The Journal of Microbiology (2010) Vol. 48, No. 3, pp. 275-283 DOI 10.1007/s12275-010-9366-8
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Isolation, Characterization, and Abundance of Filamentous Members of *Caldilineae* in Activated Sludge

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(Received November 26, 2009 / Accepted January 19, 2010)

***Chloroflexi* are currently believed to serve as backbone forming agents in the activated sludge of wastewater treatment plants (WWTPs). In this study, we isolated and characterized filamentous bacteria in the class *Caldilineae* of the phylum *Chloroflexi* in municipal WWTPs. Diversity analysis using *Chloroflexi*-specific 16S rRNA gene clone libraries showed that 97% of the clones belonged to the subdivision *Anaerolineae* comprising the two classes *Anaerolineae* (95%) and *Caldilineae* (2%). Clones of *Caldilineae* were related to a thermophilic filament *Caldilinea aerophila* with 93% 16S rRNA gene sequence similarity. We obtained filamentous isolates classified into the class *Caldilineae* showing the best match to *C. aerophila* with 89% 16S rRNA gene sequence similarity. Isolates showed no ability to assimilate glucose or N-acetylglucosamine or to degrade biopolymers which were observed in filamentous *Chloroflexi* of WWTPs. The assessment of relative abundance based on quantitative PCR of the 16S rRNA gene indicated that members of the class *Caldilineae* comprised 12-19% of the *Chloroflexi* in the activated sludge. Additionally, fluorescence *in situ* hybridization experiments showed that diverse filamentous *Caldilineae* inhabit the activated sludge of municipal WWTPs. These findings yield insight into the role of filamentous mesophilic *Caldilinea* in stabilizing flocs of activated sludge in a wide range of WWTPs.**

***Keywords*:** filaments, activated sludge, *Chloroflexi*, *Caldilineae*, fluorescence *in situ* hybridization, quantitative PCR

The phylum *Chloroflexi* was proposed by Garrity and Holt (2001) to succeed the previous 'green non-sulfur bacteria' (Woese, 1987) which comprised four well-represented classes, *Anaerolineae*, *Dehalococcoidetes*, *Chloroflexi*, and *Thermomicrobia* (Hugenholtz and Stackebrandt, 2004). Despite efforts to isolate microorganisms from various environments, only a few cultured representatives are reported in the phylum *Chloroflexi*. Phototrophic thermophilic filamentous bacteria are included in the class *Chloroflexi*: *Chloroflexus* (Pierson and Castenholz, 1974), *Oscillochloris* (Gorlenko and Pivovarova, 1977), and *Roseiflexus* (Hanada *et al.*, 2002). In the class *Dehalococcoidetes*, anaerobic dehalogenating bacteria have been isolated from aquifers contaminated with chlorinated pollutants (Grostern and Edwards, 2006). Thermophilic filamentous microorganisms comprise the class *Thermomicrobia* (Oyaizu *et al.*, 1987; Hensel *et al.*, 1989). Only recently, thermophilic filamentous organisms in the subdivision of *Anaerolineae* containing the two classes of *Anaerolineae* and *Caldilineae* were isolated from anaerobic thermophilic granular sludge and a hot spring (Sekiguchi *et al.*, 2003; Yamada *et al.*, 2006).

Recent molecular investigations from various environments greatly extended the diversity of *Chloroflexi*. Novel lineages of *Chloroflexi* were revealed from alpine tundra wet meadow soil environments (Costello and Schmidt, 2006). Diverse 16S rRNA gene clones of *Chloroflexi* were found in caves with relatively limited nutrients and organic matter, stable low temperatures, high humidity, and high mineral concentrations which provide selective ecological niches for highly specialized *Chloroflexi* (Zhou *et al.*, 2007). Clones related to *Chloroflexi* were reported to be one of the major bacteria in deep subsurface sediments of the ocean floor (Huber *et al.*, 2006). Using analysis of 16S rRNA gene clone libraries and studies of fluorescence *in situ* hybridization (FISH), filamentous *Chloroflexi* have been shown to be cosmopolitan members of various wastewater treatment processes as constituents of the backbone of activated sludge (Bossier and Verstraete, 1996; Seviour and Blackall, 1999; Björnsson *et al.*, 2002).

Filamentous *Chloroflexi* are important factors in the operation of wastewater treatment plants (WWTPs) since they are the predominant floc-stabilizing bacteria of activated sludge (Björnsson *et al.*, 2002; Kohno *et al*., 2002; Sekiguchi *et al.*, 2003; Levantesi *et al.*, 2006; Kragelund *et al.*, 2007; Miura *et al.*, 2007; Speirs *et al.*, 2009). Clones affiliated with the subdivision *Anaerolineae* (esp. class *Anaerolineae*) are the most abundant in the *Chloroflexi*-specific 16S rRNA gene libraries of activated sludge (Hugenholtz *et al.*, 1998; Sekiguchi *et al.*, 1999; Björnsson *et al.*, 2002; Juretschko *et al.*, 2002). Members of the class *Caldilineae* are known to comprise a major fraction of the *Chloroflexi* community and to prevent membrane fouling in WWTPs (Miura *et al.*, 2007). This indicates that the filamentous bacterial community of the two classes *Anaerolineae* and *Caldilineae* could be dynamically changeable depending on

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the operational conditions of WWTPs. Despite dominance in WWTPs, aerobic mesophilic filamentous bacteria of the two classes *Anaerolineae* and *Caldilineae* have not yet been isolated from any activated sludge from WWTPs and their physiology is largely unknown.

