogenetic group dominated the consumption of all DOM, suggesting that the participation of a diverse assemblage of bacteria is essential for the complete degradation of complex DOM in the ocean. Cottrell and Kirchman[18] also found that *N*-acetyl-D-glucosamine (NAG), which constitutes the largest pools of amino sugars in the ocean, was preferentially utilized by *alpha-Proteobacteria* and member of the *Cytophaga-Flavobacterium* group. Riemann and Azam[68] demonstrated that all isolates within the *Vibrionaceae* group of *delta-Proteobacteria* could also take up NAG and were all facultative anaerobes. This result suggests that a substantial fraction of bacteria in the ocean are facultative anaerobes that might be predominantly responsible for degradation of marine dissolved organic matter. The newly discovered low-temperature marine Archaea, now known to be abundant in marine environments, are also capable of assimilating dissolved amino acids at low concentrations[63].

Quantitative MAR-FISH

MAR is usually considered as qualitative or semi-quantitative, especially when [^{14}C], which is a relatively strong beta-emitter, is used[70]. However, Nielsen *et al.*[47] have recently proposed quantitative microautoradiography (QMAR) and FISH. The QMAR-FISH has a resolution of a single cell and is based on an improved fixation protocol

(preventing the leakage of tritium-labeled compounds from cells[42]) and the use of an internal standard of bacteria with known specific radioactivity). The leakage decreased the number of silver grains formed on the bacteria cell, which consequently lowered the resolution of MAR. The modified fixation protocol was the immediate immobilization on gelatin-coated cover glass after fixation in 4% PFA for 3 h and then storing at −20°C until use. With this technique, the substrate affinity (Ks) for uptake of acetate by two filamentous bacteria, the 'Candidatus *Meganema perideroedes*' and the *Thiothrix* spp., was directly determined in complex environments *in situ* without previous cultivation or enrichment[47]. This technique revealed further insight into the activity variations between different species of interest and within a single species. The filaments within both probe-defined populations had threefold variation in activity between the different filaments, demonstrating a large variation in activity level within a single population in a complex system.

CTC-FISH

Reliable methods to detect the viable fraction of microorganisms in complex environments are of great interest in microbial ecology. The available methods with the single-cell resolution are FISH, microautoradiography (MAR), and reduction of redox dye 5-cyano-2,3-tolyl-tetrazolium chloride (CTC)[69] and *p*-iodonitrotetrazolium (INT). Bacterial activity at the single cell level can be analyzed using terazorium salts (e.g., CTC and INT) as indicators of bacterial respiration. In theory, FISH reflects the recent activity state of the cell, but this depends very much on species and growth conditions. For MAR, the active bacteria can be directly detected in complex microbial communities. The main problem is, however, to find substrate(s) that can be taken up by all active bacteria. The CTC reduction method is commonly applied to determine respiratory activity and validity of bacteria in complex microbial systems[22,39]. All three of these methods were used simultaneously to investigate viability and activity on a single-cell level of *Thiothrix* filaments and single floc-forming bacteria in activated sludge[50]. Their study demonstrated that the signals from MAR and FISH analyses correlated well, whereas only 65% of the MAR- and FISH-positive *Thiothrix* cells were also CTC positive. For single floc-forming bacteria, only 41% of the MAR- and FISH-positive cells were detectable by CTC reduction. Weakly MAR-positive filamentous *Thiothrix* cells and many single cells, even being MAR- and FISH-positive, were not detected by CTC. Based on these results, it could be concluded that CTC targeted only the most active cells and underestimated the real number of active cells. This conclusion was in agreement with the previous studies suggesting that the CTC was poisonous to some of the cells[78] and that universal 16S rRNA probe counts indicated a population with at least minimal metabolic activity[39].

Other methods for determination of microbial structure and function

The 16S rRNA approach suffers from numerous biases introduced in the DNA extraction, PCR amplification, and cloning procedures. Therefore, quantitative data on the microbial community composition can only be obtained if the 16S rRNA approach is combined with quantitative *in situ* hybridization analysis. A limitation of MAR-FISH is, however, that FISH targets rRNA. The genes encoding this macromolecule are highly conserved and cannot be used to discriminate at the same phylogenetic resolution as functional genes or intergenic spacer regions. Thus, resolution of the FISH technique is somewhere near the species level at best. In addition, phylogeny based on rRNA sequence alone is rarely a reliable indicator of microbial function. In recent years, some techniques other than MAR-FISH have been developed, which allow more comprehensive simultaneous monitoring of the diversity and substrate uptake patterns of complex microbial communities. Stable isotope probing (SIP)[67] and Isotope microarray[1,64] techniques are briefly reviewed in the following section.

