

# Ecophysiological Interaction between Nitrifying Bacteria and Heterotrophic Bacteria in Autotrophic Nitrifying Biofilms as Determined by Microautoradiography-Fluorescence In Situ Hybridization

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**Ecophysiological interactions between the community members (i.e., nitrifiers and heterotrophic bacteria) in a carbon-limited autotrophic nitrifying biofilm fed only  $\text{NH}_4^+$  as an energy source were investigated by using a full-cycle 16S rRNA approach followed by microautoradiography (MAR)-fluorescence in situ hybridization (FISH). Phylogenetic differentiation (identification) of heterotrophic bacteria was performed by 16S rRNA gene sequence analysis, and FISH probes were designed to determine the community structure and the spatial organization (i.e., niche differentiation) in the biofilm. FISH analysis showed that this autotrophic nitrifying biofilm was composed of 50% nitrifying bacteria (ammonia-oxidizing bacteria [AOB] and nitrite-oxidizing bacteria [NOB]) and 50% heterotrophic bacteria, and the distribution was as follows: members of the alpha subclass of the class *Proteobacteria* ( $\alpha$ -*Proteobacteria*), 23%;  $\gamma$ -*Proteobacteria*, 13%; green nonsulfur bacteria (GNSB), 9%; *Cytophaga-Flavobacterium-Bacteroides* (CFB) division, 2%; and unidentified (organisms that could not be hybridized with any probe except EUB338), 3%. These results indicated that a pair of nitrifiers (AOB and NOB) supported a heterotrophic bacterium via production of soluble microbial products (SMP). MAR-FISH revealed that the heterotrophic bacterial community was composed of bacteria that were phylogenetically and metabolically diverse and to some extent metabolically redundant, which ensured the stability of the ecosystem as a biofilm.  $\alpha$ - and  $\gamma$ -*Proteobacteria* dominated the utilization of [ $^{14}\text{C}$ ]acetic acid and [ $^{14}\text{C}$ ]amino acids in this biofilm. Despite their low abundance (ca. 2%) in the biofilm community, members of the CFB cluster accounted for the largest fraction (ca. 64%) of the bacterial community consuming *N*-acetyl-D-[ $^{14}\text{C}$ ]glucosamine (NAG). The GNSB accounted for 9% of the [ $^{14}\text{C}$ ]amino acid-consuming bacteria and 27% of the [ $^{14}\text{C}$ ]NAG-consuming bacteria but did not utilize [ $^{14}\text{C}$ ]acetic acid. Bacteria classified in the unidentified group accounted for 6% of the total heterotrophic bacteria and could utilize all organic substrates, including NAG. This showed that there was an efficient food web (carbon metabolism) in the autotrophic nitrifying biofilm community, which ensured maximum utilization of SMP produced by nitrifiers and prevented buildup of metabolites or waste materials of nitrifiers to significant levels.**

Microbial nitrification followed by denitrification for nitrogen removal is becoming more important due to strict nitrogen discharge regulations. Aerobic biofilm systems have been used for nitrogen removal because of a biomass retention time sufficiently long to achieve reliable nitrification. In such biofilm systems, the competitive interaction between heterotrophs and nitrifiers for dissolved oxygen and space is well known (31, 32, 33). In the presence of organic carbon, nitrifiers are usually outcompeted by heterotrophs due to the low growth rate and low growth yield of the former organisms. Autotrophic nitrifiers reduce inorganic carbon to form organic carbon in cell mass, produce and release soluble microbial products (SMP) into solution from substrate metabolism (usually with biomass growth), and decay biomass (41). Therefore, they also interact through the exchange of organic matter. Coexistence of a high level of heterotrophs with nitrifiers has been found often in

autotrophic nitrifying biofilms cultured without an external organic carbon supply (34, 35).

Differences in usage of various components of organic matter (i.e., SMP) may be correlated with the diversity and distribution of heterotrophs in the biofilm. Different phylogenetic groups of heterotrophs may be responsible for mineralizing different low- and high-molecular-weight organic compounds produced or released by nitrifiers in an autotrophic nitrifying biofilm. The diversity and distribution of major groups of heterotrophs and their relative contributions to organic carbon utilization in autotrophic nitrifying biofilms are, however, poorly understood. A better understanding of the ecophysiological interaction between nitrifiers and heterotrophs is required to reveal factors that control the efficiency and stability of microbial nitrification and to improve the performance of the process.

The combined microautoradiography (MAR)-fluorescence in situ hybridization (FISH) approach developed by Lee et al. (21) and Ouverney and Fuhrman (37) allows workers to simultaneously analyze in situ the phylogenetic identities and the specific substrate uptake patterns of various cultivable or uncultivable bacteria in complex microbial communities at the

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TABLE 1. Incubation conditions

Expt <sup>a</sup>	Nonradioactive substrate(s)		Radioactive substrates		Incubation time (h)
	Ammonium concn (mM)	Bicarbonate concn (mM)	Compound	Concn ( $\mu$ M)	
1	1	— <sup>b</sup>	Sodium [ <sup>14</sup> C]bicarbonate	86	4
2	1	0.5	[1- <sup>14</sup> C]acetic acid	82	4
3	1	0.5	L-Amino acid mixture <sup>c</sup>	100	4
4	1	0.5	[ <sup>14</sup> C]NAG	88	4

<sup>a</sup> Nonradioactive acetic acid, NAG, and amino acids were not added in all experiments.

<sup>b</sup> —, Nonradioactive bicarbonate was not added.

<sup>c</sup> The L-amino acid mixture was uniformly labeled with <sup>14</sup>C.

single-cell level of resolution (12). This approach has been used to analyze specific microbial activities and functions in various microbial communities, such as activated sludge (10, 28, 29, 30), marine samples (9, 37, 38, 40), freshwater sediments (13, 14), and sewer biofilms (16). Little is known about how a biofilm community functions as a biological unit (in other words, what pathways a biofilm community uses to maximize utilization of the metabolites of nitrifiers and how it uses them to prevent buildup of waste materials of nitrifiers to significant levels).

Therefore, we used a full-cycle 16S rRNA approach and MAR-FISH to determine the patterns of utilization of organic matter by the major phylogenetic groups of heterotrophs comprising an autotrophic nitrifying biofilm community to which no organic carbon substrate was added. 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 community. First, phylogenetic differentiation (identification) of coexisting heterotrophic bacteria was performed by 16S rRNA gene sequence analysis, and new oligonucleotide probes for FISH were designed to determine the community structure and the spatial organization. Second, the substrate uptake patterns for seven major phylogenetic groups were determined by MAR-FISH with three radiolabeled organic substrates. We used *N*-acetyl-D-glucosamine (NAG) as a major structural component of bacterial cells and amino acids and acetate as representative organic carbon constituents of SMP for the MAR-FISH analysis.

