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Diversity, Localization, and Physiological Properties of Filamentous Microbes Belonging to *Chloroflexi* Subphylum I in Mesophilic and Thermophilic Methanogenic Sludge Granules

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We previously reported that the thermophilic filamentous anaerobe *Anaerolinea thermophila*, which is the first cultured representative of subphylum I of the bacterial phylum *Chloroflexi*, not only was one of the predominant constituents of thermophilic sludge granules but also was a causative agent of filamentous sludge bulking in a thermophilic (55°C) upflow anaerobic sludge blanket (UASB) reactor in which high-strength organic wastewater was treated (Y. Sekiguchi, H. Takahashi, Y. Kamagata, A. Ohashi, and H. Harada, Appl. Environ. Microbiol. 67:5740–5749, 2001). To further elucidate the ecology and function of *Anaerolinea*-type filamentous microbes in UASB sludge granules, we surveyed the diversity, distribution, and physiological properties of *Chloroflexi* subphylum I microbes residing in UASB granules. Five different types of mesophilic and thermophilic UASB sludge were used to analyze the *Chloroflexi* subphylum I populations. 16S rRNA gene cloning-based analyses using a 16S rRNA gene-targeted *Chloroflexi*-specific PCR primer set revealed that all clonal sequences were affiliated with the *Chloroflexi* subphylum I group and that a number of different phylotypes were present in each clone library, suggesting the ubiquity and vast genetic diversity of these populations in UASB sludge granules. Subsequent fluorescence in situ hybridization (FISH) of the three different types of mesophilic sludge granules using a *Chloroflexi*-specific probe suggested that all probe-reactive cells had a filamentous morphology and were widely distributed within the sludge granules. The FISH observations also indicated that the *Chloroflexi* subphylum I bacteria were not always the predominant populations within mesophilic sludge granules, in contrast to thermophilic sludge granules. We isolated two mesophilic strains and one thermophilic strain belonging to the *Chloroflexi* subphylum I group. The physiological properties of these isolates suggested that these populations may contribute to the degradation of carbohydrates and other cellular components, such as amino acids, in the bioreactors.

Granulation of sludge is a common phenomenon in upflow anaerobic sludge blanket (UASB) reactors. Sludge granules are formed within a UASB reactor vessel as a result of the self-immobilization properties of microbial cells. These sludge granules are thought to be spherical biofilms, which are composed of a number of different metabolic groups of anaerobes that lead to the complete degradation of organic matter into mainly methane and carbon dioxide (18, 24). These unique biofilms have been intensively studied, and a number of unique phenomena have been reported (23). One of the interesting features of the granules is the mechanism of formation (granulation) and disintegration (e.g., bulking) of granular sludge. Because the granulation is caused by endogenous microbial activities, it has attracted the attention of engineers and microbiologists, who have made efforts to explain the process. In addition, the deterioration of the settling properties of granular sludge is an important aspect of UASB sludge. Once the sludge granules within a reactor become fluffy (bulking) or disintegrate, they do not properly settle, and consequently the reactor cannot accept further treatment at high loading rates. The current view is that specific types of microorganisms play significant roles in both the formation and disintegration of sludge granules (23, 24).

Among the various types of important microbes that may contribute to the granule structure (such as *Methanosaeta* species) (8, 20, 25), the filamentous microbes affiliated with subphylum I of the bacterial phylum *Chloroflexi* have recently been recognized as a group that can affect granulation and bulking (25, 28). In sludge granules that settled well (particularly granules of thermophilic UASB sludges [in reactors operated at 55 to 60°C]), it was frequently observed that long, thin filamentous microbes, which apparently were different from *Methanosaeta*, predominated on the surface of the granules, forming a web-like structure (25, 31, 33). This finding suggests that these organisms have an important role in granulation and in preservation of the granule structure. In a previous study, we found that almost all of the thin filamentous cells present on the surface of thermophilic UASB sludge granules were members of *Chloroflexi* subphylum I (25). In addition, an identical population of this subphylum caused the formation of fluffy granules, triggering the filamentous bulking of sludge granules (28). These observations suggest that it is important to control

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TABLE 1. Operating conditions of the UASB reactors used in this study

Reactor	Vol (m^3)	Temp (°C)	Type of wastewater	COD load (kg COD/m^3/day)	Influent COD concn (mg/liter)	COD removal efficiency (%)
AMG	0.013	37	Artificial[a]	6	2,000	>95
BMG	>900	30–36	Actual (from a beer factory)	0.34	200–300	>90
CMG	>400	35–40	Actual (from a sugar-manufacturing factory)	9	2,000	90–95
ATG	0.013	55	Artificial[a]	10	3,000	>95
STG	0.013	55	Actual (from a shochu-manufacturing factory)	100	10,000	>95

[a] Artificial wastewater contained sucrose, acetate, propionate, and peptone (chemical oxygen demand ratio, 4.5:2.25:2.25:1).

the growth of the *Chloroflexi* subphylum I-type filamentous cells, not only to sustain granule formation but also to prevent the bulking of sludge granules in thermophilic UASB processes. Therefore, much attention should be paid to the physiology and ecology of *Chloroflexi* subphylum I-type microbes in UASB granules. However, in spite of the importance of *Chloroflexi* subphylum I populations within sludge granules, the physiology and in situ function of these microbes remain largely unknown because this subphylum has long been recognized as a typical clone cluster containing a number of environmental 16S rRNA gene clones with no cultured representatives (11). Recently, a thermophilic anaerobe, *Anaerolinea thermophila*, was isolated from a thermophilic UASB sludge as the first cultured representative of this subphylum (28, 29). However, much of the ecology, physiology, and in situ function of the microbes that have *Chloroflexi* subphylum I-type 16S rRNA gene clone sequences, particularly bacteria in mesophilic sludges, is not yet understood.

In this study, in order to elucidate the diversity, distribution, and roles of *Chloroflexi* subphylum I-type bacteria involved in UASB sludge granules, we used a polyphasic approach based on molecular methods and cultivation. We first surveyed the genetic diversity of microorganisms belonging to the phylum *Chloroflexi* present in five different types of mesophilic and thermophilic sludge granules based on 16S rRNA genes. Next, the localization of these microbial populations within sludge granules was visualized by 16S rRNA-targeted fluorescence in situ hybridization (FISH) with confocal laser scanning microscopy. Finally, to elucidate the physiology and in situ function of *Chloroflexi* subphylum I cells, we attempted to cultivate and isolate the *Chloroflexi* subphylum I species which were present in the sludges.

