Ecophysiological Role and Function of Uncultured *Chloroflexi* in an Anammox Reactor

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Abstract
The coexistence of uncultured heterotrophic bacteria belonging to the phylum *Chloroflexi* has often been observed in anaerobic ammonium oxidation (anammox) reactors fed with synthetic nutrient medium without organic carbon compounds. To determine if coexisting *Chloroflexi* in anammox reactors scavenge organic matter derived from anammox bacterial cells, the present study was conducted to investigate the substrate uptake pattern of the uncultured *Chloroflexi* present in an anammox reactor and to clarify if they take up microbial products derived from anammox bacterial cells. To accomplish this, combined microautoradiography and fluorescence *in situ* hybridization (MAR-FISH) was conducted. Phylogenetic analysis revealed that 36% of the clones analyzed in this study were affiliated with *Chloroflexi*. The sequence similarities to *Anaerolinea thermophila* and *Caldilinea aerophila* within the phylum *Chloroflexi* were only 81.0-88.7% and 80.3-83.8%, respectively. The uncultured *Chloroflexi* were found to incorporate sucrose, glucose, and N-acetyl-glucosamine. The ^{14}C-tracing experiment revealed that the uncultured *Chloroflexi* were clearly MAR-positive, indicating the utilization of decaying anammox bacterial cell materials. Taken together, these results indicate that coexisting uncultured *Chloroflexi* in anammox reactors scavenge organic compounds derived from anammox bacterial cells.

Keywords
Anammox; *Chloroflexi*; MAR-FISH; phylogenetic analysis; substrate uptake pattern

INTRODUCTION

The anaerobic ammonium oxidation (anammox) process, in which ammonium and nitrite are directly oxidized to nitrogen gas under anoxic conditions, is a cost-effective and low energy-consuming alternative to conventional nitrogen removal processes (van Dongen et al., 2001). Culture-independent molecular methods have revealed the coexistence of uncultured heterotrophic bacteria with anammox bacteria belonging to the phyla *Chloroflexi*, *Chlorobi*, *Proteobacteria*, *Bacteroidetes*, and/or Candidate divisions, even though the anammox reactors were fed with synthetic wastewater containing NH_4^+ as the sole electron donor and no organic carbon compounds (Strous et al., 2006; Li et al., 2009; Cho et al., 2010; Kindaichi et al., 2011a).

Among these bacteria, members of *Chloroflexi*, especially in 'subphylum I', have often been detected from various lab-scale anammox reactors (Strous et al., 2006; Li et al., 2009; Cho et al., 2010). Despite the *Chloroflexi* phylum comprising genetically diverse members and the ecophysiological significance of *Chloroflexi* in wastewater treatment systems including anammox reactors, the basic ecophysiology of this group is still largely unknown because only a few pure cultures of organisms belonging to 'subphylum I' have been investigated to date (Yamada and Sekiguchi, 2009). Accordingly, a better understanding of reactor performance, including the microbial community structures and ecophysiology in biofilms/granules, will lead to optimization

and efficient design of the anammox process.

In previous studies, combined microautoradiography and fluorescence *in situ* hybridization (MAR-FISH) revealed that uncultured *Chloroflexi* might play an important role in degradation of the microbial products in autotrophic nitrifying biofilms and membrane bio-reactors (Kindaichi et al., 2004; Okabe et al., 2005; Miura et al., 2007; Okabe et al., 2010). It has been suggested that coexisting *Chloroflexi* in anammox reactors also scavenge organic matter derived from anammox bacterial cells. However, these MAR-FISH experiments were performed under oxic condition. In addition, the ecophysiology of *Chloroflexi* previously reported may not necessarily reflect the ecophysiology of *Chloroflexi* in the anammox reactors due to the anammox condition (i.e., anoxic and high nitrite concentration conditions). Therefore, the present study was conducted to determine the substrate uptake pattern of uncultured *Chloroflexi* present in the anammox reactor and to clarify whether they also take up the microbial products derived from anammox bacterial cells based on the MAR-FISH approach. To achieve these goals, the anammox reactor was operated with synthetic wastewater and maintained for more than 2 years to construct a stable microbial community structure. Phylogenetic analysis of the uncultured *Chloroflexi* based on 16S rRNA gene sequencing was also conducted.