#### MATERIALS AND METHODS

**Biofilm samples.** Autotrophic nitrifying biofilms were cultured with synthetic medium in a partially submerged rotating disk reactor consisting of five polymethylmethacrylate disks. The autotrophic nitrifying biofilms were first cultured with primary settling tank effluent from the Soseigawa municipal wastewater treatment plant (Sapporo, Japan) for 2 to 3 days and then were cultured with synthetic nutrient medium that contained no organic matter. The nutrient medium was composed of  $\text{NH}_4\text{Cl}$  (3.6 mM),  $\text{NaHCO}_3$  (17.8 mM),  $\text{K}_2\text{HPO}_4$  (0.4 mM),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.41 mM), and  $\text{NaCl}$  (1.25 mM), and the pH was  $7.8 \pm 0.2$ . Distilled water was used to dilute the medium, which contained no detectable dissolved organic carbon. The reactor volume was 1,400  $\text{cm}^3$ . The total biofilm area was 2,545  $\text{cm}^2$ . The temperature was maintained at 25°C. The disk rotational speed of the 18-cm-diameter disk was fixed at 14 rpm. The hydraulic retention time of the reactor was 6 h. Biofilm samples were obtained from the rotating disk reactor after more than 3 months of operation.

**DNA extraction and PCR amplification.** DNA was extracted from a biofilm sample (approximately 0.2 ml) with a Fast DNA spin kit (Bio 101, Qbiogene Inc., Carlsbad, Calif.), as described in the manufacturer's instructions. 16S rRNA gene fragments from isolated total DNA of the biofilm sample were amplified with *Taq* DNA polymerase (TaKaRa Bio Inc., Ohtsu, Japan) by using bacterial primer

sets 11f (20) and 1492r (46). The conditions used for the PCR were as follows: 5 min of initial denaturation at 94°C and 20 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C. Final extension was carried out for 4 min at 72°C. The PCR products were electrophoresed on a 1% (wt/vol) agarose gel.

**Cloning and sequencing of the 16S rRNA gene and phylogenetic analysis.** The purified PCR products were ligated into the pGEM-T vector cloning system (Promega, Tokyo, Japan). The ligated products were transformed into *Escherichia coli* JM109 competent cells (Promega). Plasmids were extracted from the cloned cells and purified with the Wizard Plus Minipreps DNA purification system (Promega). Nucleotide sequencing was performed with an automatic sequencer (Prism 310 genetic analyzer; Applied Biosystems). All sequences were checked for chimeric artifacts by the Chimera Check program from the Ribosomal Database Project (22). Partial sequences (approximately 500 bp) were compared with similar sequences of the reference organisms by a BLAST search (2). Sequences with 97% or greater similarity were grouped into operational taxonomic units (OTUs) by the Similarity Matrix program from the Ribosomal Database Project (22). Nearly complete sequencing of the 16S rRNA gene of each representative OTU was performed, and the sequences were aligned with the CLUSTAL W package (44). A phylogenetic tree was constructed by the neighbor-joining method (42). Bootstrap resampling analysis of 100 replicates was performed to estimate the confidence of tree topologies.

**Incubation with radioactive substrates.** The following inorganic and organic substrates, labeled with radioisotopes, were used: (i) sodium [<sup>14</sup>C]bicarbonate (specific activity, 58 mCi  $\text{mmol}^{-1}$ ), as a carbon source for nitrifiers; (ii) [1-<sup>14</sup>C]acetic acid (sodium salt; specific activity, 61 mCi  $\text{mmol}^{-1}$ ), as a low-molecular-weight organic substrate produced through decomposition of complex organic compounds; (iii) an L-amino acid mixture uniformly labeled with <sup>14</sup>C (specific activity, 50 mCi  $\text{mmol}^{-1}$ ), as a low-molecular-weight organic substrate produced through decomposition of proteins; and (iv) *N*-acetyl-D-[1-<sup>14</sup>C]glucosamine ([<sup>14</sup>C]NAG) (specific activity, 57 mCi  $\text{mmol}^{-1}$ ), as a major constituent of the bacterial cell wall. Radioactive chemicals were purchased from Amersham Biosciences (Little Chalfont, United Kingdom) and ICN Biomedical Inc. (Irvine, Calif.).

Four different experiments with four different types of radioactively labeled substrate were conducted (Table 1). The homogenized biofilm samples were diluted to obtain a final concentration of 3 g of volatile suspended solids per liter with a basal medium (0.4 mM  $\text{K}_2\text{HPO}_4$ , 0.41 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.25 mM  $\text{NaCl}$ ; pH 8.0). Although the in situ spatial organization of the microbial community was disturbed, the homogenization was essential for the quantitative analysis described below. For each experiment, 1.9-ml portions of a diluted biofilm sample were transferred to 10-ml serum bottles. The cultures were supplemented with unlabeled ammonium (1 mM) and bicarbonate (0.5 mM) and with radioactively labeled inorganic and organic substrates (final radioactivity, 10  $\mu$ Ci per 2-ml culture). When radioactively labeled bicarbonate was used, unlabeled bicarbonate was not added. The final concentrations of radioactively labeled acetic acid, bicarbonate, NAG, and amino acids were 82, 86, 88, and 100  $\mu$ M, respectively. Unlabeled acetic acid, NAG, and amino acids were not added in any experiment. The final total volume of each culture was 2 ml. The vials were sealed with gas-tight rubber stoppers and aerobically incubated for 4 h with shaking at 100 rpm at 25°C. The incubation time, 4 h, was determined in preliminary experiments to minimize changes in the microbial community structure and cascade utilization of <sup>14</sup>CO<sub>2</sub> derived from oxidation of radiolabeled organic substrates during the incubation. Biofilm samples pasteurized at 70°C for 15 min were incubated in the same way with the radioactive and nonradioactive substrates in experiments performed at the same time as experiments 1 to 4 as a control to test for possible adsorption phenomena and chemography.

TABLE 2. 16S and 23S rRNA-targeted oligonucleotide probes used

Probe <sup>a</sup>	Sequence (5' to 3')	FA (%) <sup>b</sup>	Specificity	Reference
EUB338	GCTGCCTCCCGTAGGAGT	— <sup>c</sup>	Most bacteria	3
BET42a <sup>d</sup>	GCCTTCCCACCTTCGTTT	35	$\beta$ - <i>Proteobacteria</i>	23
Nso190	CGATCCCCTGCTTTTCTCC	35 <sup>e</sup>	Ammonia oxidizers	26
GAM42a <sup>f</sup>	GCCTTCCCACATCGTTT	35	$\gamma$ - <i>Proteobacteria</i>	23
ALF1b	CGTTCGYTCTGAGCCAG	20	Many $\alpha$ - <i>Proteobacteria</i> , including <i>Nitrospira</i>	23
CF319 a/b	TGGTCCGTRTCTCAGTAC	35	CFB, including OTU 7	24
S*-CFB-0655-a-A-18	CGCTCACCTCCACAACAT	20	OTU 8 of CFB	This study
S*-CFB-0730-a-A-18	TACAGKCTAGYAAGCTGC	20	OTU 9 of CFB	This study
Ntspa1026	AGCACGCTGGTATTGCTA	20	<i>Nitrospira moscoviensis</i>	18
GNSB-941	AAACCACACGCTCCGCT	35	Phylum <i>Chloroflexi</i> (GNSB)	11
CFX1223	CCATTGTAGCGTGTGTGTMG	35	Phylum <i>Chloroflexi</i> (GNSB)	6
S*-GNS-0667-a-A-18	CACCCSGAATTCCACRTT	35	OTU 11 and 12 of GNSB	This study
S*-Oplitu-0744-a-A-18	GCCTGAGCGTCAGCAATT	5	OTU 13 of <i>Verrucomicrobia</i>	This study

<sup>a</sup> Probes designed in this study were named according to the recommendations of Alm et al. (1).