MATERIALS AND METHODS

Operation of UASB reactors. Granular sludges were collected from five UASB reactors that had been operated under either mesophilic conditions (37°C) (reactors AMG, BMG, and CMG) or thermophilic conditions (55°C) (reactors ATG and STG) (Table 1). The AMG and ATG reactors had been fed with an artificial wastewater containing sucrose, volatile fatty acids, yeast extract, and trace elements (27) over 5 years of operation. The BMG, CMG, and STG reactors had received actual organic wastewaters; the BMG reactor received wastewater from a beer factory, the CMG reactor received treated wastewater discharged from a sugar-processing plant, and the STG reactor was fed with wastewater from a factory manufacturing shochu (a Japanese distilled liquor made from sweet potato). All of the reactors exhibited good performance in terms of chemical oxygen demand (COD) removal (with removal efficiencies of 90 to 95%), as well as methane formation, on the sampling dates.

Microorganisms, media, and cultivation. The following organisms were used in this study. Three thin filamentous bacterial strains, strains IMO-1, KIBI-1, and YMTK-2, were enriched and isolated in this study. *Methanospirillum hungatei* DSM 864 and *Methanothermobacter thermautotrophicus* DSM 1053 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The cultures used for enrichment and isolation of the three filamentous strains were prepared as described previously (26). All cultivation was performed in 50-ml serum vials containing 20 ml of medium (pH at 25°C, 7.2) under an 80% (vol/vol) N_2–20% (vol/vol) CO_2 atmosphere without shaking. Cultivation of strain IMO-1 was performed at 55°C, and cultivation of the other two filamentous strains (KIBI-1 and YMTK-2) was carried out at 37°C. *M. hungatei* and *M. thermautotrophicus* were inoculated into the medium mentioned above, and hydrogen was added to the gas phase (80% [vol/vol] N_2–20% [vol/vol] CO_2) in the vials as an energy source. These cultures were incubated at 37 or 55°C. For cocultivation, strain IMO-1 cells were cultivated in the presence of *M. thermautotrophicus* cells, and strains KIBI-1 and YMTK-2 were incubated with *M. hungatei* cells in medium supplemented with sucrose (20 mM) and yeast extract (0.1%) (2% inoculum).

The purity of the isolates obtained in this study was routinely examined using microscopy and incubation of cultures in medium containing 0.1% yeast extract and a mixture of carbohydrates (glucose, fructose, arabinose, and sucrose, each at a concentration of 2 mM) at 37°C for mesophilic strains and at 55°C for the thermophilic strain.

Construction of 16S rRNA gene clone libraries for members of the phylum *Chloroflexi* in five different types of granular sludge. DNA extraction from sludge samples was performed based on the method of Miller et al. (21), with slight modifications. Sludge granules were first rinsed with 1× phosphate-buffered saline (120 mM NaCl, 4 mM NaH_2PO_4, 10 mM Na_2HPO_4) and then manually homogenized. The sludge samples were then suspended in 300 μl of phosphate buffer (100 mM NaH_2PO_4, pH 8.0). After 5 s of sonication, the suspensions were collected in 2-ml screw-cap plastic vials containing 1 g of sterile 0.1-mm-diameter zirconium-silica beads with 300 μl of lysis buffer (100 mM NaCl, 500 mM Tris-HCl [pH 8.0], 5% sodium dodecyl sulfate), and the tubes were then filled with chloroform. The crude nucleic acids were extracted by a bead-beating procedure (1 min at 3,800 rpm with a Mini Bead Beater [Biospec Products]). To purify DNA for further analysis, phenol-chloroform treatment and RNA digestion were performed as described previously (27).

PCR amplification of 16S rRNA genes and subsequent cloning were performed as reported previously (27), with slight modifications. For construction of 16S rRNA gene clone libraries from the sludge samples, we used a *Chloroflexi*-specific primer set for PCR amplification of 16S rRNA genes consisting of prokaryote-specific primer UNIV530F (5′-GTGCCAGCMGCCGCGG-3′; *Escherichia coli* positions 530 to 546) (17) and *Chloroflexi*-specific primer GNSB941R (5′-AAACCACACGCTCCGCT-3′; *E. coli* positions 941 to 958) (6). PCR amplification was performed as follows: initial denaturation at 95°C for 9 min, followed by 35 cycles of 95°C for 40 s, 65°C for 30 s, and 72°C for 1 min. PCR products were purified with a Microspin column (Amersham Pharmacia Biotech) and were subjected to cloning using a TA cloning kit (Novagen). Ten clonal rRNA genes were randomly picked from each clone library and were subjected to further analysis.

DNA extraction and amplification of 16S rRNA genes from pure cultures. DNA extraction from pure cultures was performed by the method of Hiraishi (9). The 16S rRNA genes were amplified by PCR as described above, except that the following primers were used: *Bacteria*-specific primer 8F (5′-AGAGTTTGATC CTGGCTCAG-3′; *E. coli* positions 8 to 27) and universal primer 1490R (5′-G GTTACCTTGTTACGACTT-3′; *E. coli* positions 1491 to 1509) (35). PCR products were purified as described above.

Sequencing and phylogenetic tree. All sequences of rRNA gene clones, as well as the sequences from pure cultures, were determined with a CEQ DTC kit-Quick start kit (Beckman Coulter) and an automated sequence analyzer (model CEQ-2000; Beckman Coulter). Sequence data were aligned with an ARB data set using the ARB program package (19), and the aligned data were manually

corrected by using the editing tool in the package. Phylogenetic trees based on 16S rRNA gene sequences were constructed by the neighbor-joining method (22) with the ARB program package. To estimate the confidence of the tree topologies, bootstrap resampling analysis (5) for 1,000 replicates was performed with the PAUP* 4.0 program package (30). Clonal sequences that exhibited 100% identity were grouped into one phylotype, and the phylotypes found in the clone libraries were designated as follows: AMG-1 to AMG-7 for the clone library from AMG sludge, BMG-1 to BMG-5 for the clone library from BMG sludge, CMG-1 to CMG-7 for the clone library from CMG sludge, ATG-1 to ATG-7 for the clone library from ATG sludge, and STG-1 to STG-6 for the clone library from STG sludge.

Fixation, sectioning of sludge granules, and in situ hybridization. Sludge samples were fixed as described previously (25). Prior to in situ hybridization, sludge samples were subjected to freeze-thaw cycles (three times) in order to increase the efficiency of penetration of oligonucleotide probes into cells (25). The fixed sludge samples were then dehydrated by successive passages through 50, 80, and 100% ethanol (20 min each) and were subsequently immersed in Tissue-Tek 4583 O.C.T. compound (Sakura) at room temperature. The immersed sludge granules were then cut into 20-μm-thick sections with a cryotome (CM-502; Sakura). The sections of sludge samples were immobilized on glass slides coated with Vectabond (Vector Laboratories) for in situ hybridization experiments.