Stable isotope probing (SIP)[67]

DNA can be labeled with the heavy stable isotopes [^{13}C] after incubation with stable isotope-labeled substrates. Heavy DNA (^{13}C-DNA) and light DNA (^{12}C-DNA) are separated by density-gradient centrifugation. The separated ^{13}C-DNA and ^{12}C-DNA from the SIP experiment were separately subjected to full-cycle rRNA analysis, which included FISH probe design and MAR-FISH. The SIP with full-cycle rRNA analysis enabled one to design specific FISH probes for active microorganisms under conditions tested based on the ^{13}C-DNA clone library. Thus, the ^{13}C-DNA clone library represents active microbial community, whereas the ^{12}C-DNA clone library represents the overall microbial community. The potential of this SIP technique has been recently reviewed elsewhere[66,89]. This year, activated sludge was subjected to SIP after incubation with ^{13}C-methanol to label the DNA of the denitrifiers[25]. This study reports the first combined use of SIP, full-cycle rRNA anal-

ysis, which includes FISH probe design, and MAR-FISH to establish a link between the phylogeny and physiology of a methanol-utilizing denitrifying bacterial community. The dominant 16S rRNA gene phylotype in the ^{13}C-DNA clone library was closely related to those of the obligate methylotrophs *Methylobacillus* and *Methylophilus* in the order *Methylophilales* of the *Beta-proteobacteria*. There was no correlation between the denitrification rate and the relative abundances of the well-known denitrifying genera *Hypomicrobium* and *Paracoccus*. MAR-FISH provided visual evidence for the *in situ* utilization of methanol by targeted microorganisms. The application of SIP together with MAR-FISH is, therefore, a very powerful and effective approach to address the *in situ* physiology of microorganisms in complex mixed-population consortia such as activated sludge and biofilms. Although the SIP is safer than MAR-FISH, there are the following concerns; (1) a rather longer incubation time is required for enough incorporation of stable isotope into DNA or RNA, which may cause substrate cross-feeding, (2) the SIP is strongly dependent on the DNA extraction efficiency, and (3) stable isotope-labeled substrates are less available on the market than radiolabeled substrates.

Isotope microarray

A new microarray method, the isotope array approach, for identifying microorganisms that consume a specific ^{14}C-labeled substrate within complex microbial communities was recently developed[1,64]. This isotope microarray approach allows simultaneous determination of community structure and specific substrate consumption by community members via direct detection of environmentally retrieved 16S rRNA on an oligonucleotide microarray. Therefore, this approach is not affected by the multiple biases associated with PCR amplification of rRNA genes but requires efficient extraction of rRNA from samples. Compared to MAR-FISH, the isotope array allows one to apply many probes in parallel, which will be of major importance if the ecophysiology of complex microbial communities is of interest. This technique can be used as a new tool to directly link all detected microorganisms with their specific activities and functions in complex environments, if stringent hybridization conditions can be achieved for all probes. For MAR-FISH, FISH probes that will be used must be previously selected based on 16S rRNA gene analysis or by trial and error. Therefore, this method is less tedious and advantageous to study the functions of uncultured microorganisms, which are usually in low abundance in complex microbial communities. However, the incorporation rate of labeled carbon into rRNA varies significantly depending on the type of labeled substrate and on the metabolism of the organisms, which determines the sensitivity of this method. The sensitivity of this method is sufficient to detect ^{14}C incorporation into the 16S rRNA of community members that make up 5–10% of the bacterial communities.

Future Researches

The recent application of 16S rRNA approaches has revealed a remarkably vast microbial diversity including many hitherto-recognized, yet uncultured species in natural and engineered environments. This species biodiversity must be related to the functional diversity. Despite the enormous potential of MAR-FISH to simultaneously examine phylogenetic identity and the *in situ* specific activity of the microorganisms within a complex microbial community at the single-cell level without the need for cultivation or isolation, it is clear that there are inherent limitations. Therefore, a logical use of MAR-FISH in combination with those of various molecular techniques, classical microbiology, and microelectrodes will provide the clearest view of the *in situ* metabolic functions of microorganisms in complex microbial communities, which is the central to microbial ecology.

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