<sup>b</sup> FA, formamide concentration in the hybridization buffer.

<sup>c</sup> The probe can be used at any formamide concentration.

<sup>d</sup> Unlabeled probe GAM42a was used as a competitor to enhance specificity.

<sup>e</sup> Although the original description of the use of Nso190 indicated that 55% formamide should be used, we experimentally confirmed that 35% formamide was sufficient to discriminate ammonia-oxidizing  $\beta$ -*proteobacteria* in our autotrophic nitrifying biofilm, which is consistent with other studies in which Nso190 was used at much lower stringencies than that described originally (39).

<sup>f</sup> Unlabeled probe BET42a was used as a competitor to enhance specificity.

**Liquid scintillation counting.** The uptake of radioactive substrates was confirmed by liquid scintillation counting in all experiments before analysis by MAR-FISH. The <sup>14</sup>C content was measured directly in a culture sample (biomass plus culture medium) and washed biomass that was prepared by centrifugation (10,000 × g for 8 min) of a 1-ml culture sample and then resuspension in 1 ml of tap water. Each 0.1-ml aliquot was added to 3 ml of scintillation liquid (Ultima Gold XR; Packard BioScience Co., Meriden, Conn.). After the samples were thoroughly mixed and stored at room temperature for 3 h, the radioactivity was determined with an Aloka model LSC-1000 liquid scintillation counter used as recommended by the manufacturer. To evaluate the uptake of radioactive substrates, the percentage of radioactive substrate incorporated into the biomass was calculated.

**Sample fixation and washing.** After incubation with radioactive and non-radioactive substrates, the samples were fixed for 3 h at 4°C by adding 2 ml of 8% paraformaldehyde, which resulted a final concentration of 4%. Subsequently, the samples were centrifuged at 10,000 × g for 8 min and washed three times with 2 ml of phosphate-buffered saline (10 mM sodium phosphate buffer, 130 mM sodium chloride; pH 7.2) to remove excess soluble radioactive substrates. After the fixation and washing steps, the samples were spotted on a gelatin-coated cover glass (16).

**Oligonucleotide probes and in situ hybridization.** The 16S and 23S rRNA-targeted oligonucleotide probes used in this study are shown in Table 2. The probes were labeled with fluorescein isothiocyanate (FITC), tetramethylrhodamine 5-isothiocyanate (TRITC), or the sulfoindocyanine dye Cy5. Some probes were designed in this study by using the PROBE\_DESIGN tool of the ARB software package (<http://www.arb-home.de/>) according to the current version (June 2002) of the 16S rRNA sequence database. The specificity of the probes was checked against the ARB database, and the optimal hybridization conditions were experimentally determined (Table 2). All probes designed in this study were named in accordance with the standard introduced by Alm et al. (1), while the names of previously described probes were not changed to avoid confusion. Dehydration and FISH were performed by using the procedure described by Amann (4) and Okabe et al. (34). The hybridization conditions used for FISH are shown in Table 2. Simultaneous hybridizations with the probes requiring different stringency conditions were performed by using a successive hybridization procedure; hybridization with the probe requiring higher stringency was performed first, and then hybridization with the probe requiring lower stringency was performed.

The microbial community composition in the autotrophic nitrifying biofilm was quantitatively analyzed by using group- and subclass-specific probes listed in Table 2. 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. The average surface fraction was determined from at least 10 representative laser-scanning microscopy (LSM) projection images of each cross sec-

tion of the biofilm samples by using image analysis software provided by Zeiss (34).

**Autoradiographic procedure.** MAR was performed directly on the cover glasses as described by Lee et al. (21). After the FISH procedure, autoradiographic liquid film emulsion (LM-1; Amersham Biosciences) was used. The optimal exposure time was adjusted to between 1 and 3 days, depending on the percentage of radioactive substrates incorporated into the biomass.

**Microscopy and enumeration by MAR-FISH.** A model LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with an Ar ion laser (458 and 488 nm) and two He-Ne ion lasers (543 and 633 nm) was used. Formation of silver grains in the autoradiographic film was observed by using the transmission mode of the system. All images were combined and processed with the standard software package provided with the LSM510 microscope. Processed images were printed by using the Photoshop 5.0 software package (Adobe Systems Inc., Mountain View, Calif.).

A MAR-positive cell was defined as a cell that was covered with more than five silver grains. The numbers of MAR-positive cells and total probe-hybridized cells were determined by directly counting a minimum of 500 silver-grain-covered cells in randomly chosen microscopic fields.

**Nucleotide sequence accession numbers.** The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of 13 clones used for the phylogenetic tree analysis are AB117705 to AB117717.

## RESULTS

**Phylogenetic analysis.** One hundred clones were randomly selected, approximately 500 bp was analyzed, and the clones were grouped into 13 OTUs on the basis of more than 97% sequence similarity within an OTU. A nearly complete 16S rRNA gene sequence of a representative clone of each OTU was analyzed, and a phylogenetic tree was generated (Fig. 1). No chimeric sequences were observed. The similarities, the closest relatives in the database, and the detection frequencies of the representative clones are shown in Table 3. The clone distribution of the clone library was as follows: genus *Nitrospira*, 39 of 100 clones; genus *Nitrosomonas* of the  $\beta$ -subclass ammonia-oxidizing bacteria (AOB), 25 of 100 clones; green nonsulfur bacteria (GNSB), 14 of 100 clones; members of the  $\gamma$  subclass of the *Proteobacteria* ( $\gamma$ -*Proteobacteria*), 9 of 100 clones; *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, 9 of 100 clones;  $\alpha$ -*Proteobacteria*, 3 of 100 clones; and member of

