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Special issue: *Significance of culturing microbes in the omics era*

Minireview

Cultivation of Uncultured *Chloroflexi* Subphyla: Significance and Ecophysiology of Formerly Uncultured *Chloroflexi* 'Subphylum I' with Natural and Biotechnological Relevance

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Cultivation-independent molecular surveys have shown members of the bacterial phylum *Chloroflexi* to be ubiquitous in various natural and artificial ecosystems. Among the subphylum-level taxa of the *Chloroflexi* known to date, the formerly uncultured 'subphylum I' had well been recognized as a typical group that contains a number of environmental gene clones with no culture representatives. In order to reveal their ecophysiology, attempts were made over the past decade to domesticate them into laboratory cultures, and significant advances have been made in cultivating strains belonging to the group. The microorganisms characterized so far include seven species in six genera, i.e., *Anaerolinea*, *Levilinea*, *Leptolinea*, *Bellilinea*, *Longilinea*, and *Caldilinea*, and were proposed to represent two classes, *Anaerolineae* and *Caldilineae*, providing solid insights into the phenotypic and genetic properties common to the group. Another subphylum-level uncultured group of the *Chloroflexi*, *i.e.*, the class *Ktedonobacteria*, has also been represented recently by a cultured strain. In addition to the results from these tangible cultures, data obtained from functional analyses of uncultured *Chloroflexi* populations by assessing substrate uptake patterns are accumulating at an encouraging rate. In this review, recent findings on the ecological significance and possible ecophysiological roles of '*Chloroflexi* subphylum I' are discussed based on findings from both the characteristics of the cultured *Chloroflexi* and molecular-based analyses.

Key words: *Chloroflexi*, *Anaerolineae*, *Caldilineae*, uncultured microorganism

Introduction

Cultivation-independent molecular methods have provided new tools to study the microbial world, enabling us to understand the actual microbial diversity that traditional cultivation-based methods have never unveiled (32). With the application of these techniques, it has become evident that the majority of microorganisms in the environment are uncultured, and that the ecophysiology of these organisms remains largely unknown. The finding of yet-to-be cultured microorganisms have driven renewed efforts in the cultivation and isolation of such microbes, because the domestication (cultivation) of microorganisms into laboratory cultures is still the best means to gain solid insights into metabolic ability and detailed genomic traits of individual microbes. In the past few years, new microorganisms have been successfully isolated that belong to uncultured taxa with environmental and biotechnological relevance, and the information of their physiology in conjugation with phylogeny has been updated (63). *Chloroflexi* subphyla are also examples where such microbial groups have recently been cultured and characterized.

The phylum *Chloroflexi*, formerly known as 'Green nonsulfur bacteria', has been recognized as a typical ubiquitous bacterial taxon containing a number of diverse environmental 16S rRNA gene clones with a limited number of cultured representatives (33, 63). Formerly, the phylum had been divided into four major subphylum (class)-level taxa on the basis of 16S rRNA/rRNA gene sequences, i.e. 'subphyla I, II, III, and IV (Fig. 1) (33), but the class *Thermomicrobia* has been reclassified into the phylum as an additional subphylum (34). The phylogenetic depth of the phylum is comparable with that of the phylum *Proteobacteria* (20). In addition to the major five subphyla, other uncultured lineages at the subphylum level were also identified (14, 63). Among the subphyla, 'subphylum III', known as the class *Chloroflexi*, has been best represented by cultured organisms belonging to the genera *Chloroflexus*, *Oscillochloris*, *Chloronema*, *Heliothrix*, *Herpetosiphon*, and *Roseiflexus*. These organisms mostly possess filamentous morphotypes, and show photoheterotrophic and/or chemolithoheterotrophic growth under mesophilic or moderately thermophilic conditions. The class *Thermomicrobia* also involves cultured organisms belonging

