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Eco-Physiological Interaction between Nitrifying Bacteria and Heterotrophic Bacteria in Autotrophic Nitrifying Biofilms As Determined by MAR-FISH

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INTRODUCTION

Microbial nitrification followed by denitrification processes for nitrogen removal are becoming more important due to strict regulations on nitrogen discharge. Aerobic biofilm systems have been used for nitrogen removal because of a sufficiently long biomass retention time to achieve reliable nitrification. In such biofilm systems, the competitive interaction between heterotrophs and nitrifiers for dissolved oxygen and space in biofilms is well known. Autotrophic nitrifiers produce and release soluble microbial products (SMP) into solution from substrate metabolism and biomass decay. Therefore, coexistence of heterotrophs in high abundance with nitrifiers has been often found in autotrophic nitrifying biofilms cultured without the external organic carbon supply. Different phylogenetic groups of heterotrophs may be responsible for mineralizing different low- and high-molecular-weight organic matter produced or released by nitrifiers in autotrophic nitrifying biofilm. The diversity and distribution of major groups of heterotrophs and their relative contributions to organic carbon utilization in autotrophic nitrifying biofilms is poorly understood. The better understandings of the eco-physiological interaction between nitrifiers and heterotrophs is required to reveal factors controlling the efficiency and stability of microbial nitrification and to improve the process performance and control.

The combined microautoradiography (MAR) and fluorescence in situ hybridization (FISH) approach allows to simultaneously analyze the in situ phylogenetic identification and the specific substrate uptake patterns of various cultivable or uncultivable bacteria within complex microbial communities at single cell resolution. Little is known about how biofilm community functions as a biological unit, in other words, how and/or what pathways biofilm community maximize the utilization of metabolites of nitrifiers and prevent built-up of metabolites or waste materials of nitrifiers at significant levels.

Therefore, we applied a full cycle 16S rRNA approach, microautoradiography combined with fluorescent in situ hybridization (MAR-FISH), to determine utilization pattern of organic matter by the major phylogenetic groups of heterotrophs comprising autotrophic nitrifying biofilm community, to which no organic carbon substrate was fed. In the present study, we hypothesized that such a simple autotrophic nitrifying biofilm was metabolically structured and functionally integrated to maximize the utilization of metabolites (i.e., SMP) of nitrifiers and the stability of the biofilm communities. First, phylogenetic differentiation (identification) of coexisting heterotrophic bacteria was performed by 16S rDNA-cloning analysis, and new oligonucleotide probes for FISH were designed to determine the community structure and the spatial organization. Second, the substrate uptake patterns among seven major phylogenetic groups were determined by MAR-FISH with three radiolabeled organic substrates.

MATERIALS AND METHODS

Autotrophic nitrifying biofilms were cultured with synthetic medium in partially submerged rotating disk reactor (RDR). The nutrient medium was composed of the following (mM): NH_4Cl (3.6), $NaHCO_3$ (17.8), K_2HPO_4 (0.4), $MgSO_4 \cdot 7H_2O$ (0.41), NaCl (1.25); pH 7.8 ± 0.2. Distilled water was used to dilute the medium, which contained no detectable dissolved organic carbon. The reactor volume was 1,400 cm^3. The total biofilm area was 2,545 cm^2. The temperature was maintained at 25°C. The hydraulic retention time (HRT) of the reactor was 6 hours. Biofilm samples were obtained from the RDR after about 3-month operation.

Figure 1 shows experimental overview and MAR-FISH techniques. Four different experiments with four different types of radioactive substrates were conducted. For each experiment, 1.9 ml portions of diluted biofilm sample were transferred to 10 ml serum bottles. Each bottle was supplemented with a radioactive substrate (final activity of 10 μCi) and nonradioactive substrates (ammonium and/or bicarbonate) and sealed with a gas-tight rubber stopper. The final total volume of each sample was 2 ml. The bottles were aerobically incubated for 4 hours by shaking at 100 rpm at 25°C. The following inorganic and organic substrates, labeled with radioisotope, were used: (i) sodium [^{14}C]bicarbonate as a carbon source for nitrifiers, (ii) [1-^{14}C]acetic acid as low-molecular-weight organic substrates produced through decomposition of complex organic compounds, (iii) L-amino acid mixture, [U-^{14}C] as low-molecular-weight organic substrates produced through decomposition of proteins, and (iv) *N*-acetyl-D-[1-^{14}C]glucosamine (NAG) as a major constituent of bacterial cell wall.

The microbial community composition in the autotrophic nitrifying biofilm was quantitatively analyzed using group- and subclass-specific probes. For determination of microbial compositions, the surface fraction of the specific probe-hybridized cell area and the EUB338 probe-hybridized cells (total biomass) was determined after simultaneous in situ hybridization with various probe sets. A MAR-positive cell was defined as a cell covered with more than 5 silver grains. The numbers of MAR-positive cells and total probe-hybridized cells were enumerated by directly counting a minimum of 500 silver-grain-covered cells in randomly chosen microscopic fields.