MATERIALS AND METHODS

Reactor

A glass column reactor (diameter, 4.5 cm; length, 13 cm; volume, 206 cm^3) with a nonwoven fabric sheet as the biofilm carrier material was used (Tsushima et al., 2007; Kindaichi et al., 2011b). The anammox biomass was obtained from a fixed-bed biofilm column reactor that was developed previously (Tsushima et al., 2007). A synthetic nutrient medium containing only NH_4^+ as an energy source was used (van de Graaf et al., 1996; Kindaichi et al., 2007). The temperature was maintained at 37°C. The initial hydraulic retention time (HRT) of the reactor was 2.0 h.

Analytical methods

The concentrations of NH_4^+, NO_2^-, and NO_3^- were determined using an ion chromatograph (HPLC 10Avp; Shimadzu Co., Kyoto, Japan) equipped with a Shin-pack IC-C1 column and a Shin-pack IC-A3 column after filtration using a 0.2-µm-membrane filter (Hatamoto et al., 2010). The total nitrogen loading and removal rates were calculated based on the concentrations of NH_4^+, NO_2^-, and NO_3^-, and the HRT.

Phylogenetic analysis

Total DNA was extracted from the anammox biofilm in the column reactor after 740 days of operation using a Fast DNA spin kit (MP Biomedicals, Irvine, CA). The 16S rRNA gene fragments were amplified using a ONE Shot LA PCR MIX kit (TaKaRa Bio Inc., Ohtsu, Japan) and a bacteria-specific primer set (11f (Kane et al., 1993) and 1390r (Zheng et al., 1996)). The PCR reaction was conducted in a mixture with a total volume of 50 µl using 1 µg of DNA as the template. The conditions used for the PCR were as follows: 5 min of initial denaturation at 95°C and 25 cycles of 30 s at 95°C, 30 s at 50°C, and 1 min at 72°C. Final extension was conducted for 5 min at 72°C. The PCR products were electrophoresed on a 1% (wt/vol) agarose gel.

The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), after which they were cloned using a TOPO XL PCR Cloning Kit (Invitrogen, Carlsbad, CA). Cloned 16S rRNA genes were randomly selected from the clone library and sequenced. DNA sequencing was performed by Dragon Genomics Center (TaKaRa Bio Inc., Yokkaichi, Japan). 16S rRNA gene sequences were classified using the FastGroupII (Yu et al., 2006) program, and

sequences showing ≥97% homology were grouped into operational taxonomic units (OTUs). The sequences related to the phylum *Chloroflexi* were imported and aligned using Integrated Aligners in the ARB software package (Ludwig et al., 2004). Phylogenetic trees were constructed using the neighbor-joining method implemented in the ARB program. Bootstrap resampling analysis for 1,000 replicates was conducted to estimate the confidence of tree topologies. The 16S rRNA gene sequence data obtained in this study were deposited in the GenBank/EMBL/DDBJ databases under accession numbers AB638620 to AB638626.