Here we studied the diversity of the two classes *Anaerolineae* and *Caldilineae*, focusing on lesser known members of the class *Caldilineae* in the activated sludge of municipal WWTPs. Subsequently, we isolated mesophilic filamentous strains of the class *Caldilineae* from activated sludge and investigated their physiological and ecological characteristics. These results represent the first abundance, physiological and ecological properties of the class *Caldilineae* predicted by a clone library, FISH, quantitative PCR (qPCR) and isolation-based studies.

Materials and Methods

Sampling and description of WWTPs

Activated sludge mixed liquor samples were collected from municipal wastewater treatment plants of Cheongju (CJ), Daejeon (DJ), Cheonan (CA), and Anyang (AY), Korea, which treat 150,000 m^3/day, 900,000 m^3/day, 24,000 m^3/day, and 300,000 m^3/day of municipal wastewater, respectively, using anaerobic-anoxic-aerobic (biological nutrients removal) processes. Sludge volume index was in the range of 80-150 ml/g. Influent BOD concentrations were in the range of 200 to 300 mg/L with BOD removal efficiencies of about 95-99%.

Sludge DNA extraction and clone library construction

DNA was extracted from each of the activated sludge samples using a Power Soil™ DNA Extraction kit (Mo Bio Laboratories, USA) according to the manufacturer's instructions. DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, USA). The 16S rRNA gene sequences of the phylum *Chloroflexi* were amplified from activated sludge flocs. PCR was performed using *EF*-Taq DNA polymerase (Solgent, Korea), the universal 16S rRNA gene forward primer (27F) (Park *et al.*, 2006) and a *Chloroflexi*-specific 16S rRNA gene reverse primer (GNSB941 or CFX1223) (Björnsson *et al.*, 2002). Conditions of the PCR were: 5 min at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 55°C, 45 sec at 72°C; 7 min at 72°C. PCR products were purified using the Gel & PCR Purification System kit (Solgent), ligated using the T&A Cloning Vector kit (Real Biotech Corporation, Taiwan) and then transformed into *Escherichia coli* DH5α cells according to the manufacturer's instructions. Putative positive clones were transferred to a 96-well plate that contained Luria broth supplemented with ampicillin (100 μg/ml). Clones were grown overnight at 37°C and stored at -70°C before screening.

Screening of 16S rRNA gene clones

Inserts of rRNA genes from recombinant clones were reamplified with vector-specific primer sets: M13F and M13R. Each amplicon was subjected to restriction fragment length polymorphism (RFLP) by enzymatic digestions with *Mnl*I (New England Biolabs, USA) endonuclease following the manufacturer's instructions. The digested fragments were electrophoresed in 3% agarose gels. After being stained with ethidium bromide, gels were photographed using an image-capture system and scanning image analyses were performed manually.

Reference strains and cultivation

The selected reference strains for the validation of FISH probes (listed in the Table 1), *Herpetosiphon geysericola* DSM 7119^T, *Herpetosiphon aurantiacus* DSM 6206^T, *Clostridium sporogenes* DSM633^T, and *Pseudomonas stutzeri* DSM4166^T were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Braunschweig, Germany; http://www.dsmz.de).

Isolation of filamentous bacteria from activated sludge

Before inoculation, flocs of activated sludge were broken using a homogenizer for 10 min (Rao and Bhat, 1971). A minimal medium, ET (from actual wastewater), was used for isolation of filamentous microorganisms. ET contained 0.001% yeast extract (Difco, USA), 0.1 mM ammonia, 0.1 mM phosphate, 1 mM bicarbonate, 0.1× trace elements and a vitamin solution in the filtered effluent of the WWTPs (pH 7.5) (Widdel and Bak, 1992). For solidification, 1.5% Noble agar (Difco) was added before autoclaving. Broken sludge inoculated on each plate was incubated under dark, aerobic conditions at 25°C. After 4 weeks incubation, filamentous colonies were successively transferred to new solid ET media under a light microscope with 40× magnification.

Morphological and phenotypic characterization of filamentous isolates

To observe the filament morphology of isolates using transmission electron microscopy (TEM), samples were fixed in 2.5% paraformaldehyde-glutaraldehyde mixture in 0.1 M phosphate buffer (pH