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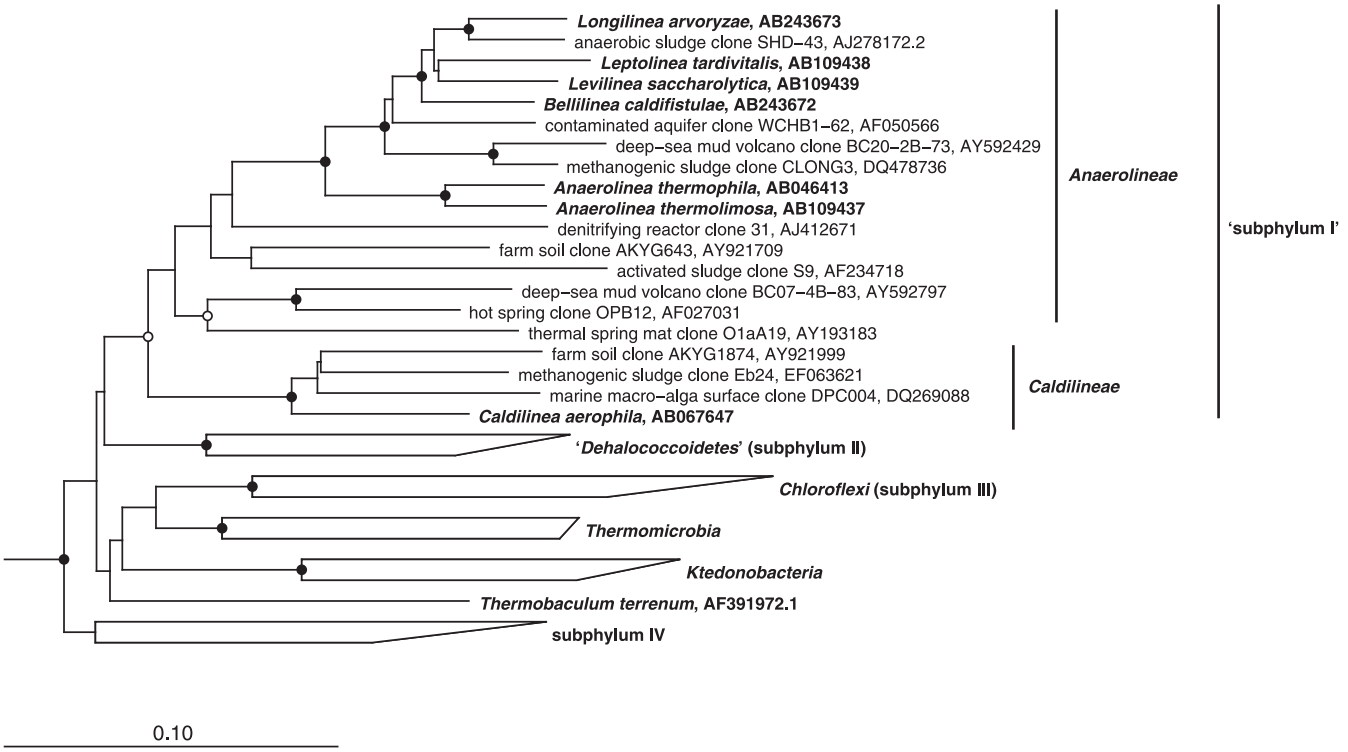

Fig. 1. Evolutionary distance dendrogram of the bacterial phylum *Chloroflexi* derived from a comparative analyses of 16S rRNA gene sequences, showing the phylogenetic relationship of the subphyla (class)-level taxa of the phylum. The sequences were aligned, and the phylogenetic tree was constructed by the neighbor-joining (NJ) method with the ARB software package (52). The fidelity of the topology of the NJ tree was also confirmed by bootstrap resampling (based on 1,000 replicates) with the neighbor-joining (PAUP* 4.0 program package) and maximum likelihood (TREEFINDER program package) methods. Nodes highly supported with bootstrap values higher than 85% by both analyses are marked as circles (filled circles, >95%; open circles, 95–85%). Nodes without symbols were not highly resolved (<85%) as specific groups in either analysis. The bar represents 10 nucleotide substitutions per 100 nucleotides.

to the genera *Thermomicrobium* and *Sphaerobacter* (34), which are rod-shaped, moderately thermophilic or hyperthermophilic, chemoheterotrophic aerobes. In addition, *Thermobaculum terrenum*, a moderately thermophilic chemolithoheterotrophic aerobe, represents a distinct lineage in the phylum, forming a new class-level taxon (9). The other three major subphyla (I, II, and IV) had been comprised solely of a variety of environmental clones except for purified, coccoid-shaped organisms, '*Dehalococcoides ethenogenes*' and related strains (e.g., (1, 53)) able to reductively dechlorinate chlorinated compounds, being classified into 'subphylum II' (class '*Dehalococcoidetes*') (31, 33). 'Subphylum I' contains the most diverse environmental clones among the four subphyla of the *Chloroflexi*; in the current 16S rRNA Ribosome Database Project (RDP) database (release 10.11), 'subphylum I' phylotypes are most frequently represented among the subphyla known to date and make up approximately >70% (>5,000 entries) of all the deposited sequences relative to the *Chloroflexi* phylum. Although the past two decades have seen a number of papers reporting the detection of '*Chloroflexi* subphylum I' in various ecosystems (see below), there had long been no description of cultivable microbes. However, in recent years, aerobic and anaerobic strains have successfully been cultivated and characterized that belong to 'subphylum I'. The microorganisms characterized so far are seven species in six genera in total, and were proposed to represent two distinct classes *Anaerolineae* and *Caldilineae*. In addition, an aerobic strain has recently been isolated and characterized that belongs to another uncultured lineage at the subphylum level in the *Chloroflexi* phylum, representing a new class, *Ktedonobacteria*.

In this review, recent findings on the ecological significance and possible ecophysiological roles of the formerly uncultured *Chloroflexi* subphyla are discussed based on findings from rRNA-based community analyses for the environment, as well as from the characteristics of recently cultured *Chloroflexi*. In addition, recent studies on the ecophysiology of these organisms in engineered systems through the evaluation of their substrate uptake pattern are described. Special emphasis is placed on the ecology and function of *Chloroflexi* 'subphylum I' members with natural and biotechnological relevance, particularly those found in waste/wastewater treatment systems.