The following 16S rRNA-targeted oligonucleotide probes were used in this study: EUB338* (a mixture of the EUB338, EUB338-I, EUB338-II, and EUB338-III probes) (1, 3) specific for *Bacteria*, ARC915 specific for *Archaea* (17), and GNSB941 specific for almost all members of the phylum *Chloroflexi* (2, 6). All probes were labeled with either Cy-3 or Cy-5 for in situ hybridization. To minimize false-positive detection of nontargeted microbes having one mismatched sequence with the GNSB941 probe, a nonlabeled probe (GNSB941*; 5′-AAACCACAAGCTCCGCT-3′; *E. coli* positions 941 to 958) was used simultaneously with GNSB941 as a masking probe (the mixture of the GNSB941 and GNSB941* probes was made by using the same number of moles of each oligonucleotide).

In situ hybridization for thin sections of granules was performed as described previously (25). The hybridization stringency was adjusted by adding formamide to the hybridization buffer (10% for EUB338*, 35% for ARC915, and 20% for GNSB941). The cells hybridized with the probes were observed with a confocal laser scanning microscope (LSM 5 PASCAL; Zeiss). For each sludge sample and each probe set, sections from at least 10 granule pellets were examined with the confocal laser scanning microscope to determine the general spatial microbial structures for each sludge sample. Typical results and images, which may represent differences among the reactors rather than differences among the granules within a reactor, are shown below.

SEM and analytical methods. Scanning electron microscopy (SEM) was performed with an Hitachi S4500 scanning electron microscope as described previously (33). Short-chain fatty acid, methane, hydrogen, and carbon dioxide contents were determined by gas chromatography as described elsewhere (12, 13). Carbohydrate contents were determined by high-pressure liquid chromatography using an SCR-101-H column (28).

Nucleotide sequence accession numbers. All 16S rRNA gene sequences of clones and isolates have been deposited in the DDBJ/EMBL/GenBank databases under accession no. AB109405 to AB109439.

RESULTS

Diversity of *Chloroflexi* in mesophilic and thermophilic UASB sludge granules. Five different types of UASB reactors (three mesophilic processes and two thermophilic processes) were used to analyze the diversity of *Chloroflexi* populations. All of the reactors contained granular-type sludge with good settling properties (mean diameter, 1 to 2 mm) and exhibited good COD removal efficiencies (more than 90%) and good methane production around the sampling dates. To elucidate the genetic diversity of *Chloroflexi* populations in these processes, 16S rRNA gene-based clone library analysis was employed. To construct libraries for the sludge samples, we first performed PCR using a *Chloroflexi*-specific primer set. Only single bands corresponding to the expected size of amplified DNA (approximately 400 bp) were obtained from all samples (data not shown). For each sample, 10 clones were randomly selected and subjected to sequencing. Subsequent phylogenetic analysis indicated that all of the clones retrieved were affiliated with subphylum I of the phylum *Chloroflexi* (Fig. 1 and Table 2). No clonal sequence could be identified as a chimeric artifact by partial treeing analysis (10).

Two clone libraries for the thermophilic sludges (ATG and STG sludges) exhibited 12 phylotypes (ATG-1 to ATG-7 and STG-1 to STG-6 [ATG-2 = STG-1]) in a total of 20 clones (Table 2). The majority of these clones were affiliated with a group called the "thermophilic cluster" (28), which is represented by the cultured *Chloroflexi* subphylum I species *A. thermophila* that we isolated in a previous study (29) (Fig. 1) (12 of the 20 clones in the ATG and STG libraries).

The clones found in three libraries for mesophilic sludges exhibited 15 distinct phylotypes (AMG-1 to AMG-7, BMG-1 to BMG-5, and CMG-1 to CMG-7 [AMG-1 = CMG-4 and BMG-4 = CMG-5]) in the 30 clones analyzed. Clones from mesophilic sludges were widely distributed within *Chloroflexi* subphylum I, and, in general, they were distantly related to each other. These findings suggest that the majority of the *Chloroflexi* populations in the UASB sludges were members of *Chloroflexi* subphylum I and that the sludge granules all contained genetically diverse members of *Chloroflexi* subphylum I populations.

Localization of *Chloroflexi* subphylum I populations in UASB sludge granules. FISH analysis of thin sections of the two thermophilic granular sludge samples (ATG and STG sludges) using the phylum-level GNSB941 probe (*Chloroflexi*-specific probe) indicated that GNSB941 probe-positive cells were abundant in the outermost layer of both types of thermophilic granules (data not shown), as shown for ATG sludge in a previous study (25). FISH analysis of sections with the EUB338* and GNSB941 probes used simultaneously suggested that the GNSB941-positive filamentous cells accounted for the majority of the EUB338*-reactive cells found in the outermost layer of each granule (data not shown). All of the GNSB941-reactive cells had thin filamentous morphotypes. SEM of both types of thermophilic sludge granules showed that these thin filamentous cells entirely covered the surface of the granules (data not shown).

FISH analyses of thin sections of mesophilic sludge granules (AMG, BMG, and CMG sludges) were performed using *Bacteria*-, *Archaea*-, and *Chloroflexi*-specific probes (EUB338*, ARC915, and GNSB941, respectively) (Fig. 2). In situ hybridization with the EUB338* and ARC915 probes for the three mesophilic sludge samples revealed a mosaic structure consisting of bacterial and archaeal microcolonies (Fig. 2A to C). In particular, the AMG and CMG sludges had a layered structure of microorganisms in sludge granules, in which the outer layer was composed mainly of bacterial cells and the inner layer was made up of composites of bacterial and archaeal cells (Fig. 2A and C). We then performed FISH analyses of the mesophilic sludge samples using the GNSB941 and ARC915 probes. GNSB941-reactive cells were most abundant in the AMG sludge, in which probe-positive cells (all the cells had a filamentous morphotype) were present mainly in the outermost layer of the sludge granules (Fig. 2D). SEM observations of AMG sludge indicated that the sludge granules were entirely covered with thin filamentous cells that were morphologically