Table 1. FISH probes and quantitative PCR primer sets used in this study

Application	Probe/Primer set	Coverage (Targeted group)	Sequence (5′→3′)	FA(%) or AT(°C)[d]	Reference or source
FISH	GNSB941[a]	*Chloroflexi*	AAACCACACGCTCCGCT	35	Gich *et al.* (2001)
	CFX1223[b]	*Chloroflexi*	CCATTGTAGGGTGTGTGTMG	35	Björnsson *et al.* (2002)
	CFX1A331[c]	*Caldilineae*	CCCCGTAGGAGTCGGGAC	30	In this study
qPCR	27F	*Caldilineae*	AGAGTTTGATCMTGGCTCAG	50	Park *et al.* (2006)
	CFX1A331R		CCCCGTAGGAGTCGGGAC	55	In this study
	518F	*Bacteria*	CCA GCA GCC GCG GTA AT	57	Muyzer *et al.* (1993)
	786R		GATTAGATACCCTGGTAG	55	Baker *et al.* (2003)
	1055YF	*Chloroflexi*	ATGGYTGTCGTCAGCT	51	Ritalahti *et al.* (2006)
	CFX1223R		CCATTGTAGGGTGTGTGTMG	54	Björnsson *et al.* (2002)

[a] Positive control, *H. geysericola* DSM 7119^T; negative control, *C. sporogenes* DSM633^T (No. of mismatch 1)
[b] Positive control, *H. aurantiacus* DSM 6206^T; negative control, *E. coli* DH5α (No. of mismatches 2)
[c] Positive control, Isolate ET1; negative control, *H. geysericola* DSM 7119^T (No. of mismatches 2), and *P. stutzeri* DSM 4166^T (No. of mismatches 2)
[d] FA, Formamide; AT, Annealing Temperature

7.2) for 2 h, postfixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in graded ethanol and propylene oxide and embedded in Epon-812. Ultra-thin sections generated with UltracutE ultramicrotome (Leica, Austria) were stained with uranyl acetate and lead citrate and examined under a CM 20 (Philips, Netherlands) electron microscope. Morphological description was based on the category of the Eikelboom and van Buijsen (1983). Gram- and Neisser-staining procedures were done as described by Magee *et al*. (1975).

To examine substrate utilization of the isolates, they were cultivated in the solid ET media (without 0.001% yeast extract) supplemented individually with 16 autoclaved or filter-sterilized compounds (see Table 2). Filament growth was observed under a light microscope (40× magnification). For determination of phototrophy, we incubated the inoculated plates under light (150 μE/m^2/s) at 25°C. For determination of anaerobic growth, the inoculated plates were incubated in the anaerobic jar (BBL, USA) with anaerobic atmosphere using the anaerobic envelope (GasPak; BBL).

Phylogenetic analysis of 16S rRNA gene sequences of filamenttous isolates

Genomic DNAs were extracted from filaments of isolates on the solid ET media and used as templates for PCR amplification and sequencing of the 16S rRNA gene described previously (Park *et al.*, 2006). PCR amplification of 16S rRNA gene from the genomic DNA of colonies was failed. We amplified the genome from a colony of the isolate using REPLI-g® Mini kit (QIAGEN, USA) reagents and following the manufacturer's protocol. The amplified genome (10 ng) was successfully used for amplification of 16S rRNA gene using 27F and 1492R (Park *et al.*, 2006). All sequences of rRNA gene clones, as well as sequences from pure cultures, were determined directly using Bigdye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems) and an ABI PRISM 3730X1 DNA Analyzer (PE Applied Biosystems). Sequences were checked for possible chimeras using the CHIMERA_CHECK program at the Ribosomal Database Project website (http://rdp8.cme.msu.edu). 16S rRNA gene sequences closely related to those of filamentous microcolonies were retrieved from the GenBank database and were aligned using the CLUSTAL X program (Thompson *et al.*, 1997). Phylogenetic trees were constructed based on neighbor joining (Saitou and Nei, 1987) and maximum parsimony method algorithms using the MEGA 3 Program (Kumar *et al.*, 2004) and PHYLIP package (Felsenstein, 1993), respectively, with bootstrap values based on 1,000 replications (Felsenstein, 1985).

Table 2. Phenotypic characteristics of the isolate ET1 and representative isolates of the two classes *Caldilineae* and *Anaerolineae* (Sekiguchi *et al.*, 2003)

Characteristics	Isolate ET1	*C. aerophila* STL-6-01	*A. thermophila* IMO-1
Cell diameter (μm)	0.5-1.0	0.7-0.8	0.2-0.3
Temperature for growth (°C)			
Range	5-30	25-50	50-60
Optimum	25	55	55
pH for growth			
Range	6.0-8.5	7.0-9.0	6.0-8.0
Optimum	7.5	7.5-8.0	7.0
O_2 respiration	+	+	-
Phototrophic growth	-	-	-
Anaerobic growth	-	-	+
Utilization			
Yeast extract	+	+	+
Casamino acids	+	-	+/-
Tryptone	-	+	+/-
Pyruvate	+	+	+/-
Lactate	+	+	-
Acetate	+	+	-
Succinate	ND	+	-
Galactose	+	+	+
Arabinose	-	-	+/-
N-acetylglucosamine	-	ND	ND
Dextrose	-	ND	ND
Biopolymer degradation			
Starch	-	+	+
Skim milk	-	ND	ND

-, Negative; +, positive; ND, not determined.
Utilization of various substrates (added at final concentrations of 10 mM unless specified) was tested in the minimal medium, ET (from actual wastewater, See 'Materials and Methods'). Yeast extract and casamino acid were added as 0.1%. The following substrates were not utilized: mannose, fructose, arabinose, xylose, ribose, ethanol, formate (5 mM), malate, alanine or serine.