Ecological significance of Chloroflexi *subphyla*

In 1998, Hugenholtz *et al.* analyzed 16S rRNA gene sequences of 5,224 cultured bacteria and 2,918 environmental gene clones retrieved from a wide range of natural and artificial ecosystems, revealing ubiquitous bacterial groups to be those of the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Planctomycetes*, *Acidobacteria*, *Verrucomicrobia*, and *Chloroflexi*, and the candidate phylum OP11 (33). In the past decade, a flood of papers reporting the molecular detection of *Chloroflexi* phylotypes in a variety of environments have been published, further supporting their

Table 1. Relative abundance of *Chloroflexi* phylotypes in natural and artificial ecosystems as determined by 16S rRNA gene-based clone library analyses

Subphyla	Natural habitats											Artificial habitats				
	Sediment				Soil			Hot spring	Freshwater	Ocean	Hypersaline lake	Wastewater treatment		Lagoon	Mine drainage	Microbial fuel cell system
	Deep seafloor	Sea	Lake	River	Agricultural	Geothermal	Meadow					Aerobic	Anaerobic			
Subphylum I (*Anaerolineae* and *Caldilineae*)	○[a]	○	●	●-●●	●●●	○	○	○-●	○	—	○-●●●	●	○-●●●	○	●	○-●●
Subphylum II ('*Dehalococcoidetes*')	○	—	—	●●	—	—	—	—	—	—	—	—	—	—	—	—
Subphylum III (*Chloroflexi*)	—	—	—	—	—	—	—	○-●	—	—	○-●●●	○	—	—	●	—
Subphylum IV	○-●●●	—	—	—	—	—	—	—	●	○	—	—	—	—	—	—
New subphyla	○	—	—	○	—	—	●	—	—	—	—	—	—	—	—	—
References	59	54	82	46	5	78	22	48	85, 99	29	50	42, 45, 56	3, 24, 58, 64, 90	97	79	39, 64

[a] Frequency of clones assigned to a subphylum of *Chloroflexi* as a percentage of the total number of bacterial sequencs analyzed: —, 0%; ○, 0.1–5%; ●, 5–15%, ●●, 15–25%, ●●●, >25%.

ubiquity in natural and engineered environments (Table 1). For example, *Chloroflexi* phylotypes were found as the most numerous bacterial group (nearly 80% of all bacterial gene clones analyzed) in an organic-rich deep subseafloor biosphere (38), where these phylotypes mainly fall into 'subphyla I, II and IV'. Similarly, *Chloroflexi* are often one of the most dominating bacterial phyla in various deep subseafloor sediments (*e.g.*, (7), see also a recent review by Fry *et al.* (27)), some of which were associated with methane hydrate-bearing sites (38, 59). Other natural environments where *Chloroflexi* phylotypes were detected in abundance are hot springs (*e.g.*, (4, 48)), hypersaline microbial mats (*e.g.*, (50)), soil (*e.g.*, agricultural soils (5, 75), geothermal soils (78), and low-temperature meadow soils (14)), sediment (*e.g.*, sea and lake (river) sediment (18, 35, 46, 54, 82, 89), and hydrothermally active sediment (79, 83)), chlorinated-solvent-contaminated aquifer sites (21, 26, 88)), oceans (*e.g.*, (6, 29, 63, 87)), and freshwater (*e.g.*, (85, 99)).

Recently, Lau *et al.* analyzed the bacterial communities of microbial mats from five hot springs (temperature: 60–65°C), indicating that 10–15% of the phylotypes detected were related with the *Chloroflexi* phylum (48). These *Chloroflexi* phylotypes fall into 'subphyla I and III'. Similarly, *Chloroflexi* phylotypes were found in a hypersaline (salinity: 8%) microbial mat as the majority of the mat constituents (21–39% of the bacterial rRNA clones analyzed were those of the *Chloroflexi* phylum) (50). These *Chloroflexi* phylotypes, again, fall into the 'subphyla I and III'. In this study, quantitative rRNA-targeted dot blot hybridization was conducted with an oligonucleotide probe specific for the phylum *Chloroflexi* (GNSB941 probe, Fig. 2, Table 2), estimating their abundance to be 22–41% of the total rRNA. These microbial mats were fueled by sunlight, and therefore photosynthetic bacteria of the class *Chloroflexi* ('subphylum III') were found in association with uncultured 'subphylum I' organisms. Costello *et al.* determined the bacterial community of a tundra wet meadow soil, where the annual soil temperature was 0.3°C and the temperature was stable throughout the year, revealing *Chloroflexi* phylotypes to make up 16% of the bacterial rRNA gene clones (14). In this case, the phylotypes found were classified into 'subphylum I' as well as other formerly and currently uncultured, previously unrecognized groups at the subphylum level, including the class *Ktedonobacteria* (see below). These findings suggest a wide range of temperature (0 to 65°C) and salinity for the habitats of *Chloroflexi*.