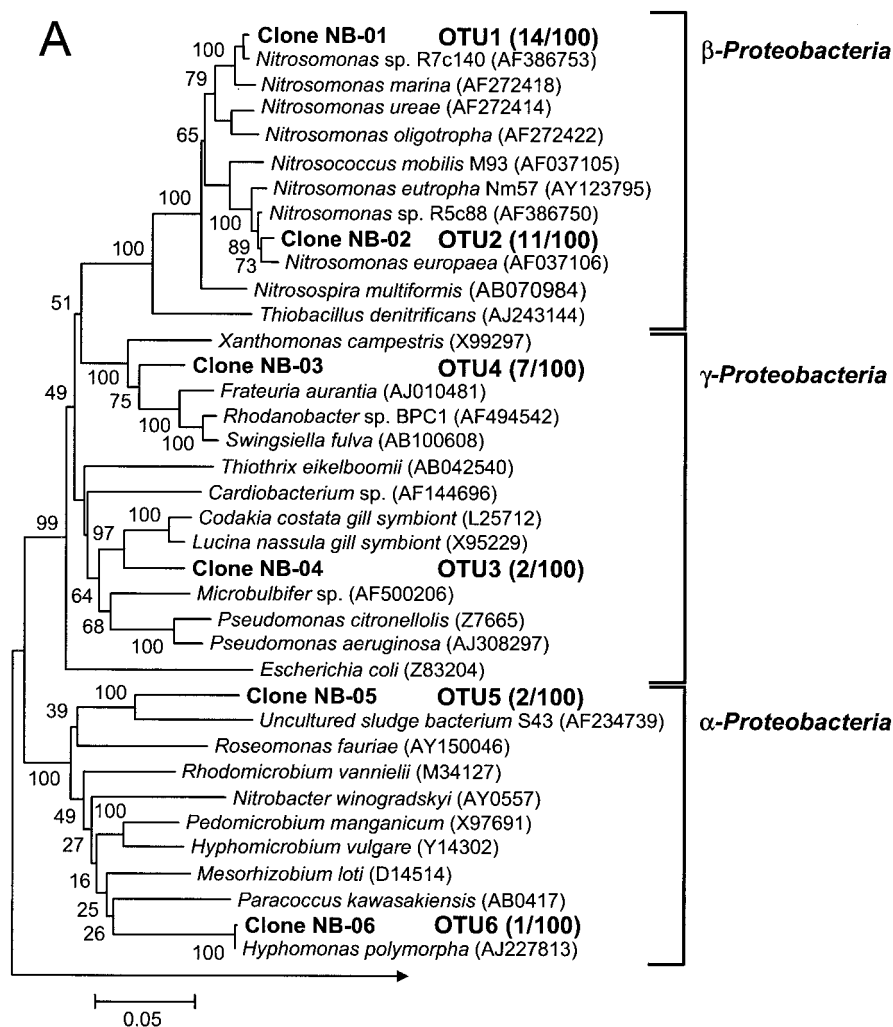


FIG. 1. Phylogenetic trees for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -Proteobacteria (A) and *Nitrospira*, *Verrucomicrobia*, GNSB, and the CFB group (B), showing the positions of the clones obtained from the autotrophic nitrifying biofilm. The tree was generated by using 1,309 bp (A) and 1,086 bp (B) of the 16S rRNA genes and the neighbor-joining method. Scale bar = 5% sequence divergence. The values at the nodes are bootstrap values (100 $\times$  resampling analysis). The *Methanobacterium congolense* sequence served as the outgroup for rooting the tree. The numbers in parentheses indicate the frequencies of appearance of identical clones in the clones analyzed.

the phylum *Verrucomicrobia*, 1 of 100 clones. Therefore, the clones affiliated with nitrifying bacteria (AOB and nitrite-oxidizing bacteria [NOB]) accounted for 64% of the clones analyzed. The sequence of OTU 10 was affiliated with the *Nitrospira* sp. belonging to subdivision I of the genus *Nitrospira*, but the level of sequence similarity to the previously isolated organism *Nitrospira moscoviensis* belonging to subdivision II of the genus *Nitrospira* (10) was only 93%. This indicated that the *Nitrospira*-like bacteria detected in the biofilm are most likely members of a novel species of *Nitrospira*. None of the clone sequences affiliated with the  $\alpha$ -subclass NOB (i.e., the genus *Nitrobacter*) were detected in the biofilm. Almost all the clones belonging to heterotrophic bacterial groups exhibited less than 96% similarity to previously identified bacteria or environmental clones that have not been isolated yet in the database (Fig. 1).

**In situ spatial organization.** The in situ spatial organization of heterotrophic bacteria and nitrifying bacteria was visualized

by FISH by using newly designed probes and the previously described group-specific probes (Table 2). The microorganisms represented by OTUs 5 and 6 could be detected with the ALF1b probe. Large rod-shaped bacterial cells occurring singly were also detected with the ALF1b probe. These cells were evenly distributed throughout the biofilm, and some of them were present in the nitrifier clusters (Fig. 2A). The microorganisms represented by OTUs 3 and 4 could be detected with the GAM42a probe. Small, long, rod-shaped bacterial cells were found around the nitrifier clusters throughout the biofilm with the GAM42a probe (Fig. 2B). Probes S\*-CFB-0655-a-A-18 (specific for OTU 8) and S\*-CFB-0730-a-A-18 (specific for OTU 9) were designed in this study for the CFB group because previously described probes CF319a/b and CFB560 (36) did not cover all clones affiliated with the members of the CFB group detected in this study. By using probes CF319a/b, S\*-CFB-0655-a-A-18, and S\*-CFB-0730-a-A-18, thin filamentous bacteria were detected around the nitrifier clusters

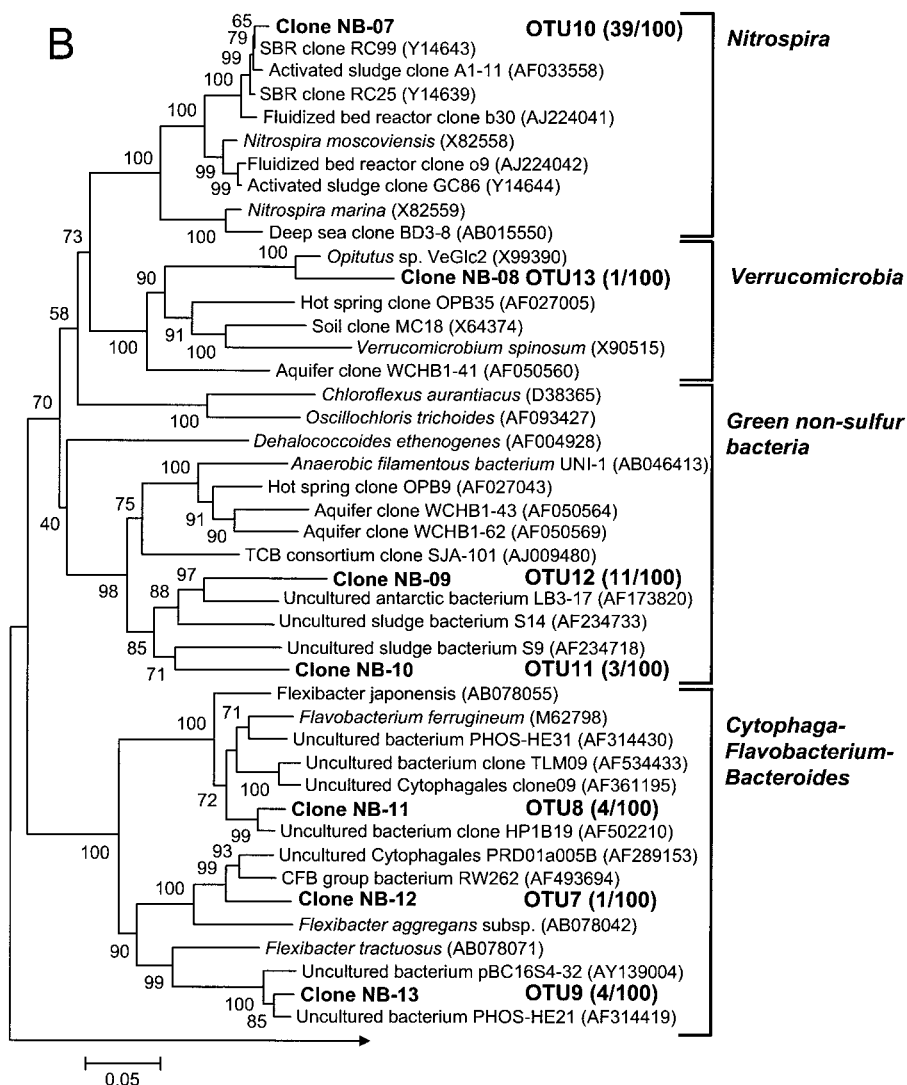


FIG. 1—Continued.