Quantification of 16S rRNA gene copy numbers using real-time PCR

All quantitative real-time PCR experiments using genomic DNAs from activated sludge and isolates were carried out using a MiniOpticon real-time PCR detection system (Bio-Rad Laboratories, USA) and built-in Opticon Monitor Software version 3.1 (Bio-Rad Laboratories). General thermal cycling parameters for real-time PCR were used: 15 min at 95°C; 40 cycles of 10 sec at 95°C, 20 sec at 55°C, and 20 sec at 72°C. Readings were taken between each cycle. The specificity for real-time PCR reactions was tested by analyzing melting curves, checking the size of reaction products using gel electrophoresis and sequencing reaction products. Copy numbers were calculated using an external standard curve that describes the relationship between a known copy number of genes and the cycle threshold (Ct) value, as previously described (Ritalahti *et al.*, 2006; Park *et al.*, 2008). Information about PCR amplification primers for 16S rRNA genes is listed in Table 1.

FISH analysis

The phylum *Chloroflexi*-specific oligonucleotide probes (Gich *et al.*, 2001; Björnsson *et al.*, 2002) were used in this study. One probe specific to the *Caldilinea* group, CFX1A331, was designed using an ARB software package (Ludwig *et al.*, 2004). Selected parameters of the probes used in this study are detailed in Table 1. Detailed information about most of these probes is given in probeBase (Loy *et al.*, 2003). Probes were commercially synthesized and 5' labeled either with the 6-Carboxy Fluorescein-aminohexyl amidite (FAM) or with the sulfoindocyanine dye (Cy3) (Thermohybaid Interactiva, Germany). Optimal stringencies of the probes were determined empirically using a previously reported method (Crocetti *et al.*, 2000) and evaluated using the reference strains and the isolate ET1 as described in the Table 1. FISH was performed on paraformaldehyde-fixed samples as described previously (Amann *et al.*, 1995; Xia *et al.*, 2008). Intact flocs of activated sludge were fixed for *in situ* localization of filaments. Following FISH, samples were observed by a fluorescence microscope (Eclipse 80i, Nikon, Japan) equipped with a digital camera (SD-5Mc, Nicon) using oil immersion objective.