MAR-FISH

MAR-FISH was conducted using the procedure described by Kindaichi et al. (2004) and Okabe et al. (2005), with slight modification. Briefly, MAR-FISH in this study consisted of the following two experiments: a substrate uptake pattern experiment and a ^{14}C-tracing experiment. The anammox biofilm samples were taken after 775 days of operation. In the substrate uptake pattern experiment, 5 ml biofilm samples (5 mg of dry weight) containing synthetic nutrient medium with NH_4^+ (100 mg-N/l) and NO_2^- (90 mg-N/l) were incubated in 10-ml serum bottles with 10 μCi of either [^{14}C(U)]protein hydrolysate, [^{14}C(U)]sucrose, D-[^{14}C(U)]glucose, or N-acetyl-D-[^{14}C]glucosamine for 24 h at 37°C under anoxic conditions. In the ^{14}C-tracing experiment, the incubation consisted of the following two stages: a ^{14}C labeling stage and a ^{14}C-tracing stage. In the ^{14}C labeling stage, 5 ml biofilm samples (15 mg of dry weight) containing the synthetic nutrient medium with NH_4^+ (200 mg-N/l) and NO_2^- (190 mg-N/l) were incubated in 10-ml serum bottles with 100 μCi of sodium [^{14}C]bicarbonate for 24 h at 37°C. In the ^{14}C-tracing stage, the ^{14}C-labeled anammox biofilm (7.5 mg of dry weight) obtained from the ^{14}C labeling stage and fresh anammox biofilm (i.e. non-labeled biomass, 15 mg of dry weight) containing the synthetic nutrient medium with NH_4^+ (200 mg-N/l) and NO_2^- (190 mg-N/l) were incubated in 10-ml serum bottles for 72 h at 37°C. Following incubation, all samples were fixed by the addition of paraformaldehyde to a final concentration of 4%, after which they were washed in phosphate buffered saline and spotted onto cover glasses as described previously (Okabe et al., 2005). The uptake of labeled organic compounds into the cells was confirmed by liquid scintillation counting (LSC-5100, Aloka Co., Ltd., Tokyo, Japan).

In situ hybridization was conducted according to the procedure described by Okabe et al. (1999). The 16S rRNA-targeted oligonucleotide probes used in this study were EUB338 (Amann et al., 1990), EUB338-II (Daims et al., 1999) and EUB338-III (Daims et al., 1999), which target most bacteria; Amx820 (Schmid et al., 2001), which targets anammox bacteria; and GNSB-941 (Gich et al., 2001), which is specific for the phylum *Chloroflexi*. To detect all bacteria, the probes (EUB338mix) were used in an equimolar mixture together with probes EUB338, EUB338II, and EUB338III. The probes were labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine 5-isothiocyanate (TRITC) at the 5' end. The cover glasses were carefully dipped in pre-warmed (43°C) LM-1 emulsion (GE Healthcare UK, Ltd., Little Chalfont, UK) and exposed to 4°C for 2 days, after which the samples were developed (3 min) in Kodak L-19 developer using the method described by Lee et al. (1999). A LSM5 PASCAL confocal laser scanning microscope (CLSM; Carl Zeiss, Oberkochen, Germany) equipped with an Ar ion laser (488 nm) and a HeNe laser (543 nm) was used to observe the samples. MAR-positive and MAR-negative cells were assessed by comparing silver grains on the top of cells with the background level.

RESULTS AND DISCUSSION

Reactor performance

Figure 1 shows the nitrogen loading and removal rates of the column reactor used in this study. After inoculation, the simultaneous oxidation of NH_4^+ and NO_2^- was observed within 20 days of

operation. During the first 100 days of operation, the nitrogen loading rate was gradually increased by increasing the NH_4^+ and NO_2^- concentrations and/or reducing the HRT to 1.0 h. After 150 days of reactor operation, the nitrogen loading rate reached 10 g-N l^{-1} day^{-1}, which was then maintained for 28 months. The average nitrogen removal rate and nitrogen removal efficiency were 7.5 g-N l^{-1} day^{-1} and 71%, respectively, after 150 days of operation. The average nitrogen stoichiometric ratio after stable operation (after 150 days) was 1:1.19:0.19 for conversion of NH_4^+ and NO_2^- to the production of NO_3^-. This stoichiometric ratio was a little different from the previously reported ratio of 1:1.32:0.26 (Strous et al. 1998). These results clearly indicated that the reactor performance was stable over 2 years.