mainly in the surface of the biofilm (Fig. 2C). The S\*-GNS-0667-a-A-18 probe was designed to cover both OTUs 11 and 12 belonging to subdivision I of the GNSB. The filamentous morphotype (thickness, ca. 0.5 to 2.0 μm), which occurred mainly around the nitrifier clusters, was detected with probes S\*-GNS-0667-a-A-18, GNSB-941, and CFX1223 (Fig. 2D). OTU 13, which is related to *Opitutus* sp. in the phylum *Verrucomicrobia* (8, 17), was not clearly detected with probe S\*-Opitu-0774-a-A-18 due to the small cell size, low fluorescence signal intensity, and low abundance.

**Community composition.** The microbial community composition in the autotrophic nitrifying biofilm was analyzed by FISH with various sets of probes (Table 2 and Fig. 3). Consistent with the results of the 16S rRNA gene sequence analysis, members of the *Nitrospira* phylum (NOB) and AOB accounted for 28 and 22% of the organisms, respectively. On the other hand, members of the α-proteobacterial, γ-proteobacterial, GNSB, and CFB groups accounted for 23, 13, 9, and 2%, respectively; these results were slightly different from the results of the 16S rRNA gene sequence analysis. The ratio of

total nitrifiers (AOB plus NOB) to all heterotrophs was approximately 1:1.

**Substrate uptake patterns for different phylogenetic groups.** The percentages of uptake (the amount of the total radioactivity incorporated in cell mass) of [<sup>14</sup>C]bicarbonate, [<sup>14</sup>C]acetic acid, <sup>14</sup>C-amino acids, and [<sup>14</sup>C]NAG after 4 h of incubation were 7, 13, 6, and 1%, respectively. The differences in uptake of substrates may be attributed to the different microbial population sizes and the substrate uptake rate of each bacterial group. The uptake of all of the radioactive substrates was not significant (less than 0.08%) when the samples were pasteurized as a control. Consequently, no silver-grain-coated cells were observed. These results indicated that the radioactive substrates were truly incorporated by the cells rather than adsorbed on the cell surface.

The patterns of uptake of organic compounds were very different for the different phylogenetic groups. The nitrifying bacteria (AOB plus NOB) took up only [<sup>14</sup>C]bicarbonate, underlining the autotrophic growth of these organisms (Fig. 4A). No MAR-positive nitrifiers were detected in the experiment

TABLE 3. Detection frequency and phylogenetic relatives of the clones analyzed

OTU <sup>a</sup>	No. of clones <sup>b</sup>	Group	Closest taxon	Accession no.
10	39	<i>Nitrospirales</i> , <i>Nitrospira</i>	<i>Nitrospira</i> sp.	Y14639
1	14	$\beta$ - <i>Proteobacteria</i> , <i>Nitrosomonas</i>	<i>Nitrosomonas</i> sp. strain R7c140	AF386753
2	11	$\beta$ - <i>Proteobacteria</i> , <i>Nitrosomonas</i>	<i>Nitrosomonas europaea</i>	AF353160
11	11	GNSB	Uncultured antarctic bacterium LB3-17	AF173820
4	7	$\gamma$ - <i>Proteobacteria</i> , <i>Xanthomonas</i>	<i>Frateuria aurantia</i>	AJ010481
9	4	CFB group	Uncultured bacterium PHOS-HE21	AF314419
8	4	CFB group	Uncultured bacterium clone HP1B19	AF502210
12	3	GNSB	Uncultured sludge bacterium S9	AF234718
3	2	$\gamma$ - <i>Proteobacteria</i>	<i>Lucina nassula</i> gill symbiont	X95229
5	2	$\alpha$ - <i>Proteobacteria</i>	Uncultured sludge bacterium S43	AF234739
6	1	$\alpha$ - <i>Proteobacteria</i> , <i>Hyphomonas</i>	<i>Hyphomonas polymorpha</i>	AJ227813
7	1	CFB group	Uncultured <i>Cytophagales</i> clone	AF289153
13	1	<i>Verrucomicrobia</i> , subdivision IV	<i>Opiritus</i> sp.	X99391

<sup>a</sup> Clones exhibiting 97% or greater similarity with each other were grouped into an OTU.

<sup>b</sup> A total of 100 clones in 13 OTUs were examined.

with the radiolabeled organic substrates due to the high background of [<sup>12</sup>C]bicarbonate, which diluted <sup>14</sup>CO<sub>2</sub> derived from oxidation of radiolabeled organic substrates during 4 h of incubation. No uptake of [<sup>14</sup>C]bicarbonate by heterotrophic bacterial groups was observed after 4 h of incubation (Table 4).  $\alpha$ -*Proteobacteria* that hybridized with probe ALF1b and  $\gamma$ -*Proteobacteria* that hybridized with probe GAM42a accounted for 35 and 42% of the total [<sup>14</sup>C]acetic acid-utilizing bacteria, respectively (Fig. 5A). <sup>14</sup>C-amino acids were utilized by all heterotrophic bacterial groups (Fig. 4C and Table 4). The relative contributions to <sup>14</sup>C-amino acid utilization reflected the community composition of heterotrophic bacteria. The GNSB that hybridized with probes S\*-GNS-0667-a-A-18, GNSB-941, and CFX1223 took up <sup>14</sup>C-amino acids and [<sup>14</sup>C]NAG (Fig. 4C and D) and accounted for 9% of the <sup>14</sup>C-amino acid-utilizing bacterial population and 27% of the [<sup>14</sup>C]NAG-utilizing bacterial population (Fig. 5A). Interestingly, the members of the CFB group that hybridized with the CF319a/b, S\*-CFB-0665-a-A-18, and S\*-CFB-0730-a-A-18 probes were a numerically dominant group of [<sup>14</sup>C]NAG-utilizing bacteria (accounting for 64% of the bacteria), even though their abundance in the biofilm was only 2% (Fig. 5A). More than 75% of the CFB group bacteria that hybridized with the specific probes took up <sup>14</sup>C-amino acids and [<sup>14</sup>C]NAG, demonstrating the high substrate specificity of the CFB organisms for amino acids and NAG (Fig. 5B). The unidentified group utilized all organic substrates used in this study (Fig. 5A). Although its abundance in the biofilm was relatively low (ca. 3% [Fig. 3]), the unidentified group could be an ecophysiological important bacterial group for carbon metabolism in the biofilm. However, the phylogenetic identity of the unidentified group was not clear in this study.