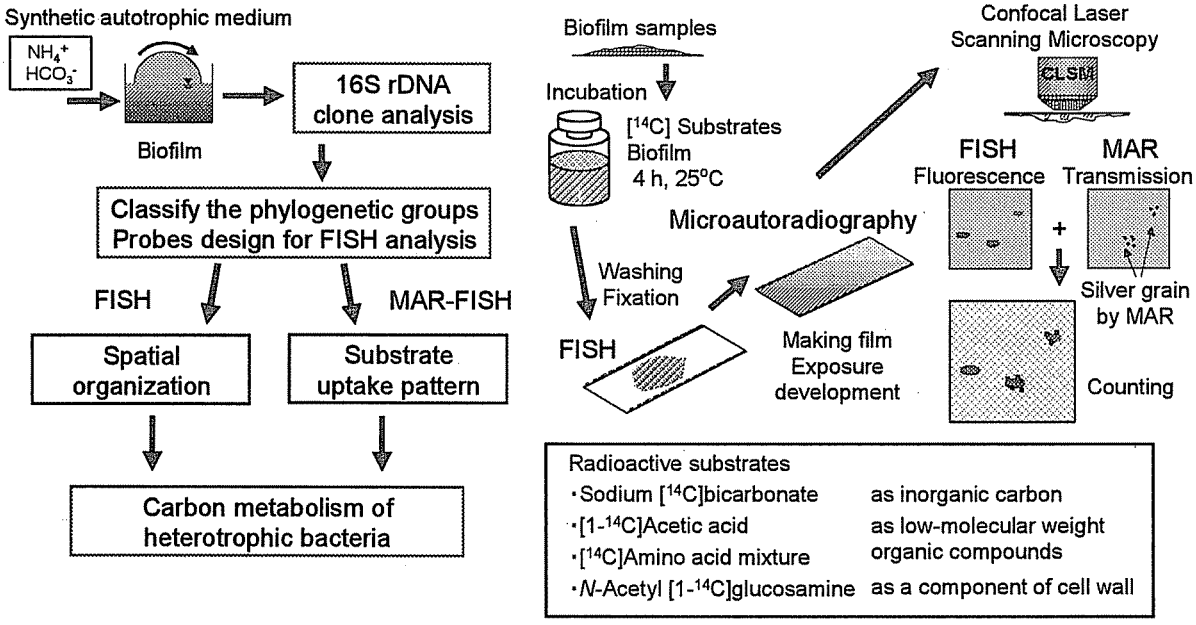

Figure 1 Experimental overview and MAR-FISH techniques.

RESULTS

Community composition

FISH analysis showed that this autotrophic nitrifying biofilm was comprised of 50% of nitrifying bacteria (ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB)) and 50% of heterotrophic bacteria, where member distribution was as follows: α-*Proteobacteria* (23%), γ-*Proteobacteria* (13%), green non-sulfur bacteria (GNSB) (9%), *Cytophaga-Flavobacterium-Bacteroides* (CFB) division (2%), and unidentified (could not be hybridized with any other probes except for EUB338) (3%). This result indicated that a pair of nitrifiers (AOB+NOB) supported a heterotrophic bacterium via production of soluble microbial products (SMP). MAR-FISH revealed that heterotrophic bacterial community was composed of phylogenetically and metabolically diverse, and to some extent metabolically redundant, which ensures the stability of ecosystems as a biofilm.

In situ spatial organization

The *in situ* spatial organization of heterotrophic bacteria and nitrifying bacteria was visualized by FISH using newly designed probes and the previously published group-specific probes. Large rod-shaped bacterial cells occurring singly were detected with ALF1b probe (specific to α-*Proteobacteria*). They were evenly distributed throughout the biofilm, and some of the cells were detected within the nitrifier cluster. Small long rod-shaped bacterial cells were found around the nitrifier clusters with the probe GAM42a (specific to γ-*Proteobacteria*). The distribution of the α- and γ-*Proteobacteria* was irrelevant to depths within the biofilm, likely owing to that the biofilm was thin to allow oxygen to fully penetrate throughout the biofilm. By using probes CF319a/b (specific to the member of CFB), thin filamentous bacteria were detected around the nitrifier's clusters mainly in the surface of the biofilm. The filamentous bacteria (ca. 0.5-2.0 μm thick) within the division of green non-sulfur bacteria (GNSB), which mainly occurred around nitrifier's clusters, was detected with probes S-*-Unbac-0667-a-A-18, GNSB-941, and CFX1223 (specific to GNSB).