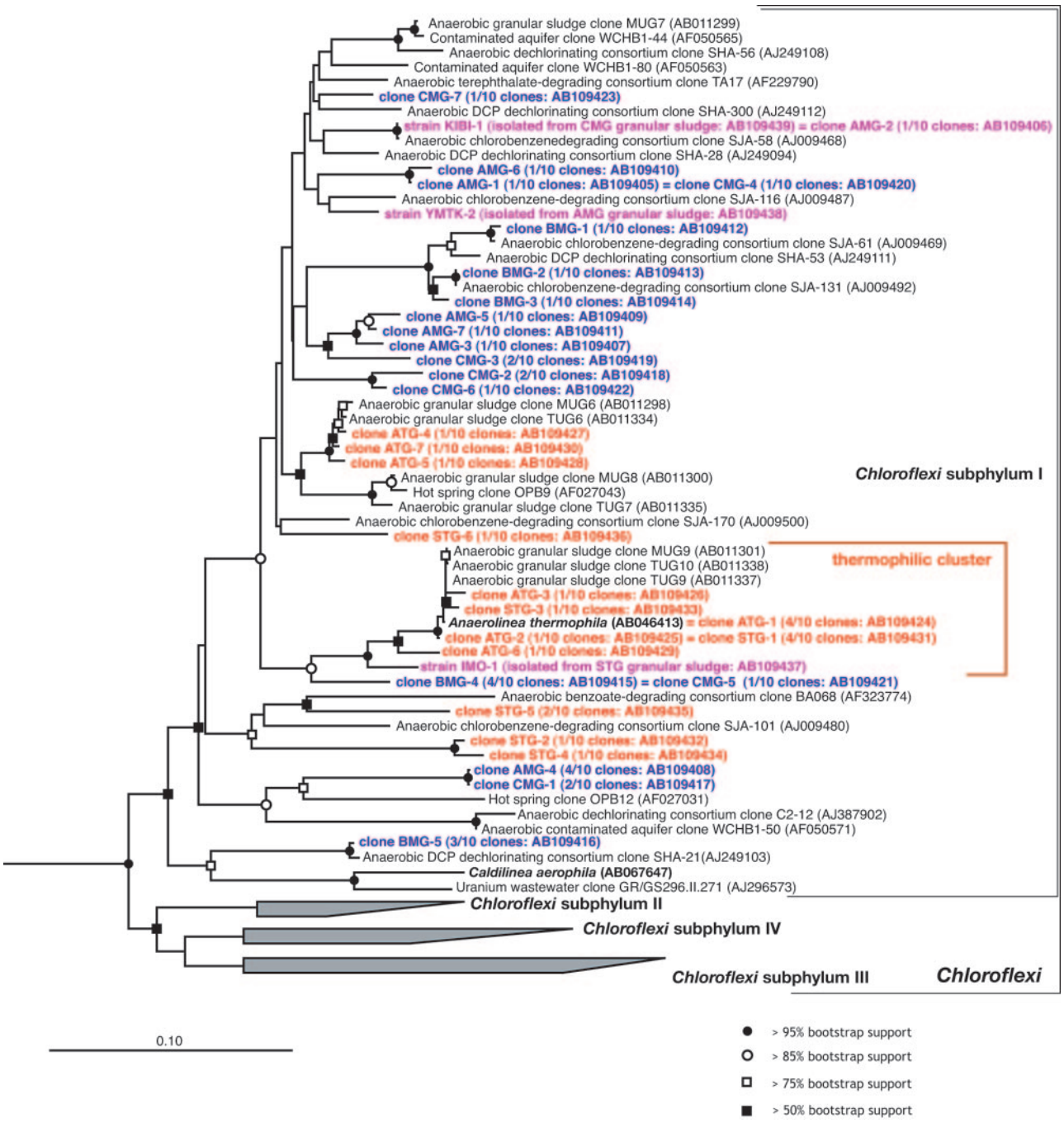

FIG. 1. Evolutionary dendrogram of clones and isolates obtained from mesophilic and thermophilic sludge granules in the bacterial phylum *Chloroflexi*, based on 16S rRNA gene sequences. The sequences were aligned, and the phylogenetic tree was constructed by the neighbor-joining method. Bar = 5 nucleotide substitutions per 100 nucleotides. 16S rRNA gene sequences of *Thermotogae* were used to root the tree. Bootstrap values greater than 50% are indicated at branch points. The accession number of each reference sequence and the origins of the environmental clones are indicated in parentheses. Our clones and isolates used in this study are indicated by boldface type; the clones retrieved from mesophilic sludges are indicated by blue, the clones retrieved from thermophilic sludges are indicated by red, and strains isolated in this study are indicated by purple.

similar to the cells found in the outermost layer of thermophilic granules (Fig. 3A and D) (the same SEM observation was made previously for sludge from a reactor with the same process as the AMG reactor [25]). In addition, FISH analysis of sections with the EUB338* and GNSB941 probes used simultaneously suggested that the GNSB941-positive filamentous cells accounted for the majority of the EUB338*-reactive cells (including the filamentous cells) found in the outermost layer of the AMG granules (data not shown).

Two other sludges (BMG and CMG sludges) also exhibited

TABLE 2. Clone libraries of *Chloroflexi* subphylum I-type 16S rRNA genes constructed from five different types of UASB sludge granules

Reactor	Reactor temp (°C)	Clone library (10 clones for each library)							
		No. of phylotypes	OTU[a]	Size (bp)	No. of clones	% Sequence similarity to *A. therinophila*	Cluster	Same sequence in other libraries and strains	Accession no.
AMG	37	7	AMG-1	407	1	85.5		CMG-4	AB109405
			AMG-2	407	1	85.5		Strain KIBI-1	AB109406
			AMG-3	408	1	86.0			AB109407
			AMG-4	403	4	82.6			AB109408
			AMG-5	407	1	85.3			AB109409
			AMG-6	407	1	84.5			AB109410
			AMG-7	407	1	86.0			AB109411
BMG	30–36	5	BMG-1	407	1	82.1			AB109412
			BMG-2	407	1	82.8			AB109413
			BMG-3	407	1	83.0			AB109414
			BMG-4	408	4	90.9		CMG-5	AB109415
			BMG-5	406	3	79.4			AB109416
CMG	35–40	7	CMG-1	404	2	82.8			AB109417
			CMG-2	407	2	87.2			AB109418
			CMG-3	407	2	88.0			AB109419
			CMG-4	407	1	85.5		AMG-1	AB109420
			CMG-5	408	1	90.9		BMG-4	AB109421
			CMG-6	407	1	86.7			AB109422
			CMG-7	407	1	86.0			AB109423
ATG	55	7	ATG-1	407	4	100.0	Thermophilic cluster	*A. thermophila*	AB109424
			ATG-2	407	1	99.8	Thermophilic cluster	STG-1	AB109425
			ATG-3	407	1	99.3	Thermophilic cluster		AB109426
			ATG-4	407	1	86.2			AB109427
			ATG-5	407	1	86.0			AB109428
			ATG-6	407	1	95.8	Thermophilic cluster		AB109429
			ATG-7	407	1	87.0			AB109430
STG	55	6	STG-1	407	4	99.8	Thermophilic cluster	ATG-2	AB109431
			STG-2	404	1	84.2			AB109432
			STG-3	407	1	99.5	Thermophilic cluster		AB109433
			STG-4	403	1	83.0			AB109434
			STG-5	408	2	82.2			AB109435
			STG-6	408	1	86.5			AB109436

[a] OTU, operational taxonomic unit.

probe-positive signals with the GNSB941 probe; all of the probe-reactive cells were again filamentous cells (this finding was confirmed by FISH observations of dispersed sludge cells [data not shown]). In the BMG sludge, GNSB941-positive filamentous cells were found mainly in the outer layer of the sludge granules (Fig. 2E). However, these populations seemed to be less abundant than those in AMG sludge. SEM observations for BMG sludge showed that the surface of the granules was not covered by thin filamentous cells; instead, *Methanosaeta*-like thick filamentous cells were found, which were long sheathed filaments with characteristic septum-like spacer plugs, as described previously for typical *Methanosaeta* cells (32) (Fig. 3B and E). In the case of the CMG sludge, GNSB941-positive filaments were widely distributed in the sludge granules. Here, the *Chloroflexi*-type populations seemed to be less significant than those of other sludges (Fig. 2F). SEM observations together with FISH observations of CMG sludge granules showed that the surface of the granules was not covered with *Chloroflexi*-type thin filamentous cells but was completely covered with unidentified bacterial filaments whose thickness was much greater than that of typical *Chloroflexi*-type cells found in sludge granules (Fig. 3C and F).