## DISCUSSION

Microbial nitrification (oxidation of NH<sub>4</sub><sup>+</sup> to NO<sub>3</sub><sup>-</sup> via NO<sub>2</sub><sup>-</sup>) is carried out by two phylogenetically unrelated groups of lithoautotrophic bacteria, the AOB and the NOB. Nitrifiers are known to produce SMP from substrate metabolism and biomass decay (5, 41). With a longer sludge retention time, like that in biofilm processes, a larger amount of SMP should be

produced (41). Since no external organic carbon was added in this experiment, SMP produced by nitrifiers provided the sole organic substrates for heterotrophic bacteria. To investigate ecophysiological interactions between nitrifying bacteria and heterotrophic bacteria in an autotrophic nitrifying biofilm fed only NH<sub>4</sub><sup>+</sup> as an electron donor, we used a full-cycle 16S rRNA approach and MAR-FISH analysis. The metabolic route of SMP was evaluated based on individual nutritional traits determined by MAR-FISH and the in situ spatial organization of each phylogenetic group as revealed by FISH.

In the MAR-FISH analysis, we used three radiolabeled organic substrates (NAG, amino acids, and acetic acid). Some of the SMP have been identified as humic and fulvic acids, polysaccharides, proteins, amino acids, nucleic acids, and structural components of cells (5). Both gram-positive and gram-negative bacteria contain NAG as a main substantial constituent of the cell wall peptidoglycan and lipopolysaccharides (5). Peptidoglycan depolymerization (i.e., cell decay) leads to liberation of NAG, which could provide a main pool of organic matter in autotrophic nitrifying biofilms. Amino acids are ubiquitous and are known to be an important source of C, N, and energy for heterotrophic bacteria in various aquatic environments, and most heterotrophically active bacteria are able to take up these compounds (38). Acetate is the major end product of fermentation of polysaccharides and proteins and thus is regarded as one of the most important and common substrates in water and wastewater treatment systems.

**Substrate uptake patterns of heterotrophs.** The patterns of uptake of low-molecular-weight dissolved organic matter differed greatly for the different phylogenetic groups. Despite of the low abundance (ca. 2%) in the biofilm community, the members of the CFB cluster accounted for the largest fraction (ca. 64%) of the bacterial community consuming NAG. Although the  $\alpha$ - and  $\gamma$ -*Proteobacteria* were numerically dominant bacterial groups, they did not utilize NAG at all.

These results are consistent with previous studies that demonstrated that some members of the CFB group degrade various refractory biomacromolecules, such as cellulose, chitin, DNA, lipids, and proteins, which should have been abundant in the biofilm, in which dead microorganisms were trapped (36). These organisms are also known for their ability to pro-

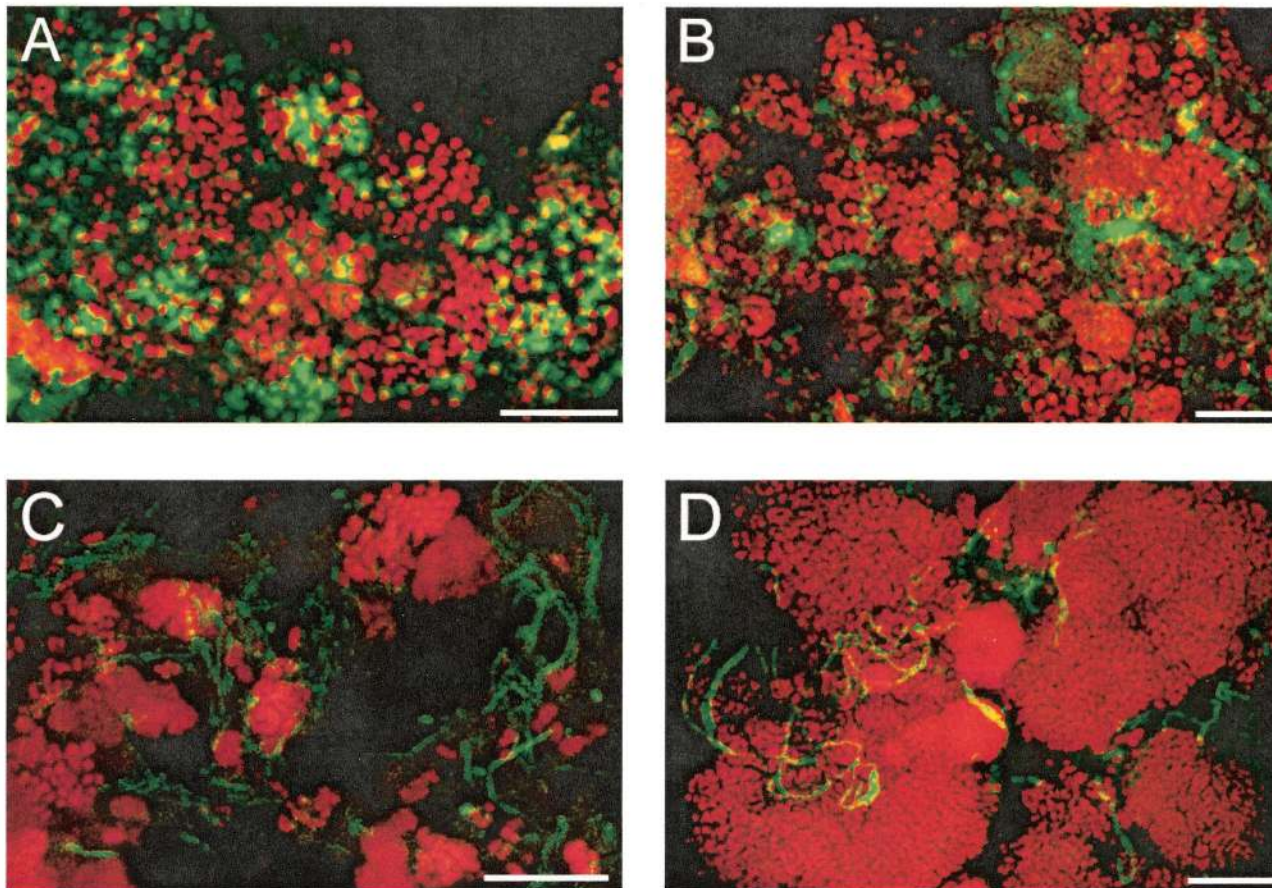


FIG. 2. Confocal laser scanning microscope images showing the in situ spatial organization of nitrifying bacteria (AOB plus NOB) and heterotrophic bacteria in the autotrophic nitrifying biofilm. (A) FISH with TRITC-labeled probes Nso190 and Ntspa1026 (specific for AOB and *Nitrospira*, respectively) and FITC-labeled probe ALF1b (specific for  $\alpha$ -*Proteobacteria*). (B) FISH with TRITC-labeled probes BET42a and Ntspa1026 (specific for  $\beta$ -*Proteobacteria* and *Nitrospira*, respectively) and FITC-labeled probe GAM42a (specific for  $\gamma$ -*Proteobacteria*). (C) FISH with TRITC-labeled probes Nso190 and Ntspa1026 (specific for AOB and *Nitrospira*, respectively) and FITC-labeled probes CF319a/b, S\*-CFB-0665-a-A-18, and S\*-CFB-0730-a-A-18 (specific for the CFB group). (D) FISH with TRITC-labeled probes Nso190 and Ntspa1026 (specific for AOB and *Nitrospira*, respectively) and FITC-labeled probes S\*-GNS-0667-a-A-18, GNSB-941, and CFX1223 (specific for GNSB). Bars = 10  $\mu$ m. The surface of the biofilm cannot be seen in the photographs.

duce exopolysaccharide slime, suggesting that they primarily form biofilms. It is, however, likely that the abundance of members of the CFB group was potentially underestimated due to a lack of CFB-specific probes that fully cover the whole CFB division and the low fluorescence intensity (i.e., low 16S rRNA content) in the biofilm.