Soil is believed to be one of the most complex environments for microbial life (15). Concerning *Chloroflexi* phylotypes in soil environments, a recent review by Janssen showed the *Chloroflexi* to be one of the most dominant phyla in soils: 32 previously published clone libraries for different soil samples were re-evaluated and the mean contribution of the *Chloroflexi* phylum to soil bacterial communities was found to be 3% (range: 0–16%) (40). Similarly, pyrosequencing of rRNA gene clone libraries (*ca.* 150,000 clones in total) for different soils further supported the significance and genetic diversity of the *Chloroflexi* population (22, 65). Concerning the *Chloroflexi* phylum in oceanic and freshwater bacterioplanktons, environmental clones belonging to 'subphylum IV' (formerly known as cluster SAR202) were found in the Sargasso sea (4% of bacterial clones) (29), and similar phylotypes were detected in different ocean samples (6). Freshwater bacterioplankton also often contain the *Chloroflexi* phylum (average: 1%, range: 0–4%), most of them affiliated with 'subphyla I and IV' (85, 99).

Chloroflexi phylotypes have also been found in abundance in artificial and engineered ecosystems, such as lagoons (*e.g.*, (97)), mine drainage (*e.g.*, (73, 79)), anaerobic sludges for waste and wastewater treatment (*e.g.*, (2, 12, 17, 19, 25, 30, 43, 51, 57, 58, 64, 66, 71, 74, 76, 90, 94)), activated sludge systems (*e.g.*, (3, 8, 13, 25, 42, 45, 47, 55, 56, 60, 62, 77, 80, 98)), and microbial fuel cell systems (39, 61). Among these ecosystems, waste/wastewater treatment facilities are perhaps the best-recognized habitat where *Chloroflexi* phylotypes reside in abundance. For example, Rivière *et al.* evaluated bacterial and archaeal community structures of seven mesophilic (29–37°C), anaerobic (methanogenic) digesters decomposing municipal sewage sludge and found the *Chloroflexi* to be the most abundant bacterial phylum (average: 32% of all the bacterial clones analyzed, range: 15–45%)