Enrichment and isolation of *Chloroflexi* subphylum I-type filaments from mesophilic and thermophilic UASB sludge granules. We then attempted to selectively cultivate GNSB941-reactive filaments from all of the sludge samples. The cultivation and isolation procedure was based on previous findings for a cultivated species of *Chloroflexi* subphylum I, *A. thermophila* (28, 29). First, we tried to find good inocula that contained a number of *Chloroflexi* cells as the major population. In the search for the inocula, various sludges retained in UASB reactors were examined by FISH using the *Chloroflexi*-specific GNSB941 probe. As a result, we found that certain types of tiny flocs (diameter, ~0.5 mm) in sludge samples contained very abundant GNSB941-positive filamentous cells (Fig. 4). These flocs, which were found to involve a high number of filamentous cells, were then subjected to weak sonication in phosphate-buffered saline in order to disperse them, were serially diluted in the medium mentioned above, and were cultivated anaerobically under the appropriate temperature conditions. In the cultivation attempts, hydrogenotrophic methanogens (*M. hungatei* for mesophilic conditions and *M. thermautotrophicus* for thermophilic conditions) were also

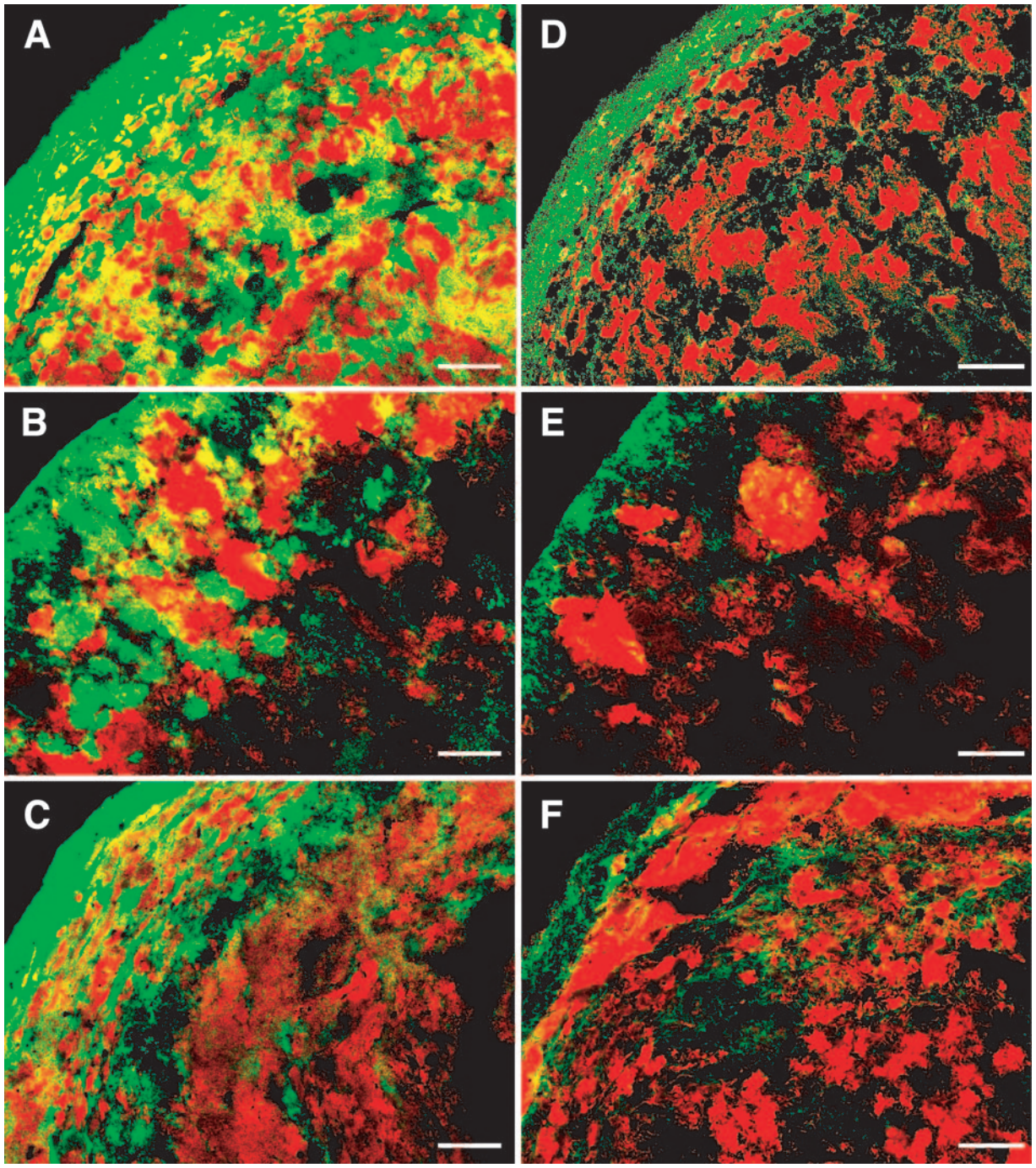

FIG. 2. Fluorescence in situ hybridization of sections from three mesophilic sludge granules, viewed using confocal laser scanning microscopy. (A and D) AMG sludge granule; (B and E) BMG sludge granule; (C and F) CMG sludge granule. The sections in panels A to C were hybridized with the Cy-3-labeled EUB338 probe, specific for the domain *Bacteria* (green), and the Cy-5-labeled ARC915 probe, specific for the domain *Archaea* (red); and the sections in panels D to F were hybridized with the Cy-3-labeled GNSB941 probe, specific for the bacterial phylum *Chloroflexi* (green), and the Cy-5-labeled ARC915 probe, specific for the domain *Archaea* (red). Bars = 50 μm.

added to each cultivation vial to enhance the hydrogen-scavenging reaction in the vials (28, 29).