Despite the low abundance (ca. 9%), the members of the GNSB cluster also played an important role in degradation of [ $^{14}$ C]NAG, accounting for 27% of the [ $^{14}$ C]NAG-consuming bacterial population (Fig. 5A). Based on 16S rRNA gene analysis, all our clone sequences were affiliated with GNSB subdivision I. Subdivision I contains the most diverse environmental clones among the four subdivisions; these clones were derived from various oxic and anoxic environments (15). It could be speculated that the GNSB could utilize a yeast extract-like substrate that includes amino acids released by the autolysis of heterotrophic bacteria in the biofilm, as suggested by Sekiguchi et al. (43).

The  $\alpha$ - and  $\gamma$ -proteobacterial populations were numerically

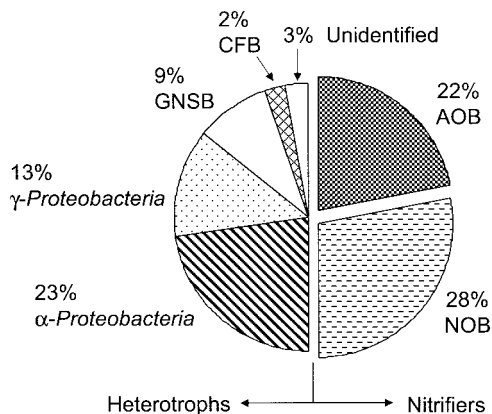


FIG. 3. Microbial community composition for the autotrophic nitrifying biofilm as determined quantitatively by FISH. The number of cells that hybridized with a probe in each microscopic field was expressed as a percentage of the total surface area of the bacteria that hybridized with the EUB338 probe. Unidentified refers to the surface area of cells detectable with probe EUB338 but not with any other specific probe.

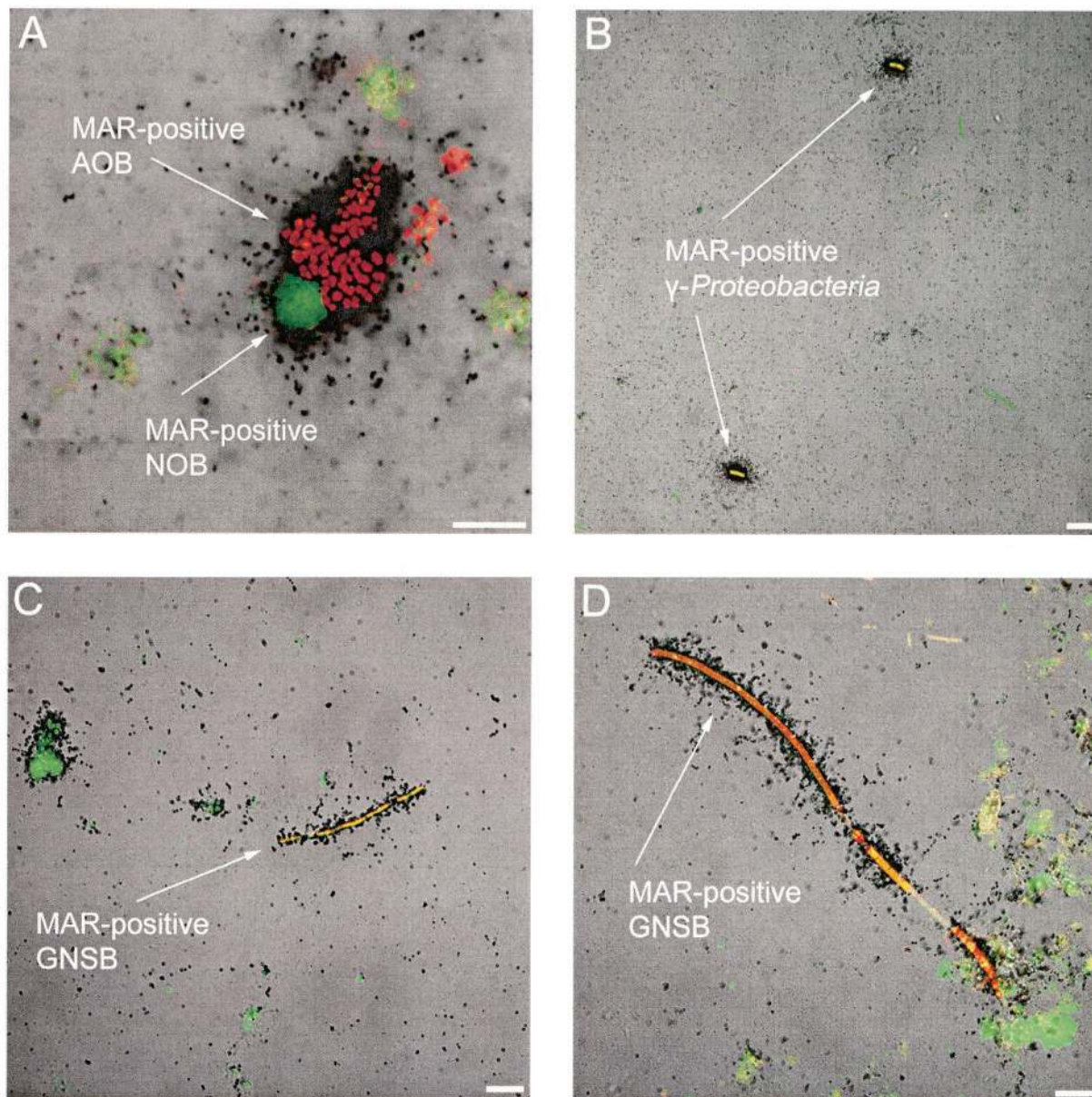


FIG. 4. Combined MAR-FISH images of homogenized autotrophic nitrifying biofilm samples. (A) Uptake of [ $^{14}\text{C}$ ]bicarbonate by nitrifying bacteria when  $\text{NH}_4^+$  was used as the sole electron donor. In situ hybridization was performed with FITC-labeled probe Ntspa1026 (green), which was used simultaneously with TRITC-labeled probe Nso190 (red). (B) Uptake of [ $^{14}\text{C}$ ]acetic acid by members of the  $\gamma$ -Proteobacteria group. In situ hybridization was performed with FITC-labeled probe EUB338 (green) and TRITC-labeled probe GAM42a (red). (C) Uptake of  $^{14}\text{C}$ -amino acids by the GNSB. In situ hybridization was performed with FITC-labeled probe EUB338 (green) and TRITC-labeled probes S\*-GNS-0667-a-A-18, GNSB-941, and CFX1223 (red). (D) Uptake of [ $^{14}\text{C}$ ]NAG by the GNSB. In situ hybridization was performed with FITC-labeled probe EUB338 (green) and TRITC-labeled probes S\*-GNS-0667-a-A-18, GNSB-941, and CFX1223 (red). Yellow signals resulted from binding of both FITC- and TRITC-labeled probes to one cell. Bars = 10  $\mu\text{m}$ .