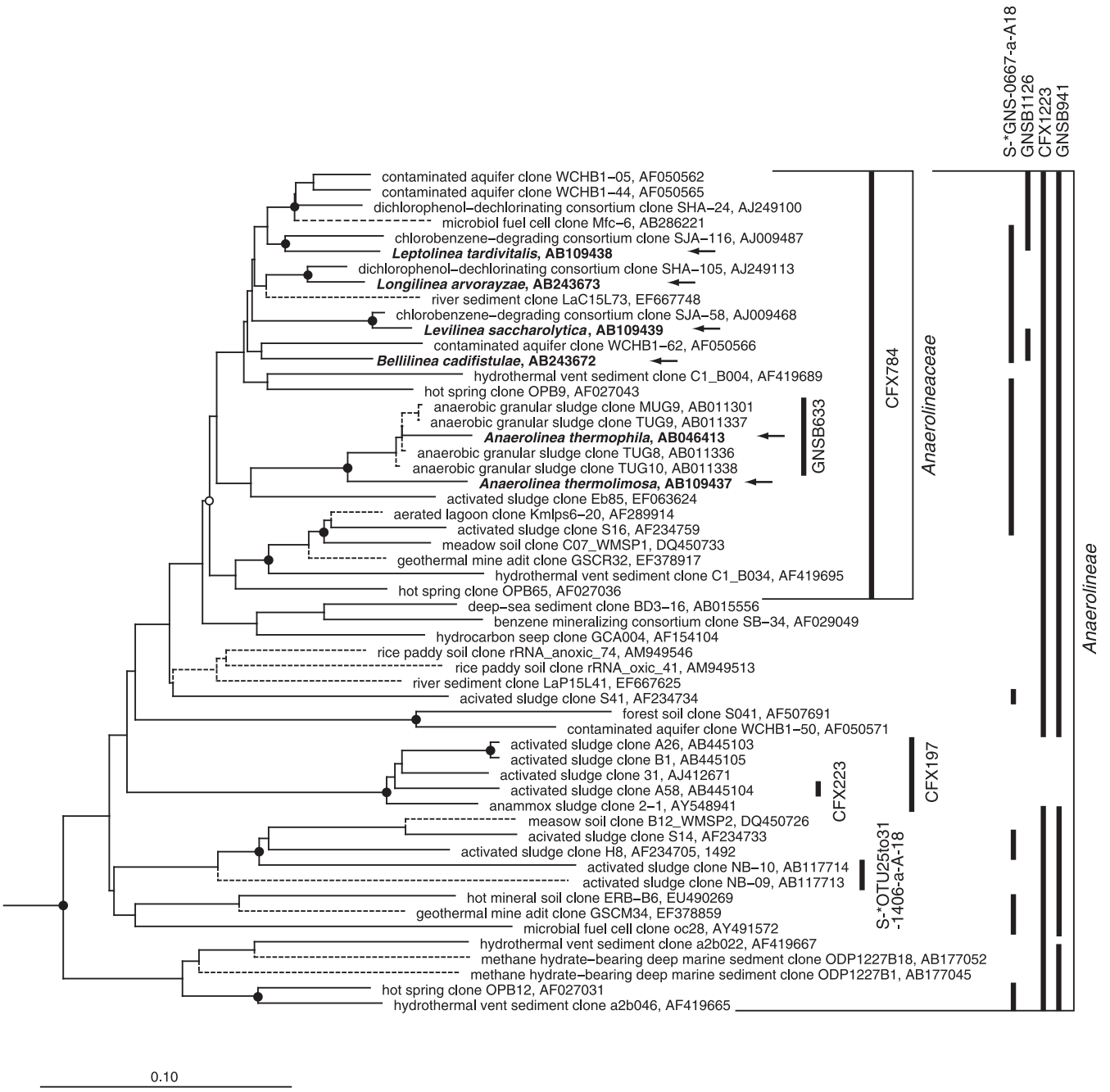

Fig. 2. Evolutionary distance dendrogram of the class *Anaerolineae* derived from a comparative analyses of 16S rRNA gene sequences, showing the phylogenetic positions of cultivated strains belonging to the class (indicated by arrow) and other related gene clones that were retrieved from a variety of environments. The sequences were aligned, and the phylogenetic tree was constructed by the NJ method with the ARB software package (52). The base tree was constructed with >1,200 nt sequences. Partial sequences of <1,200 nt (highlighted with dotted branches) were inserted into the base tree using the parsimony insertion tool of the ARB program. 16S rRNA gene sequences of members affiliated with the phylum *Thermotogae* were used as outgroups (not shown in the tree). The bar represents 10 nucleotide substitutions per 100 nucleotides. The fidelity of the topology of the NJ tree was also confirmed by bootstrap resampling (based on 1,000 replicates) with the neighbor-joining (PAUP* 4.0 program package) and maximum likelihood (TREEFINDER program package) methods. Nodes highly supported with bootstrap values higher than 85% by both analyses are marked as circles (filled circles, >95%; open circles, 95–85%). Nodes without symbols were not highly resolved (<85%) as specific groups in either analysis. Probe specificity is shown to the right of the figure with the probe name; the bars indicate a perfect match between the probe and target sequences.

(64). All the *Chloroflexi* phylotypes detected were affiliated with 'subphylum I'. Chouari *et al.* conducted quantitative rRNA-targeted dot blot hybridization with an oligonucleotide probe specific to the phylum *Chloroflexi* (GNSB1126 probe, Fig. 2, Table 2) for a mesophilic (33°C), anaerobic digester for sewage sludge, estimating their abundance to be 20% of the total rRNA (12). Similarly, Narihiro *et al.* estimated the microbial diversity of twelve different types of mesophilic (35–40°C), anaerobic (methanogenic) sludges treating organic wastewaters, detecting *Chloroflexi* phylotypes as a predominant phylum (average: 12% of the bacterial clones analyzed, range: 0–32%) (58). The abundance of

Table 2. 16S rRNA-tageting oligonucleotide probes used for in situ detection of the *Chloroflexi* phylum and 'subphylum I'

Oligonucleotide	Target group	Probe sequences (5'-3')	Target site (*E. coli* position)	Length (nt)	References
GNSB941	virtually all members of the phylum *Chloroflexi*	AAACCACACGCTCCGCT	941–957	17	28
CFX1223	virtually all members of the phylum *Chloroflexi*	CCATTGTAGCGTGTGTGTMG	1223–1242	20	8
GNSB1126	members of the class *Anaerolineae*	AACACACAGCGAGGG	1112–1126	15	12
CFX784	members of the class *Anaerolineae*	ACCGGGGTCTCTAATCCC	784–801	18	8
GNSB633	*Anaerolinea thermophila*	TAGCCCGCCAGTCTTGAACG	633–652	18	68
S-*OTU25to31-1406-a-A-18	uncultured phylotypes of the class *Anaerolineae*	CCAGCTCCCATGACGTGA	1406–1423	18	42
S-*GNS-0667-a-A18	uncultured phylotypes of the class *Anaerolineae*	CACCCSGAATTCCACRTT	667–684	18	45
CFX197	uncultured phylotypes of the class *Anaerolineae*	TCCCGGAGCGCCTGAACT	197–214	18	80
CFX223	uncultured phylotypes of the class *Anaerolineae*	GGTGCTGGCTCCTCCCAG	223–240	18	80

Groups targeted by the probes are shown in Fig. 2.

these phylotypes varied depending on wastewater type, and all of them fell into 'subphylum I'. Similar phylotypes ('subphylum I') have been frequently found in anaerobic sludges that had treated wastewaters containing compounds recalcitrant to biodegradation, such as phenol (11% of bacterial clones) (24), phthalates (4–7%, (51, 90)), 4-methylbenzoate, (7%, (90)), 2,4-dinitroanisole and n-methyl-4-nitroaniline (36–42%, (3)).