First, we collected filamentous flocs from two thermophilic reactors (ATG and STG) and incubated them at 55°C in serially diluted cultures. In the cultures with lower dilutions, irrelevant microorganisms, such as short rods and cocci, grew within 1 to 2 days after inoculation. However, growth of thin filamentous microbes and F_{420}-autofluorescent rods resembling *M. thermautotrophicus* was observed with higher dilutions of cultures within 1 to 2 weeks after inoculation with an STG sample (data not shown). In situ hybridization of the filamentous cells grown using the GNSB941 probe resulted in detec-

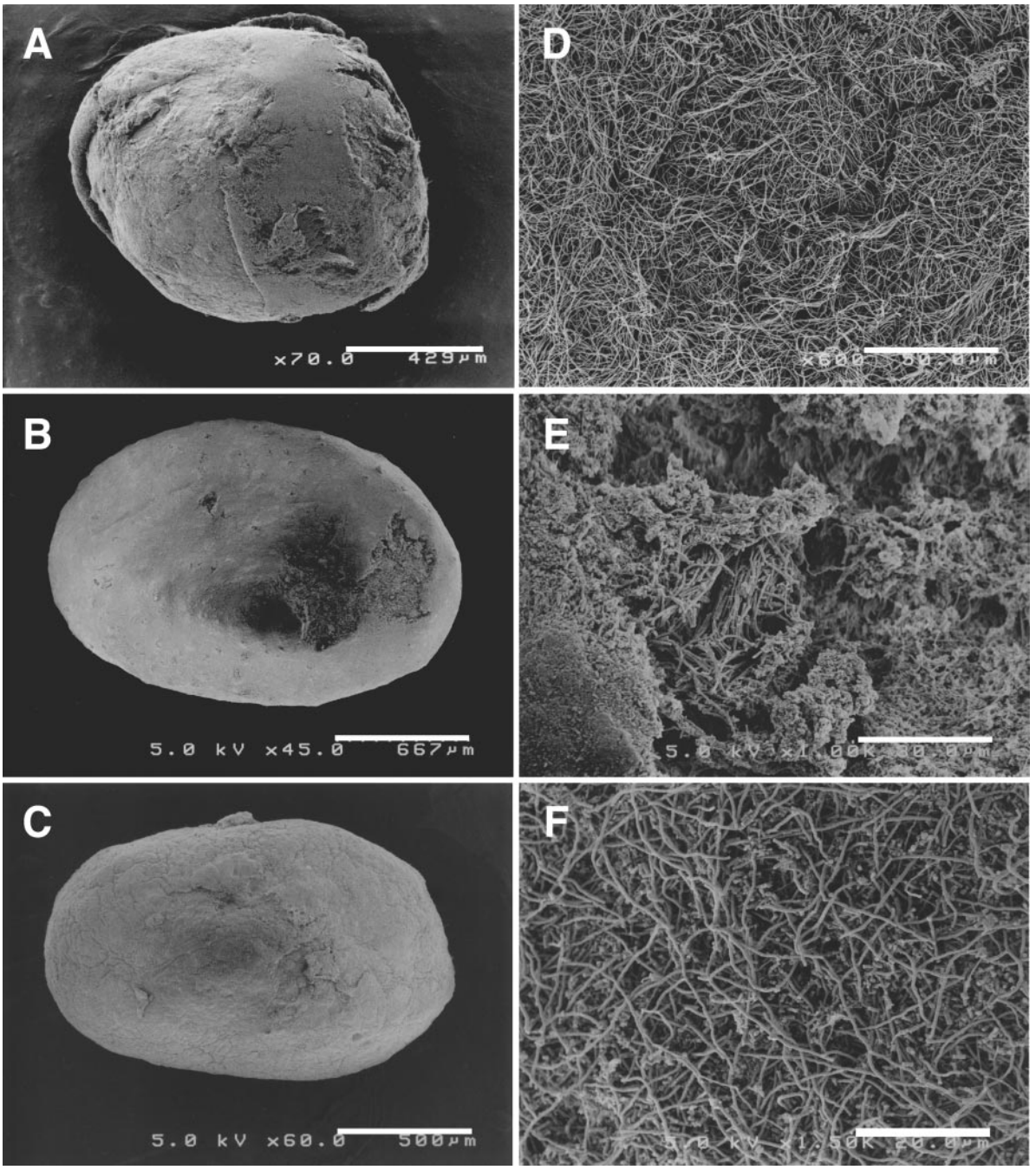

FIG. 3. Scanning electron micrographs of whole sludge granules (A to C) and the surfaces of the sludge granules (D to F). (A and D) AMG sludge granule; (B and E) BMG sludge granule; (C and F) CMG sludge granule.

tion of thin filamentous microbes (data not shown), indicating that the cells enriched at higher dilutions were affiliated with the phylum *Chloroflexi*. However, positive results were not obtained for cultures with ATG sludge in five cultivation attempts.

The strategy that was used for thermophilic sludges was also employed for mesophilic samples, except that *M. hungatei* was used as the partner methanogen instead of *M. thermautotrophicus* and all of the cultures were incubated at 37°C. As in the thermophilic cultivation, enrichments of GNSB941-reactive thin filamentous microbes and *M. hungatei*-like methanogens were established in cultures at higher dilutions from AMG and CMG granules. However, cultivation of *Chloroflexi* cells with BMG sludge was not successful in five cultivation attempts.

The three successful enrichments were purified further by successive transfers into fresh media and were then cultivated several times in sucrose yeast extract medium supplemented with 2-bromoethane sulfonate (final concentration, 5 mM) in order to eliminate partner methanogens. All cultures were subjected to roll tube isolation with the same medium (me-

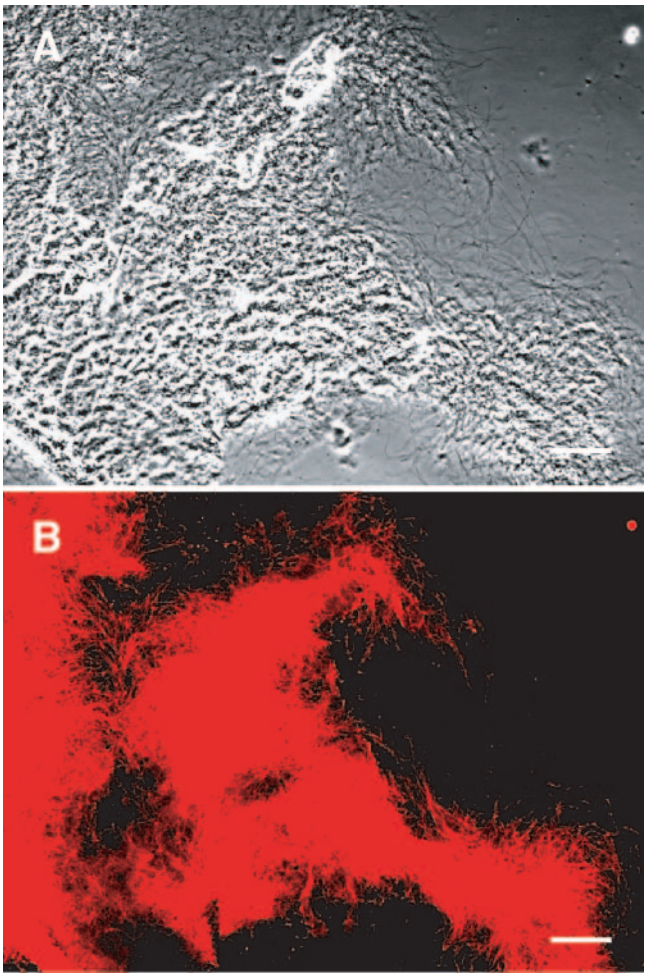

FIG. 4. Micrographs of a floc found in the AMG reactor. (A) Phase-contrast and (B) fluorescence micrographs of the same field, showing that all filamentous cells in the floc hybridized with the *Chloroflexi*-specific probe GNSB941. Bar = 50 μm.