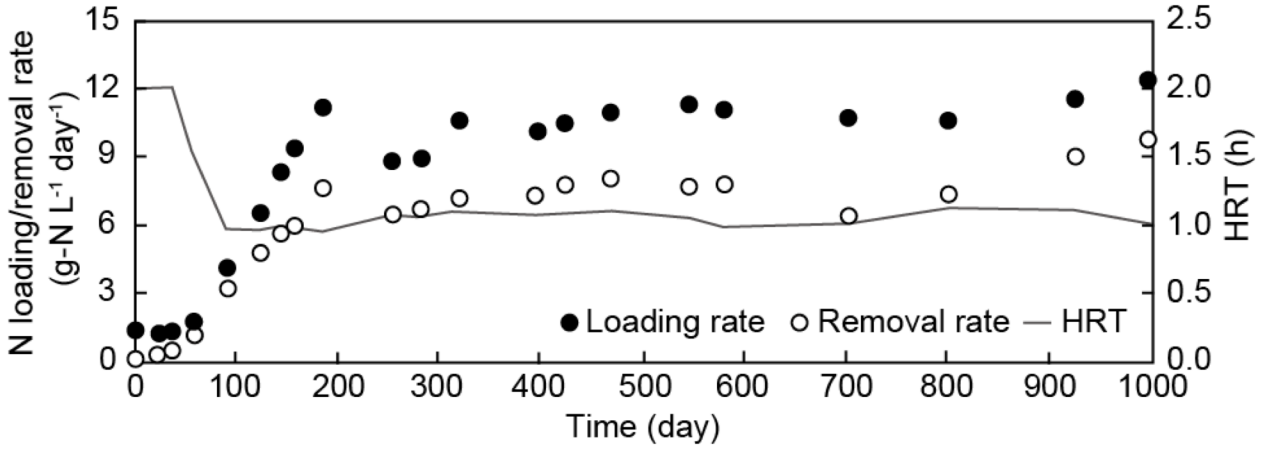

Figure 1 Nitrogen loading and removal rates of the column reactor.

Phylogenetic analysis

Ninety-one clones were obtained from the clone library and then grouped into OTUs based on 97% or higher sequence similarity. The phylum-level distribution of the clone was as follows: *Chloroflexi*, 33 of 91 clones; *Betaproteobacteria*, 26 of 91 clones; *Chlorobi*, 20 of 91 clones; and others, 12 of 91 clones. Therefore, the clones affiliated with the *Chloroflexi* accounted for 36% of the clones analyzed. Figure 2 shows the phylogenetic tree of the 33 clones (7 OTUs) obtained from the reactor within the phylum *Chloroflexi*. All OTUs belonged to either *Anaerolineae* or *Caldilineae* within 'subphylum I' (Fig. 2). The level of sequence similarity to the isolated bacteria *Anaerolinea thermophila* and *Caldilinea aerophila* was only 81.0-88.7% and 80.3-83.8%, respectively. These results indicate that the clones detected in this biofilm were most likely different species from the isolated bacteria at the genus level; thus, the physiological characteristics of these uncultured *Chloroflexi* are not clear. The most frequently detected clone, HUY-B09, was closely related to that of an uncultured *Anaerolineae* bacterium, clone UAR5, with 99% similarity. This uncultured bacterium clone was also obtained in a lab-scale up-flow anammox reactor that was fed with a synthetic nutrient medium without any organic carbon sources (Cho et al., 2010). However, probe GNSB-941 had two mismatches when compared with clones HUY-B04 and -H05; therefore, we conducted the following MAR-FISH experiments targeting the other five OTUs.