dominant heterotrophic populations in the biofilm. High levels of  $\alpha$ -Proteobacteria in an autotrophic nitrifying biofilm and in oligotrophic river biofilms have been reported previously (25, 31). It is probable that  $\alpha$ - and  $\gamma$ -Proteobacteria primarily utilize low-molecular-weight fatty acids produced from degradation of SMP produced by nitrifiers. Some  $\alpha$ -Proteobacteria strains also could take up NAG (9, 40). The most frequently detected clone sequences were affiliated

with the *Hyphomonas* cluster of  $\alpha$ -Proteobacteria (98% similarity) and the *Xanthomonas* cluster of  $\gamma$ -Proteobacteria (91 to 95% similarity) (Fig. 1A). *Hyphomonas* spp. catabolize proteins, peptides, and/or amino acids for energy and growth and form biofilms by production of extracellular polymeric substances (45). *Xanthomonas* spp. are able to utilize L-arabinose that is a constituent of the bacterial cell wall under aerobic and microaerophilic conditions (7).



TABLE 4. Summary of patterns of uptake of radioactive substrates by phylogenetic groups

Group	Uptake of:			
	Bicarbonate	Acetic acid	Amino acids	NAG
AOB	+ <sup>a</sup>	—	—	—
NOB	+	—	—	—
<i>α-Proteobacteria</i>	—	+	+	—
<i>γ-Proteobacteria</i>	—	+	+	—
GNSB	—	—	+	+
CFB	—	—	+	+
Unidentified	—	+	+	+

<sup>a</sup> +, MAR positive; —, MAR negative.

**Carbon metabolism in the autotrophic nitrifying biofilm.** It is thought that the primary degraders directly colonize or occur around the nitrifier clusters that liberate (excrete) SMP. They then recruit other species to form metabolically structured and functionally integrated biofilm communities that ensure maximum utilization of metabolites (i.e., SMP) of nitrifiers. The in situ spatial organization of the nitrifier clusters and heterotrophs in the biofilm was visualized by FISH (Fig. 2). In this study, GNSB were detected mainly around nitrifier clusters in a biofilm for the first time (6, 19). We also found that there was a tendency for members of the CFB group, GNSB, and *γ-Proteobacteria* to be more closely associated with the nitrifier clusters. However, we could not determine whether there was a clear relationship between the spatial organization of different groups of heterotrophs and their physiological roles in carbon metabolism in the biofilm.

The ratio of nitrifiers (AOB plus NOB) to heterotrophs was approximately 1:1 in the biofilm. According to the kinetic parameters for SMP production by nitrifiers (41), we estimated that only about 1.5 mg of chemical oxygen demand per liter of SMP could be produced from 3.6 mM NH<sub>4</sub><sup>+</sup> in the feed. Therefore, the carbon metabolism in the biofilm must be efficient to support heterotrophic bacteria (ca. 10<sup>8</sup> to 10<sup>9</sup> cells cm<sup>-3</sup>) with this small amount of SMP. SMP have proved to be major components of organic matter in effluents from biological wastewater treatment processes (27). Our knowledge regarding SMP of nitrifiers is, however, far from complete, and much work is still required to fully understand their contribution to the growth of heterotrophic bacteria.

In conclusion, a full-cycle 16S rRNA approach followed by MAR-FISH revealed that *α*- and *γ*-*Proteobacteria* mainly utilized low-molecular-weight organic matter, like [<sup>14</sup>C]acetic acid and [<sup>14</sup>C]-amino acids, whereas the GNSB and CFB group were specialized and decomposed [<sup>14</sup>C]NAG despite low abundance in the autotrophic nitrifying biofilm. The phylogenetically diverse and (to some extent) metabolically redundant heterotrophic bacterial community could have ensured effective utilization of SMP produced by nitrifiers, which may have created a functionally structured stable biofilm ecosystem. To determine how the radioactivity of [<sup>14</sup>C]bicarbonate incorporated by nitrifiers is transferred to major phylogenetic groups of heterotrophs, the fate of the radioisotopically labeled atom of [<sup>14</sup>C]bicarbonate should be directly traced by MAR-FISH in the future.

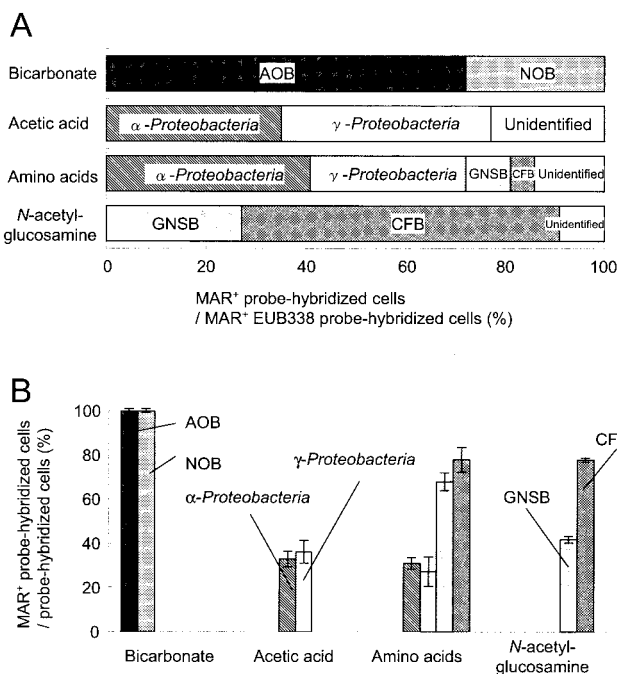


FIG. 5. Patterns of substrate uptake by the major phylogenetic bacterial groups identified by using group-specific probes. (A) Fractions of group-specific-probe-hybridized cells that simultaneously took up radioactive substrates (MAR-positive cells), expressed as percentages of the total bacteria that were detected with probe EUB338 and simultaneously took up radioactive substrates. (B) Fractions of probe-hybridized and MAR-positive cells, expressed as percentages of each group-specific-probe-hybridized cell count. AOB, AOB that hybridized with probe Nso190; NOB, NOB that hybridized with probe Ntspa1026; *α-Proteobacteria* hybridized with probe ALF1b; *γ-Proteobacteria* hybridized with probe GAM42a; GNSB, GNSB that hybridized with probes S\*-GNS-0667-a-A-18, GNSB-941, and CFX1223; CFB, members of the CFB group that hybridized with probes CF319a/b, S\*-CFB-0665-a-A-18, and S\*-CFB-0730-a-A-18. The error bars indicate the standard errors ( $n = 10$ ).

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