Chloroflexi phylotypes are often present in activated sludge systems; phylogenetic analysis of activated sludge clones belonging to the *Chloroflexi* phylum indicated that they are affiliated with 'subphyla I and III' (8, 47). These clones were most abundant in submerged membrane bioreactors treating municipal wastewater (*Chloroflexi* phylum-specific probes, GNSB941 and CFX1223, were used for in situ detection and the probe-reactive cells accounted for 14–26% of the total, (56)). The phylotypes found in the systems again fall into 'subphyla I and III'. Nitrifying systems were also shown to contain these *Chloroflexi* in abundance; Kindaichi *et al.* found that phylotypes belonging to 'subphylum I' were dominant (13% of bacterial clones analyzed) in nitrifying biofilms formed in a submerged rotating disk reactor (45). *Chloroflexi* cells were found in abundance in nitrifying-denitrifying systems, in which uncultured 'subphylum I' cells (as detected using the probe S-*OTU25to31-1406-a-A-18, Fig. 2) accounted for 16% of the cells (42). Another example of these engineered ecosystems is the microbial fuel cell. The microbial fuel cell is a bio-electrochemical system that generates electric power from organic matter, in which *Chloroflexi* phylotypes are often found. For example, a phylotype affiliated with 'subphylum I' was one of the most dominant (17% of bacterial clones) in a microbial fuel cell system fed with cellulose (39).

These molecular inventories of *Chloroflexi* phylotypes in a wide range of natural and human-made ecosystems strongly suggest the ecological significance and physiological breadth of these organisms, playing indispensable roles in such habitats.

Cultivation of uncultured Chloroflexi at the subphylum level

The classes *Anaerolineae* and *Caldilineae* as '*Chloroflexi* subphylum I'. Phylotypes affiliated with 'subphylum I' are detected in a wide range of environments, and the group now contains the most diverse rRNA gene sequences among known subphyla with strong natural and biotechnological relevance. To unveil their physiology, attempts were made to cultivate them, and the first pure culture representing the group, *i.e.*, *Anaerolinea thermophila*, was obtained in 2001 from a thermophilic (55°C) anaerobic sludge treating organic wastewater (71, 72). Simultaneously, the second tangible organism of the group, *Caldilinea aerophila*, was obtained from a hot spring (72). Later, a new thermophilic species of the genus *Anaerolinea* and four species of four genera, *i.e.*, *Levilinea*, *Leptolinea*, *Bellilinea*, and *Longilinea*, were successfully cultivated and characterized (92, 93). To our knowledge, these seven species are only the cultivated organisms of 'subphylum I'.

The morphology, physiology, and genetic properties of cultivated strains of the two classes are shown in Table 3. The strains are anaerobic or aerobic, mesophilic or moderately thermophilic, multicellular filamentous, chemolithoorganoheterotrophic organisms degrading carbohydrates and amino acids (or peptides). No growth was found in the dissimilatory reduction of nitrate and sulfate. It may not be appropriate to conclude that 'subphylum I' is comprised solely of such heterotrophs, but based on the unveiled physiological traits of these microorganisms, common features that make 'subphylum I' recalcitrant to isolation are likely to be (1) a relatively slow growth rate compared to commonly cultivable microbes and/or (2) the need to associate with other microbes (syntrophy) for efficient growth. In fact, the *Anaerolinea*-type anaerobes cultivated so far are all very slow growers (doubling time: 45–92 hrs), and hence are easily outcompeted by fast-growing heterotrophic anaerobes like *Clostridia*- and *Thermoanaerobacter*-type cells. We actually found that irrelevant fast-growing microbes immediately outcompeted *Anaerolinea*-type cells when we

Table 3. Characteristics of cultivated species belonging to classes *Anaerolineae* and *Caldilineae* in the phylum *Chloroflexi*