For FISH of activated sludge flocs, paraformaldehyde-fixed samples

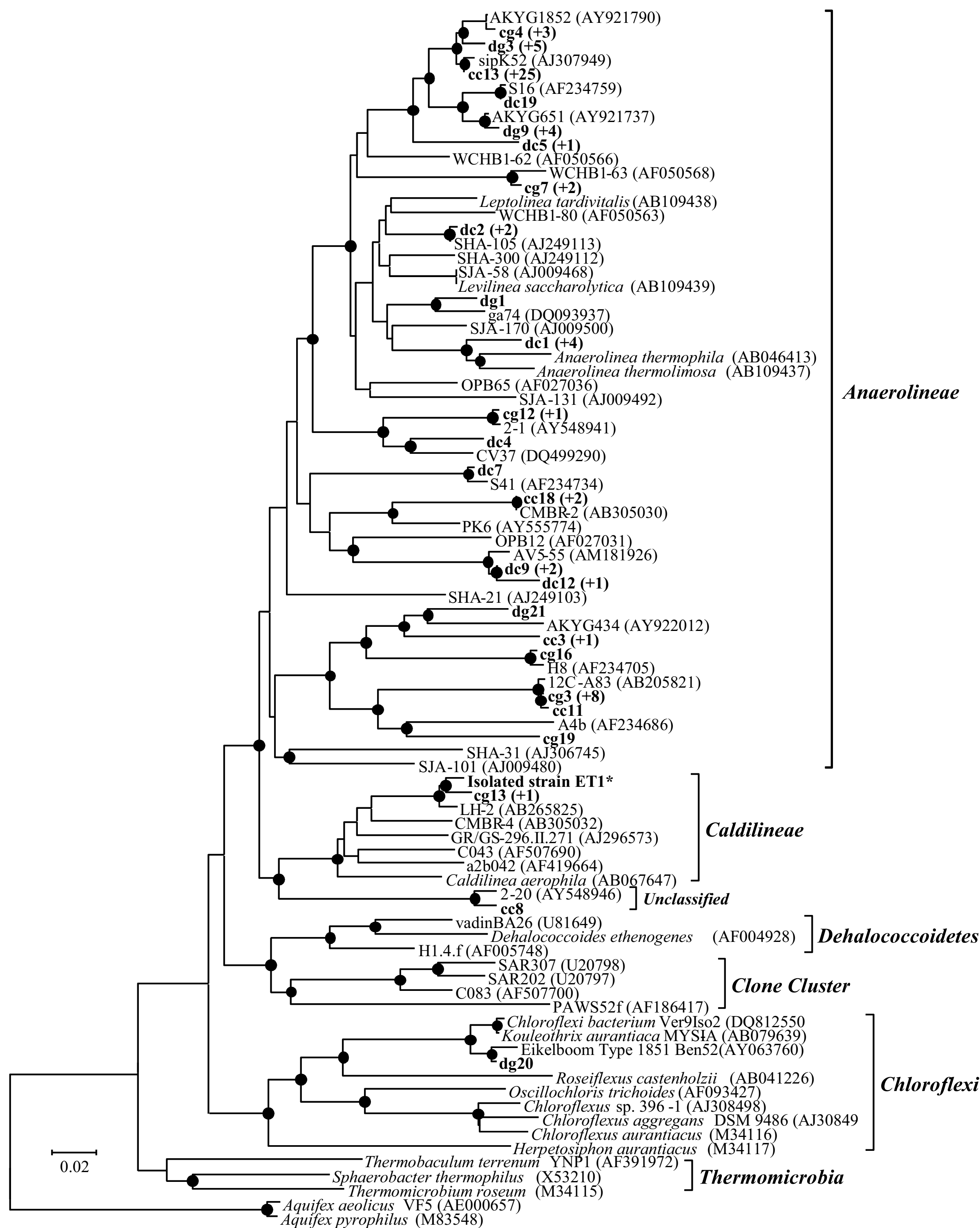

Fig. 1. Phylogenetic tree of the phylum *Chloroflexi* based on 16S rRNA gene sequences from environmental clones and isolates. 16S rRNA gene sequence of the *A. aeolicus* (AE000657) and *A. pyrophilus* (M83548) was used as an outgroup. Clones of 16S rRNA gene sequences obtained in this study are indicated with boldface type. The first letter 'c' or 'd' in the clone name indicates clones from the WWTPs of Cheongju and Daejeon, respectively. The second letter 'g' or 'c' in the clone name indicates clones made using reverse primer GNSB941 and CFX1223, respectively. The number in the parentheses following the clone name indicates the total number of redundant clones with a cutoff value of 97% 16S rRNA gene similarity. The accession number of each reference sequence is indicated in parentheses. The tree was generated using the neighbor joining and maximum parsimony method. Numbers at the nodes are bootstrap values (1,000 replicates) with more than 50% bootstrap support. Bar indicates 2 nucleotide substitutions per 100 nucleotides.

were hybridized with *Chloroflexi* phylum-targeting (CFXMIX) and *Caldilinea* group-targeting (CFX1A331) probes. CFXMIX was composed of equal amounts of the phylum *Chloroflexi* probes GNSB941 and CFX1223 labeled with the same fluorochrome (Gich *et al.*, 2001). For quantification of filament of the phylum *Chlorflexi* and class *Caldilineae*, the length of filaments from five different FISH images from the each sludge was determined and averaged.

Nucleotide sequences accession numbers

All partial 16S rRNA gene sequences determined in this study were deposited in the GenBank database under the accession numbers EU875524-EU875587.

Results and Discussion

Diversity analysis of *Chloroflexi*

Diversity of the phylum *Chloroflexi* in the activated sludge of two metropolitan areas municipal WWTPs was analyzed using *Chloroflexi*-specific 16S rRNA gene clone libraries. To increase the coverage of *Chloroflexi* diversities, two clone libraries were constructed using a universal bacterial 16S rRNA gene forward primer (27F) (Park *et al.*, 2006) and a *Chloroflexi*-specific reverse primer (GNSB941 or CFX1223) (Björnsson *et al.*, 2002). A total of 87 recombinant clones were randomly selected and their rRNA gene inserts were subjected to RFLP analysis resulting in 63 different RFLP types. As shown in Fig. 1, all of the clones were related to *Chloroflexi* and were divided into the classes of *Anaerolineae* (95%), *Caldilineae* (2%), *Chloroflexi* (1%), and unclassified (1%). These results correspond with the report of Björnsson *et al*. (2002) that most of the phylum *Chloroflexi* clones of activated sludge mixed liquor are related to group I (the subdivision *Anaerolineae*). In our libraries, the major clones (about 31%) belonging to the class *Anaerolineae* (a representative clone, cc13) were related to those (clone sipK52 in Fig. 1) detected at a cold sulfidic spring which were associated with novel *Euryarchaeota* (Rudolph *et al.*, 2001). Two clones of the libraries were related to the class *Caldilineae* (Fig. 1).

Miura *et al*. (2007) found that carbohydrate-degrading filamentous strains of the phylum *Chloroflexi* were dominant and important in preventing membrane fouling of the membrane bioreactor. In their study, clones affiliated with the two classes *Anaerolineae* and *Caldilineae* were 74% and 50% of total *Chloroflexi* clones, respectively. This indicates that the composition of filamentous *Chloroflexi* in flocs from the activated sludge process are different from those of the membrane reactor. This implies that operation conditions of WWTPs could significantly contribute to changes in the composition of filaments in the subdivision *Anaerolineae*.

Cultivation of filamentous *Chloroflexi*

Isolation of the major filamentous *Chloroflexi* is a prerequisite for elucidation of physiological and ecological roles of filamentous bacteria in flocs of activated sludge. Despite widespread molecular observations of aerobic mesophilic filamentous bacteria, they have not been isolated among the subdivision *Anaerolineae* from activated sludge. We observed that diverse morphologies of filamentous microcolonies initially appeared on solid ET media on which diluted broken sludge flocs were spread. Growth of filamentous microcolonies was observed with a light microscope with 40× magnification.