dium containing sucrose, yeast extract, and 2-bromoethane sulfonate). In the case of the thermophilic enrichment (enrichment from the STG sludge), tiny colonies that were 0.1 to 0.2 mm in diameter were formed in the agar medium within 2 weeks after inoculation. Similar colonies were also formed in mesophilic enrichments (enrichment from the AMG and CMG sludges) within 1 month after inoculation. All three filamentous strains were eventually obtained in pure-culture form using a repeated roll tube isolation procedure. We designated these isolates strains IMO-1 (isolated from the STG sludge at 55°C), YMTK-2 (isolated from the AMG sludge at 37°C), and KIBI-1 (obtained from the CMG sludge at 37°C) (Fig. 5). Phylogenetic analysis of these isolates based on 16S rRNA gene sequences revealed that all of them belong to *Chloroflexi* subphylum I. At present, the organism that is most closely related to these strains and has been cultivated and characterized is *A. thermophila* (the levels of similarity of the 16S rRNA genes of strains KIBI-1, YMTK-2, and IMO-1 with the 16S rRNA genes of *A. thermophila* are 88%, 88%, and 93%, respectively). The levels of sequence similarity of the 16S rRNA genes between the isolates were found to be less than 89%; these organisms were distantly related to each other and occupied different phylogenetic positions in the subphylum (Fig. 1). In the clone library analyses mentioned above, a strain KIBI-1-type phylotype (AMG-2 clone) was retrieved from the AMG sludge, although the strain had a different original source (strain KIBI-1 was isolated from CMG sludge). Neither the same phylotype of strain IMO-1 nor the same phylotype of strain YMTK-2 was recovered from any clone library in this study.

Partial characterization of filamentous isolates belonging to the phylum *Chloroflexi*. All of the isolates were strictly anaerobic, thin and filamentous, slowly growing, heterotrophic microorganisms (Fig. 5). Strain IMO-1 was a thermophilic organism that grew well at temperatures around 55°C, but no growth was observed at temperatures above 60°C or below 40°C. Strains YMTK-2 and KIBI-1 were mesophilic anaerobes; good

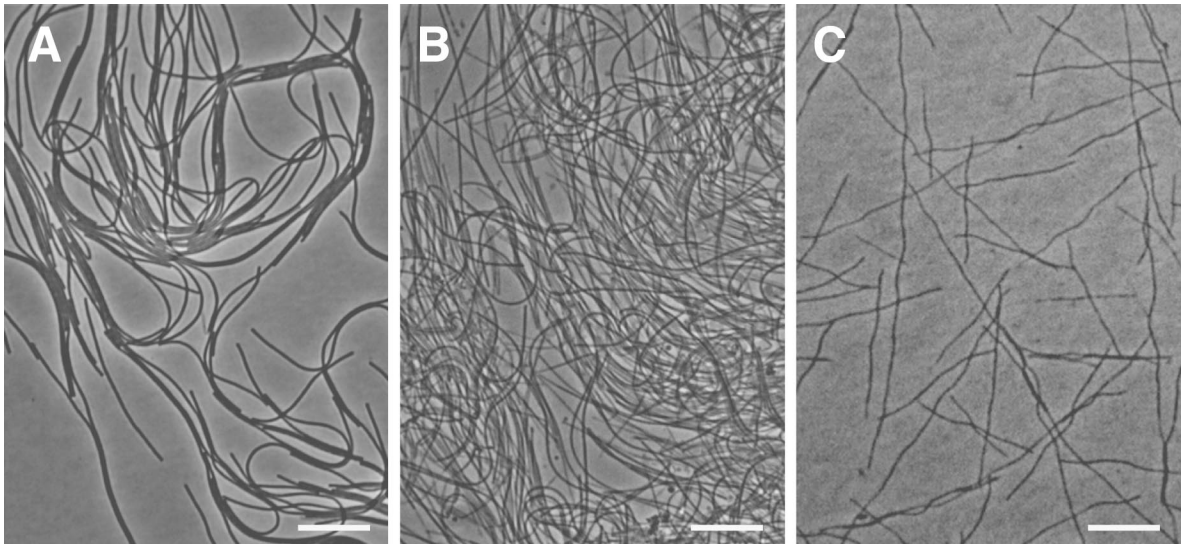

FIG. 5. Phase-contrast micrographs showing the cell morphology of mesophilic and thermophilic isolates affiliated with *Chloroflexi* subphylum I. (A) Strain IMO-1. Bar = 10 μm. (B) Strain KIBI-1. Bar = 10 μm. (C) Strain YMTK-2. Bar = 20 μm.

growth was observed at temperatures around 37°C, and there was no growth at temperatures above 50°C or below 25°C for both strains, indicating that these organisms are the first cultured mesophilic species of *Chloroflexi* subphylum I. They required yeast extract for growth. Strains KIBI-1 and IMO-1 could utilize carbohydrates such as glucose, fructose, and sucrose (20 mM) in the presence of yeast extract (doubling times, 56 and 48 h). However, no growth occurred in the absence of yeast extract. Strain YMTK-2 grew in yeast extract medium (doubling time, 50 h). Strain YMTK-2 did not grow in medium with glucose, fructose, and sucrose (20 mM) without yeast extract. These strains produced mainly hydrogen, acetate, and carbon dioxide from these substrates. The growth of strain IMO-1 was found to stagnate after a certain amount of hydrogen had accumulated in medium supplemented with sucrose and yeast extract. Cocultivation with *M. thermautotrophicus* significantly stimulated the growth of strain IMO-1; the growth rate of the coculture was five times higher than that of the pure culture (doubling time in coculture, 10 h). The cultivated *Chloroflexi* subphylum I species *A. thermophila* had a similar response to cocultivation with *M. thermautotrophicus*. However, the growth rates of the two mesophilic strains did not change significantly in cocultivations with *M. hungatei* (data not shown).