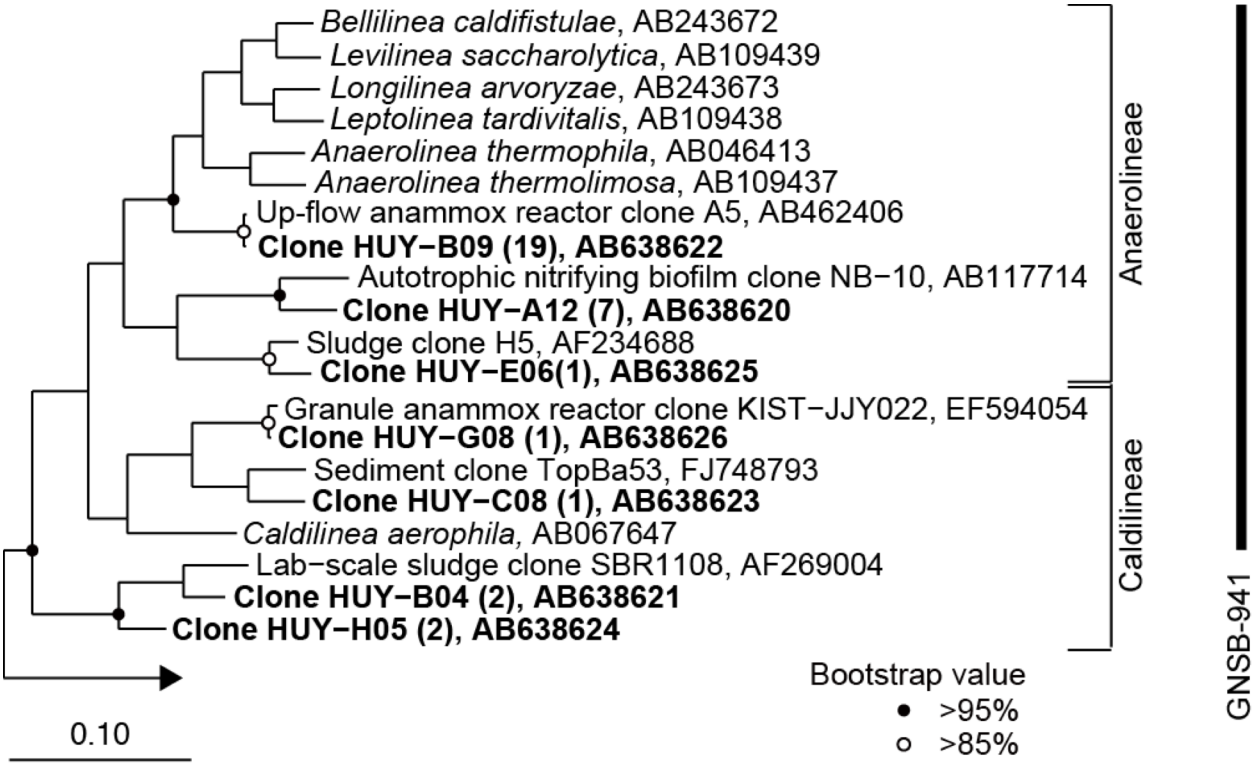

Figure 2 Phylogenetic tree of clones obtained from the anammox reactor and related bacteria within the *Chloroflexi*. The GenBank/EMBL/DDBJ accession numbers are also indicated. The numbers in parentheses indicate the frequencies of the appearance of identical clones analyzed. The scale bar represents the number of nucleotide changes per sequence position. The symbols at each branch point show the bootstrap values obtained from 1,000 resamplings. The *Chloroflexus aurantiacus* (AJ308501) sequence served as an outgroup to rooting the tree. The black line indicates the specificity of the phylum-level probe, GNSB-941, with no mismatches.

Substrate uptake pattern of *Chloroflexi*

The uptake of labeled organic compounds after 24 h of incubation is shown in Table 1. No MAR-positive cells were observed when the samples were pasteurized as a control. It should be noted that no anammox bacteria that took up organic carbon were observed in this experiment. However, in this substrate uptake pattern experiment, uncultured *Chloroflexi* were able to consume sucrose, glucose, and N-acetyl-glucosamine under anoxic conditions. The differences in the uptake of substrates may be attributed to the different microbial population sizes and the substrate uptake rate of each species. However, it is difficult to determine which species actually utilized each substrate. The uncultured *Chloroflexi* have the ability to utilize N-acetyl-glucosamine under anoxic conditions (NO_2^- is used as the electron acceptor), although uptake of N-acetyl-glucosamine by filamentous *Chloroflexi* has been observed under aerobic conditions in a biofilm (Kindaichi et al., 2004), in activated sludge (Kragelund et al., 2007) and under anoxic conditions (NO_3^- as the electron