Colony PCR and sequence analysis of 16S rRNA genes from 17 microcolonies revealed that the filamentous colonies were classified as *Chloroflexi* (8), *Firmicutes* (8), and *Actinomycetes* (1) (data not shown). During transfer of these filamentous colonies to fresh solid ET media, growth of most strains could not be maintained. Finally, we obtained seven strains that could be successively transferred monthly for 1 year. Tests to check for bacterial contamination in the axenic cultures could be performed with any liquid media since the filamentous isolates did not show growth in any liquid media, including nutrient media. Growth was only observed by monitoring the elongation of filaments (Meletiadis *et al.*, 2001) under the microscope as shown in Fig. 2. These isolates exhibited more than 99.2% 16S rRNA gene sequence similarity to each other.

The closest cultivated relative was *Caldilinea aerophila* STL-6-O1 (with 89.4% 16S rRNA gene sequence similarity) which was the only isolate of the class *Caldilineae* originating from a hot spring (Sekiguchi *et al.*, 2003). Our isolates showed close relationships with the two clones of the class *Caldilineae* (97.4% 16S rRNA gene sequence similarity) retrieved from activated sludge flocs in this study (see Fig. 1). Further, our isolates showed ca. 93% 16S rRNA gene sequence similarity

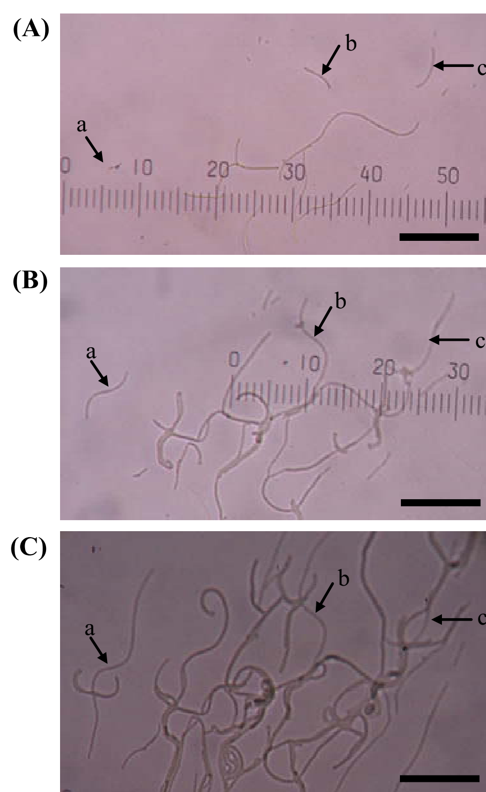

Fig. 2. Photomicrographs of growth of strain ET1 on solid ET media. Photographs were taken after (A) inoculation, (B) 2 days of incubation, and (C) 5 days of incubation. During incubation, the lengths of filaments of strain ET1 were bidirectionally elongated. Pictures were taken under 40× magnification. Arrows indicate the same position for pairwise comparison of filament growth. Scale bar in each image: 100 μm.

with dominant filamentous bacteria (clone CMBR-4) of the class *Caldilineae* (Miura *et al.*, 2007). Our isolates described here are the first aerobic mesophilic filamentous bacteria of the subdivision *Anaerolineae* to be retrieved from activated-sludge processes of municipal WWTPs.

Characteristics of filamentous isolates

Physiological properties were determined using the isolate ET1 as a representative. The sizes of the colonies ranged from 1-2 mm in diameter after 2 weeks of incubation. Observing the colony on the ET plate was difficult since it was small and transparent and not convex. We did not observe any breakages or branches during growth (Fig. 2). Using the rate of elongation of filaments on the solid ET media, we determined a specific growth rate of ca. 0.67 d^{-1} on solid ET media. Cells were Gram-negative, non-motile, and non-spore forming. Growth was not observed above 37°C. The pH range for growth was 6.0-8.5. A detailed morphological study was performed using TEM (Fig. 3). The diameter of the filaments was about 0.5-1.0 μm. Septum-like structures were rarely observed (Fig. 3A). A weak intracellular membrane-like structure was observed (Fig. 3C). Filaments were not covered by a sheath, negative in Neisser and Gram stain. Based on the Eikelboom criteria (Eikelboom and van Buijsen, 1983), the morphology of the isolate was determined to be close to *Haliscomenobacter hydrossis* which was a filamentous bacterium of the phylum *Bacteroidetes*/*Chlorobi.* There were various types of inclusion bodies in the filaments (Figs. 3B, C, and D). However, *C. aerophila* STL-6-O1 had regular septa but no vivid inclusion bodies (Sekiguchi *et al.*, 2003). Strain ET1's ability to accumulate diverse storage materials might be necessary for adapting to the fluctuating (starvation) environments of anaerobic-anoxic-aerobic (biological nutrients removal) processes of WWTPs. We could not observe any growth during incubation in the liquid media.