Characteristic	Class *Anaerolineae*						Class *Caldilineae*
	Anaerolinea thermophila	*Anaerolinea thermolimosa*	*Levilinea saccarolytica*	*Leptolinea tardivitalis*	*Bellilinea caldifistulae*	*Longilinea arvoryzae*	*Caldilinea aerophila*
Type strain	strain UNI-1^T	strain IMO-1^T	strain KIBI-1^T	strain YMTK-2^T	strain GOMI-1^T	strain KOME-1^T	strain STL-6-O1^T
Cell diameter (μm)	0.2–0.3	0.3–0.4	0.4–0.5	0.15–0.2	0.2–0.4	0.4–0.6	0.7–0.8
Temperature range (°C)	50–60	42–55	25–50	25–50	45–65	30–40	37–65
Optimum growth temperature (°C)	55	50	37–40	37	55	37	55
pH range	6.0–8.0	6.5–7.5	6.0–7.2	6.0–7.2	6.0–8.5	5.0–7.5	7.0–9.0
Optimum growth pH	around 7.0	around 7.0	around 7.0	around 7.0	around 7.0	around 7.0	around 7.5–8.0
Doubling time (h)	72 (48)*	48 (10)*	56 (56)*	50 (50)*	45 (29)*	92 (38)*	5 (N.D)*
O_2 respiration	–	–	–	–	–	–	+
Major cellular fatty acids	$C_{16:0}$, $C_{15:0}$, $C_{14:0}$	ai-$C_{17:0}$, i-$C_{15:0}$, $C_{16:0}$	$C_{14:0}$, i-$C_{15:0}$, $C_{16:0}$	Branched $C_{17:0}$, $C_{16:0}$, $C_{14:0}$	$C_{16:0}$, $C_{14:0}$, i-$C_{15:0}$	i-$C_{15:0}$, ai-$C_{15:0}$, $C_{14:0}$,	$C_{18:0}$, $C_{16:0}$, $C_{17:0}$
Major quinone	–	–	–	–	–	–	MK-10
DNA G+C content (mol%)	54.5	53.3	59.5	48.2	54.7	54.5	59.0
Utilization in the presence of yeast extract of:							
Tryptone	±	+	+	+	±	+	+
Betain	ND	–	±	+	–	–	ND
Pyruvate	±	+	+	±	+	–	+
Glucose	+	+	+	+	+	–	+
Mannose	+	+	±	+	+	–	–
Galactose	+	+	±	±	+	–	ND
Fructose	+	+	+	+	+	±	–
Arabinose	±	+	–	±	+	–	–
Xylose	±	+	+	+	±	–	–
Ribose	±	+	+	+	+	–	–
Pectin	±	±	±	+	+	+	ND
Starch	+	±	–	±	–	–	+
Isolation source	Thermophilic UASB sludge	Thermophilic UASB sludge	Mesophilic UASB sludge	Mesophilic UASB sludge	Thermophilic anaerobic sludge	Rice paddy soil	Hot spring
Reference	71, 72	93, 94	93, 94	93, 94	92	92	72

* Doubling time in parentheses indicates that for sytrophic growth with hydrogenotrophic methanogens.
–, Negative; ±, variable; +, positive; ND, not determined. Only differences found among the strains are listed. All strains showed the following characteristics: multicellular filamentous morphology; growth under anaerobic conditions (fermentation).

attempted to isolate them (70, 71). This is probably the primary reason why many attempts to isolate 'subphylum I' organisms have failed. Consequently, selecting appropriate inocula, in which 'subphylum I'-type cells are highly abundant, is one of the keys to success (71). In fact, the cultivation and isolation of *Anaerolinea thermophila* was possible only when we used spine-like structures of sludge granules as the inoculum, in which *Anaerolinea* cells were highly concentrated. In the isolation, highly enriched portions of *Anaerolinea* cells were found by fluorescence *in situ* hybridization (FISH) with the probe GNSB633 (Fig. 2, Table 2), and were carefully washed and serially diluted in liquid medium. In this case, the fast-growing anaerobes outgrew the *Anaerolinea* cells in lower dilutions, but the *Anaerolinea* grew slowly in the highest dilution, in which growth was also checked as determined by FISH with GNSB633. Such rRNA-directed cultivation may be also important for cultivating uncultured cells. In fact, four strains of the genera *Anaerolinea*, *Levilinea* and *Leptolinea* were successfully isolated by this approach, with rRNA-directed cultivation using inocula that contain 'subphylum I' cells in abundance (71, 94).

Another approach that eliminates irrelevant fast-growing heterotrophic microbes (i.e., 'subphylum I' cells) is to establish primary enrichment cultures that allow the growth of other slow growing bacteria, such as syntrophic propionate-oxidizers (37). When we constructed methanogenic, syntrophic propionate-degrading enrichment cultures, we found that they contained GNSB941 probe-positive filamentous cells as a concomitant population (92). Therefore, the enrichment cultures were transferred to anaerobic media that support the growth of *Anaerolinea*-type cells, resulting in the cultivation and isolation of two additional anaerobes of the genera *Bellilinea* and *Longilinea* (92). In primary enrichment cultures, they might survive on certain remnants from the propionate-oxidizing community. Similarly, *Caldilinea aerophila* was isolated from a primary aerobic enrichment culture to focus on the isolation of chemolithotrophic thermophiles (72). For the primary enrichment culture, thiosulfate was used as the sole energy source, and the cultured cells were subsequently transferred to an aerobic organic medium, resulting in the cultivation and isolation of *C. aerophila*. Similar to *Bellilinea* and *Longilinea*, *C. aerophila* survived on remnants from the community that formed during the