Table 1. Substrate uptake pattern of *Chloroflexi* determined by MAR-FISH

Substrate	MAR-positive (%)*
^{14}C-protein hydrolysate	0
^{14}C-sucrose	25
^{14}C-glucose	70
^{14}C-N-acetyl-glucosamine	65

* A minimum of 20 filamentous bacteria was investigated visually to estimate the percentage of GNSB-941-probe defined filamentous bacteria that were clearly MAR-positive.

acceptor) in a membrane bioreactor (Miura et al., 2007). Since this monosaccharide is a component of the cell wall of most bacteria and continuously released when cell decay of anammox bacteria occurs in the biofilm, it is possible that the uncultured *Chloroflexi* consume organic compounds derived from anammox bacteria under anoxic conditions.

Fate of ^{14}C incorporated into anammox bacteria

To label only anammox cells in the biofilm, homogenized biofilm samples were incubated in synthetic nutrient medium (without any organic carbon source) with [^{14}C]bicarbonate. In preliminary experiments, we confirmed that the combination of 24 h of incubation and a 2-day exposure time were the optimum conditions for detection of a sufficient amount of labeled anammox bacterial cells without substrate cross-feeding from anammox bacteria to the uncultured *Chloroflexi*. In the ^{14}C labeling stage, MAR-FISH revealed that almost all anammox bacterial cells were strongly MAR-positive, but no-MAR-positive *Chloroflexi* were observed (Fig. 3A-3C). Liquid scintillation counting reveled that anammox bacteria incorporated approximately 16% of the [^{14}C]bicarbonate added during 24 h of incubation when compared with a pasteurized control (0.15% uptake). In the ^{14}C-tracing stage, uncultured *Chloroflexi* that could incorporate ^{14}C-labeled microbial products derived from anammox bacterial cells were directly visualized by MAR-FISH (Fig. 3D-3F). The uncultured *Chloroflexi* were clearly MAR-positive after 72 h of incubation with