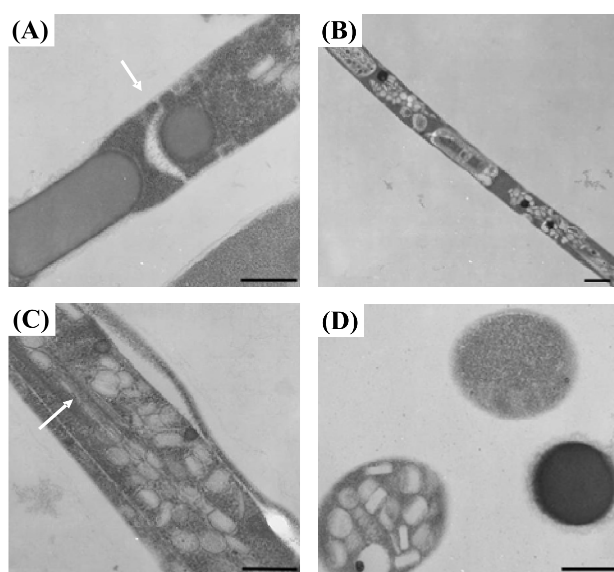

Fig. 3. Transmission electron micrographs of ultrathin sections of strain ET1. (A), (B), (C), and (D) show cross sections of filamentous cells of strain ET1. The arrow in (A) indicates the position of a septum. The arrow in (C) indicates intracellular membrane-like structures. Scale bar in each image: 500 nm.

The isolate could grow heterotrophically in aerobic conditions but could not grow either fermentatively or respiratively in the presence of nitrate or sulfate as sole electron acceptors under anaerobic atmospheres. Light did not stimulate growth of the isolate. We did not observe any autotrophic growth with reduced sulfur (thiosulfate, elemental sulfur, and sulfite) or nitrogen compounds (ammonia and nitrite) as sole electron donors.

Physiological properties of our isolate were not similar to the *Caldilineae* phylotypes of the membrane reactor (Miura *et al.*, 2007) considering the limited ability of ET1 to assimilate various carbohydrates. Assimilation of glucose and N-acetylglucosamine was observed in the filamentous *Chloroflexi* by microautoradiography-coupled FISH experiments (Miura *et al.*, 2007), but those assimilations were not observed in our isolate (see Table 2). A potential role in macromolecule degradation was suggested in filamentous *Chloroflexi* (Björnsson *et al.*, 2002; Okabe *et al.*, 2005). Filamentous bacteria of the unclassified *Chloroflexi*, *Kouleothrix aurantiaca*, isolated from bulking sludge also showed good growth on carbohydrates and biopolymer (Beer *et al.*, 2002; Kohno *et al*., 2002). In contrast to these filamentous *Chloroflexi*, our isolate exhibited limited abilities to degrade biopolymers.

Abundance of filamentous *Caldilineae* in activated sludge

Our results of the cultivation-independent molecular survey and cultivation of filamentous *Chloroflexi* indicate that the class *Caldilineae* could possibly be filamentous members of flocs in activated sludge. To quantitatively support this idea, abundance and localization of the class *Caldilineae* was studied using qPCR and FISH techniques.

qPCR : Although the clones of the class *Caldilineae* were retrieved from the 16S rRNA gene clone libraries of activated sludge, abundance could not be estimated directly due to potential PCR amplification bias (Hugenholtz and Goebel, 2001; Acinas *et al.*, 2005). To determine the relative abundance of *Chloroflexi*, the copy numbers of 16S rRNA gene of the phylum *Chloroflexi* and class *Caldilineae* were quantified in the activated sludge using group specific PCR primers (Table 1). The relative abundance of the 16S rRNA gene copies of the phylum *Chloroflexi* in four activated sludge (CJ, DJ, CA, and AY) were 3.9-11.8% of the total copies of the domain *Bacteria* (Fig. 4). The values of *Chloroflexi* count are high although the plant was operated without any problem of bulking. It is possible that some of the 16S rRNA genes might be originated from non-filamentous *Chloroflexi*. *Caldilineae* comprised 12-19% of the total *Chloroflexi*, which was much higher than the value (2%) calculated from the clone library analysis. However, we could not ascertain that all these were from filamentous microorganisms.

FISH : Although qPCR analysis gives an exact number of copies of target genes in a sample, we could not obtain information about the morphology of target gene-containing microorganisms. FISH analysis was conducted to investigate the morphology and distribution of filamentous *Caldilineae* in activated sludge flocs. We designed and evaluated new probes targeting the class *Caldilineae* and obtained a specific probe

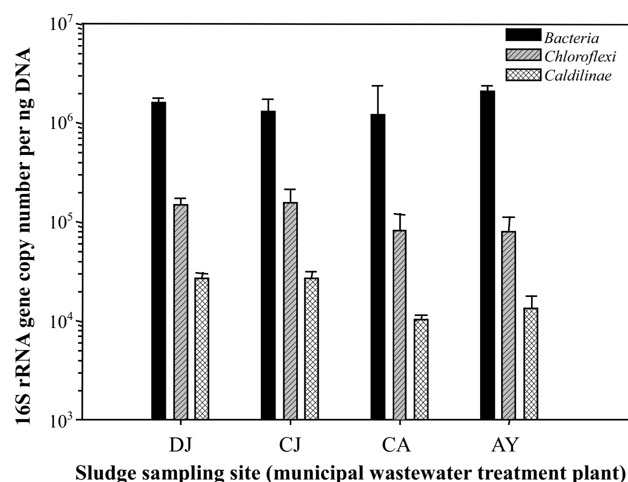

Fig. 4. Relative abundance of the phylum *Chloroflexi* and class *Caldilineae* found in the WWTPs of Cheongju (CJ), Daejeon (DJ), Cheonan (CA), and Anyang (AY) as determined by estimating the copy number of the 16S rRNA gene. The copy number of the 16S rRNA gene was quantified by real-time PCR. The error bar represents the standard deviation of quadruple PCR reactions.