Substrate uptake patterns of different phylogenetic groups

To evaluate substrate uptake patterns of various phylogenetic groups of heterotrophic bacteria, MAR-FISH was performed with seven group-specific probes (i.e., AOB, NOB, α-*Proteobacteria*, γ-*Proteobacteria*, GNSB, CFB, and Unidentified). Uptake patterns of organic compounds greatly differed among the phylogenetic groups (Table 1). The nitrifying bacteria (AOB+NOB) took up only [^{14}C]bicarbonate, underlining the autotrophic growth. No uptake of [^{14}C]bicarbonate by any other groups of heterotrophic bacteria was observed for 4-h incubation. Members of the α- and γ-*Proteobacteria* dominated the utilization of [^{14}C]acetic acid and [^{14}C]amino acids in this biofilm. Despite of the low abundance (ca. 2%) in the biofilm, members of the CFB cluster accounted for the largest fraction (ca. 64%) of bacteria consuming *N*-acetyl-D-[1-^{14}C]glucosamine (NAG). Members of the GNSB utilized only [^{14}C]amino acids. These results indicated that members of the α- and γ-*Proteobacteria* might be specialized to utilize low-molecular-weight organic matter like [^{14}C]acetic acid and [^{14}C]amino acids in this biofilm. The unidentified group took up all organic substrates used in this study. Although its abundance in the biofilm was relatively low (ca. 3%), the "unidentified" group could be eco-physiologically an important bacterial group for carbon cycle in this biofilm. However, detailed phylogenetic identification of this "unidentified" group was not clear in this study.

Table 1 Summary of uptake pattern of radioactive substrates by phylogenetic groups

	Bicarbonate	Acetic acid	Amino acids	N-acetyl-glucosamine
Ammonia-oxidizing bacteria	+	-	-	-
Nitrite-oxidizing bacteria	+	-	-	-
α-Proteobacteria	-	+	+	-
γ-Proteobacteria	-	+	+	-
Green non-sulfur bacteria	-	-	+	-
Cytophaga-Flavobacterium-Bacteroieds	-	-	+	+
Unidentified	-	+	+	+

Uptake of organic matter derived from nitrifiers

Furthermore, we investigated the fate of the radioisotopically labeled atom of [^{14}C]bicarbonate by MAR-FISH. [^{14}C]bicarbonate was first incorporated in nitrifying bacterial cells or in unknown components of SMP, which were subsequently released and incorporated by (or transferred to) mainly filamentous heterotrophic bacteria classified as "unidentified" group. The nitrifiers (AOB+NOB) accounted for more than 60% of total bacteria and constituted all MAR-positive bacteria after 1 day of incubation. In contrast, MAR-positive filamentous bacteria (i.e., classified as "unidentified" group) were first detected after 5 days of incubation. The population of MAR-positive filamentous bacteria increased with time, while the populations of total bacteria decreased by approximately 50% after 25 days of incubation due to cell decay. This result clearly demonstrated that [^{14}C]bicarbonate originally incorporated in nitrifying bacterial cells were subsequently utilized by mainly filamentous heterotrophic bacteria in the "unidentified" group. This evidenced that an efficient food web (carbon flow) existed in the autotrophic nitrifying biofilm community to assure the maximum utilization of metabolites of nitrifiers and to prevent built-up of metabolites or waste materials of nitrifiers at significant levels.

CONCLUSION

Heterotrophic bacterial community was composed of phylogenetically and metabolically diverse, and to some extent metabolically redundant. This diversity and redundancy ensures the stability of ecosystems and effective carbon metabolism as a biofilm (Figure 2).

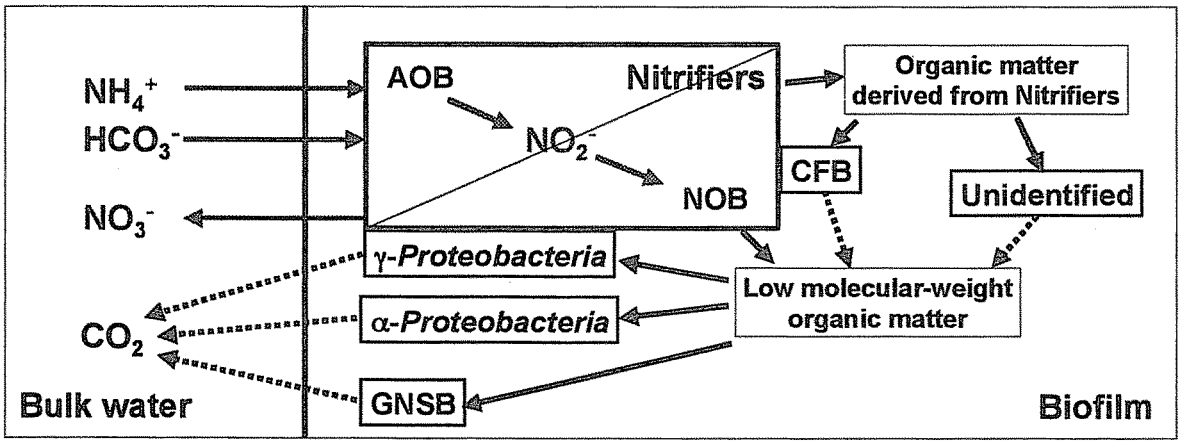

Figure 2 Carbon flow in an autotrophic nitrifying biofilm.