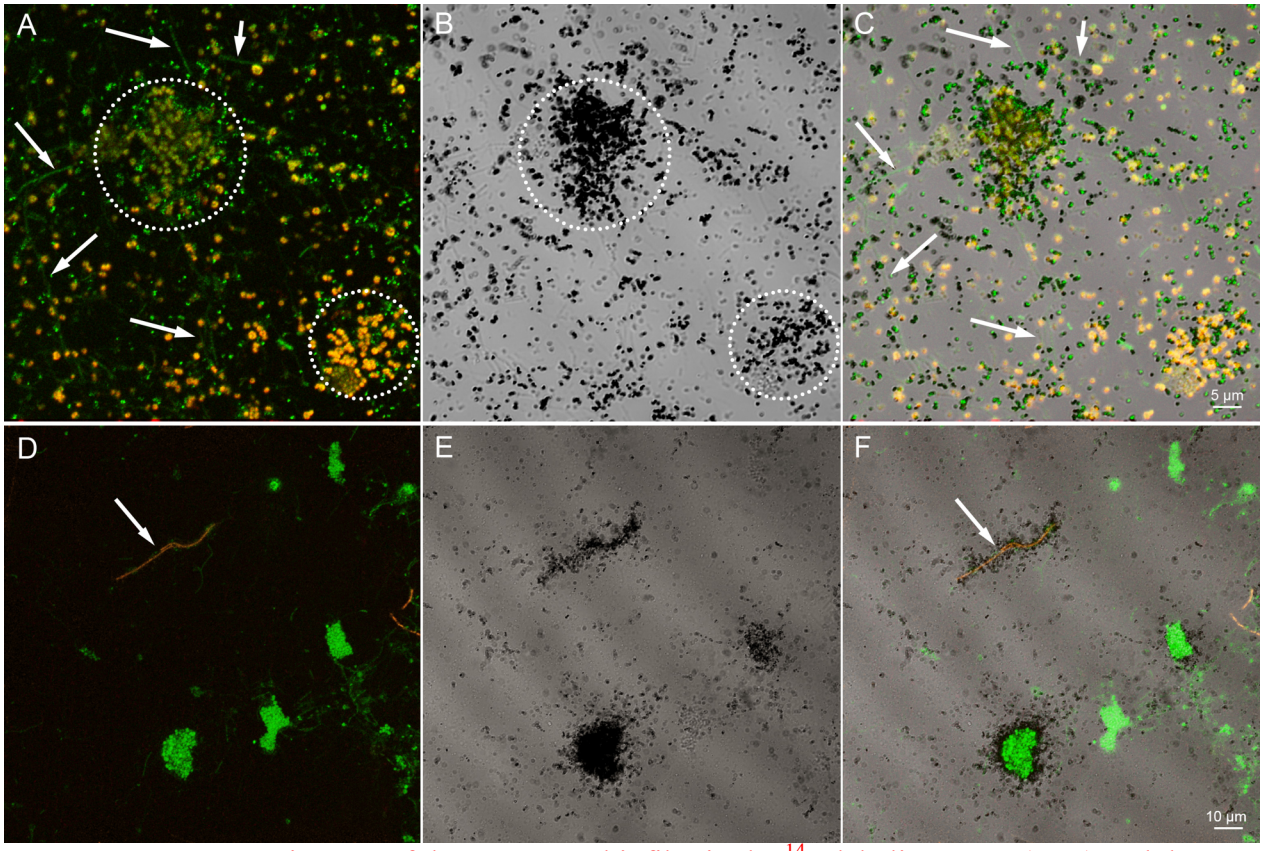

Figure 3 MAR-FISH images of the anammox biofilm in the ^{14}C labeling stage (A-C) and the ^{14}C-tracing stage (D-F) after 2 days of exposure. *In situ* hybridization was conducted using FITC-labeled EUB338mix (green) and TRITC-labeled Amx820 probes (red) (A and C), and FITC-labeled EUB338mix (green) and TRITC-labeled GNSB-941 probes (red) (D and F). Panel B and E are the bright-field MAR image, corresponding to the FISH image. Panel C and F are the overlay image. Panel A-C shows that anammox cells were labeled (dotted circle in A and B) but filamentous *Chloroflexi* were not labeled (arrows in A and C). Panel D-F shows that filamentous *Chloroflexi* took up organic compounds derived from anammox bacterial cells (arrows in D and F).

^{14}C-labeled anammox cells, indicating the preferential utilization of the decaying anammox bacteria cell materials (Fig. 3D-3F). MAR-FISH also revealed that, with the exception of *Chloroflexi*, no MAR-positive filamentous bacteria were observed (data not shown). Taken together, these results indicate that coexisting uncultured *Chloroflexi* in anammox reactors scavenge organic compounds derived from anammox bacterial cells. These results are comparable with the previous study that coexisting *Chloroflexi* in autotrophic nitrifying biofilms scavenged organic compounds derived from nitrifying bacteria. However, it was difficult to quantify the MAR-positive *Chloroflexi* owing to their low levels (<10% of total bacteria) in the biofilm. Accordingly, more studies are needed to elucidate the ecophysiological role and function of uncultured *Chloroflexi* in the biofilm using species-specific probes and whether the other coexisting bacteria in this biofilm could consume organic compounds derived from anammox bacterial cells.

CONCLUSIONS

The uncultured *Chloroflexi* coexisted in an anammox reactor fed with synthetic nutrient medium without organic carbon compounds over 2 years. In a substrate uptake pattern experiment, the uncultured *Chloroflexi* were found to be able to consume sucrose, glucose, and N-acetyl-glucosamine under anoxic conditions. MAR-FISH using ^{14}C-labeled anammox cells revealed that the coexisting *Chloroflexi* degraded and utilized cellular compounds derived from the dead biomass and metabolites of anammox bacteria, indicating that the coexisting *Chloroflexi* are specialists for degrading structural cell components in the anammox reactor and that they might prevent the accumulation of organic waste products in the biofilm.

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