(CFX1A331, see Table 1). Most of the filaments could be hybridized with a *Chloroflexi*-specific probe mix (Fig. 5). Relatively thicker filaments of the phylum *Chloroflexi* (Fig. 5) were determined to be members of *Caldilineae*. Among *Caldilineae*-like filaments, some exhibited visible string-of-beads morphology. These results indicated that strains of the class *Caldilineae* composed the filaments of floc in the activated sludge of WWTPs. Quantitation based on FISH showed that about 9-17% of total *Chloroflexi* filaments (CJ 17±5%, DJ 13±5%, CA 9±3%, and AY 10±4%, Data from the mean of three independent measurements, $p<0.05$) were from *Caldilinea* which is slightly lower than those obtained using qPCR.

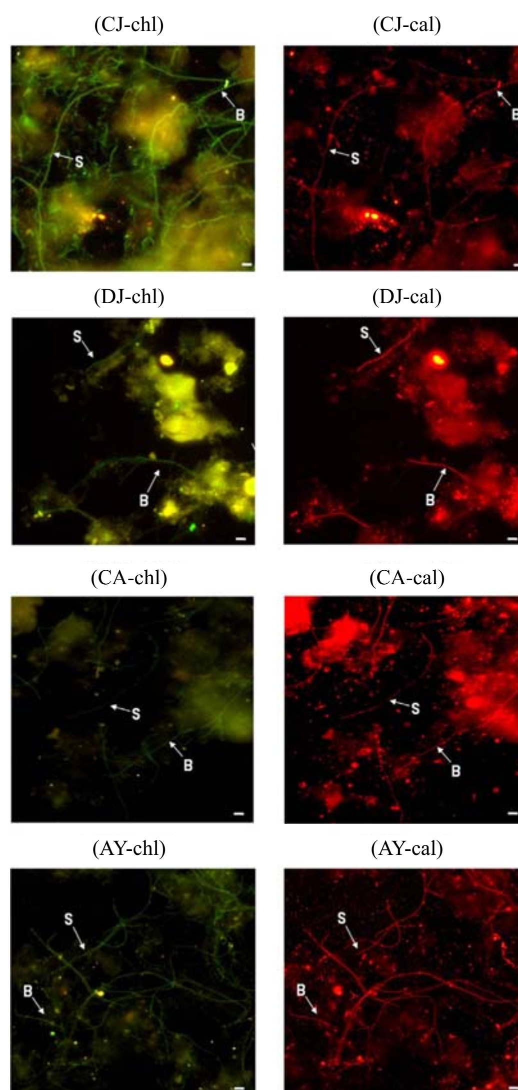

Fig. 5. FISH of activated sludge flocs from municipal WWTPs visualized with fluorescent microscopy. FISH images are obtained with activated sludge of Cheongju (CJ), Daejeon (DJ), Cheonan (CA), and Anyang (AY) municipal WWTPs. Images in left side panel (*CJ-chl*, *DJ-chl*, *CA-chl*, and *AY-chl*) were fluorescence signals from the FAM-labeled probe mix of GNSB941 and CFX1223 specific for the phylum *Chloroflexi* (green). At the same location, images in right side panel (*CJ-cal*, *DJ-cal*, *CA-cal*, and *AY-cal*) were observed by changing the filter for fluorescence signals from the Cy-3-labeled CFX1A331 probe, specific for the class *Caldilineae* (red). Arrows with S and B indicate representative filaments with smooth type and string-of-beads type morphologies, respectively. Scale bar in each image: 5 μm.

Conclusion

Members of the subdivision *Anaerolineae* were dominant in the activated sludge of municipal WWTPs based on a 16S rRNA gene library analysis. The mesophilic filamentous strain of the class *Caldilineae* isolated in this study grew heterotrophically under aerobic conditions with a limited ability to assimilate carbohydrates and degrade biopolymers. qPCR and FISH analysis showed that *Caldilineae* inhabited activated sludge of municipal WWTPs as backbone-forming filaments. Further work on this isolate is required to elucidate the roles of filamentous *Chloroflexi* in sludge stabilization and sludge bulking in WWTPs.

Acknowledgements

This work was supported by a Ministry of Science and Technology grant (R01-2007-000-20806-0) and the National Research Foundation of Korea (NRF) grant (2009-0087901) funded by the Korea government (MEST). We are grateful for the comments and suggestions of the two anonymous reviewers and editor.

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