DISCUSSION

***Chloroflexi* populations in thermophilic sludge granules.** In this study, we reconfirmed that the thin filamentous cells on the surface of thermophilic granules are members of the *Chloroflexi*, in particular *Chloroflexi* subphylum I. Because thermophilic sludge granules that settle well are always found to be entirely covered with these types of filamentous cells, it has been suggested that the filaments are one of the essential constituents for granulation of thermophilic sludge (25, 31, 33). In a previous study, these thin filamentous cells were found to belong to subphylum I of the bacterial phylum *Chloroflexi* using the full-cycle rRNA approach (25). In this study, we used probe GNSB941, which is a *Chloroflexi*-specific probe at the phylum level, to visualize these cells in sludge granules, and we found that *Chloroflexi*-type filamentous cells were abundant on the surfaces of two different types of thermophilic sludge granules, one of which was used to treat actual distillery wastewater. These observations supported the hypothesis that *Chloroflexi*-type filamentous populations (particularly *Chloroflexi* subphylum I populations) ubiquitously reside in thermophilic sludge granules. These findings also further supported the importance of *Chloroflexi* subphylum I-type filamentous cells in thermophilic UASB processes in terms of granular structure preservation and the degradation of organic compounds in waste streams under thermophilic conditions.

***Chloroflexi* populations in mesophilic sludge granules.** More importantly, this study showed that *Chloroflexi* populations found in mesophilic sludge granules have some similarity to populations found in thermophilic sludge granules, because (i) all of the mesophilic and thermophilic sludges contained significant genetically diverse *Chloroflexi*-type populations; (ii) all of the *Chloroflexi* 16S rRNA gene clones detected were affiliated with *Chloroflexi* subphylum I in both types of sludge; (iii) all cells positive with a *Chloroflexi*-specific probe had filamentous morphotypes; and (iv) in some mesophilic sludges *Chloroflexi*-type filamentous populations completely covered the surfaces of the granules, as they did with thermophilic granules. A number of *Chloroflexi* subphylum I-type genes were previously detected by 16S rRNA gene clone-based microbial population analysis of mesophilic granular sludges in UASB processes, especially a process involved in the treatment of artificial wastewater mainly comprised of sucrose, acetate, propionate, and yeast extract (27). *Chloroflexi* subphylum I-type populations were also detected in mesophilic UASB reactors used to treat terephthalate-containing (37) or 4-methylebenzoate-containing (36) wastewaters by 16S rRNA gene-based clone library analysis. Furthermore, similar findings were also obtained by 16S rRNA gene retrieval from anaerobic sludges in fluidized bed reactors treating ethanol-plus-sulfate-rich wastewater (15), lactate-plus-sulfate-rich wastewater (15), trichlorobenzene-containing wastewater (34), or wine distillery wastewater (7). These previous reports, along with our findings, strongly suggest that *Chloroflexi* subphylum I-type microbes reside in various types of mesophilically grown anaerobic sludges as one of the fundamental constituents.

In our previous study, certain types of mesophilic granules were found to be covered with thin filamentous cells, as has been found for thermophilic granules (25); the thin filaments were also clearly shown to be affiliated with *Chloroflexi* in this study. However, it was frequently observed previously that mesophilic sludge granules that settled well were formed without thin filamentous cells on the surface of the granules (8, 20). In fact, for some of the mesophilic sludges examined in this study *Chloroflexi* cells were less abundant within the sludge granules. These findings may suggest that *Chloroflexi*-type thin filamentous cells are not essential constituents of mesophilic granules; i.e., the role of *Chloroflexi* subphylum I cells in mesophilic sludge granules may not be as significant as the role of these cells in thermophilic sludge granules. In addition, the occurrence of sludge bulking in mesophilic UASB reactors caused by *Chloroflexi* subphylum I filaments is unknown, although mesophilic sludge bulking in a contact process due to the outgrowth of certain thin filamentous cells has been reported (4).

Morphology, physiological properties, and functions of *Chloroflexi* subphylum I cells. Based on the spatial distribution within sludge granules observed in this study, as well as in a previous study (25), we suggest that the majority of the *Chloroflexi* cells metabolize primary substrates in wastewater, such as carbohydrates, since these cells were observed mainly on the surface of the sludge granules, as in the case of *A. thermophila* (28, 29). In addition, some of these microbes may be heterotrophs decomposing organic matter from cells, such as amino acids, because some of the cells were widely distributed inside the sludge granules, as shown in Fig. 2D and F. These predicted phenotypic features were in good agreement with the actual physiological properties of *Chloroflexi* subphylum I strains examined in this study; i.e., all strains isolated in this study were heterotrophs that decompose either carbohydrates or cellular matter (yeast extract). Furthermore, all the *Chloroflexi* subphylum I isolates have some common morphological and physiological traits of the genus *Anaerolinea*, such as filamentous morphology, heterotrophic and anaerobic growth, low growth rates, and a requirement for yeast extract for

growth (28, 29), although they are genetically distantly related to each other, as inferred from the 16S rRNA genes. These morphological and physiological traits may suggest some aspects of the common phenotypic features of *Chloroflexi* subphylum I cells that can be found in sludge granules.

Chloroflexi subphylum I-type cells are abundant not only in anaerobic sludges but also in aerobic sludges. For example, recent molecular studies have revealed remarkable abundance and distribution of microbes belonging to *Chloroflexi* subphylum I in activated sludge systems, and whole-cell in situ hybridization analyses for these microbes suggested that members of subphylum I all have a filamentous morphotype and that there is a wide range of thicknesses (2, 14, 16). These observations are consistent with our finding that all of the GNSB941-positive cells in the sludge samples, as well as the three newly isolated strains, are filamentous, further suggesting a common morphological trait of *Chloroflexi* subphylum I. Filamentous morphotypes are known to be important for making granulation successful (as shown in the case of the genus *Methanosaeta*) (8, 20, 25) and seem to be superior to other morphotypes for retaining their own cells within granule-type structures, thus possibly making the organisms some of the fundamental constituents of sludge granules.

Conclusions. In summary, we found that organisms affiliated with the bacterial taxon *Chloroflexi* subphylum I are ubiquitous microbial populations in mesophilic and thermophilic sludge granules. In particular, we were able to visualize *Chloroflexi* cells (possibly *Chloroflexi* subphylum I cells) in mesophilic sludge granules, which resulted in detection of filamentous cells on the surface of or inside sludge granules. Through isolation of *Chloroflexi* subphylum I microbes, important aspects of *Chloroflexi* subphylum I cells, such as their general morphology and physiological traits, were elucidated. Importantly, we suggest that the in situ physiological functions of *Chloroflexi* subphylum I cells in anaerobic sludge are carbohydrate degradation and cellular matter degradation. Future elucidation of the physiological properties of these isolates in detail may contribute to a deeper understanding of *Chloroflexi* subphylum I organisms in various ecosystems.

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