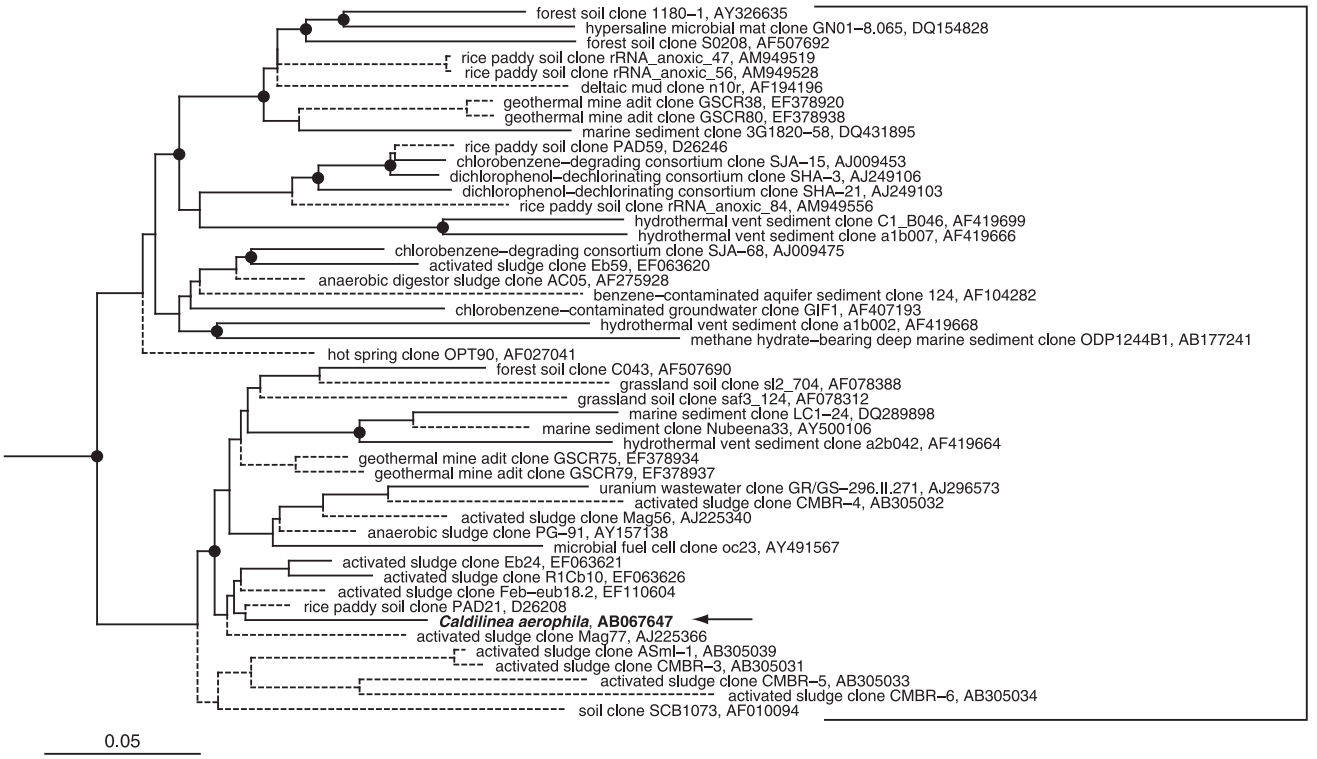

Fig. 3. Evolutionary distance dendrogram of the class *Caldilineae* derived from a comparative analysis of 16S rRNA gene sequences, showing the phylogenetic positions of *Caldilinea aerophila* (indicated by arrow) and other related gene clones that were retrieved from a variety of environments. The tree was constructed and marked as described in the legend of Fig. 2. The bar represents 5 nucleotide substitutions per 100 nucleotides.

primary enrichment, because *C. aerophila* cannot utilize thiosulfate as an energy source. In contrast, inoculating the original sample (hot spring microbial mat) directly into the same aerobic, organic medium resulted in the cultivation of typical fast growers like *Thermus*-type cells (72). These cases also demonstrated that the elimination of fast-growers from an inoculum is indispensable to the cultivation of 'subphylum I'.

Co-cultivation is an additional strategy. In the enrichment of *Anaerolinea*-type anaerobes, hydrogenotrophic methanogens were added beforehand, which stimulated the growth of 'subphylum I' anaerobes (94). Interestingly, some 'subphylum I' anaerobes produced hydrogen as an end product of fermentation, and grew more rapidly when co-cultivated with hydrogenotrophic methanogens (Table 3), indicating that they are "semi-syntrophic" bacteria requiring a hydrogen-scavenging partner for efficient growth (71, 72, 92, 93). Because microorganisms rarely live in pure cultures, this approach may be generally applicable to uncultured strains, whereby in situ conditions are appropriately mimicked, using co-cultivation for example.

These results indicated that it is still feasible to apply traditional cultivation techniques to the isolation of unseen microbes, if they are used thoughtfully in combination with molecular tools, and with carefully selected inocula, which contain sufficient amounts of targeted cells. By using a combination of these approaches, more novel microbes belonging to 'subphylum I' may be obtained.

Concerning the higher taxonomy of these organisms, the monophyly of 'subphylum I' was evaluated in detail based on the 16S rRNA gene sequences of these cultured organisms and environmental gene sequences, and the group was found not to be a monophyletic taxon (93), as suggested by Hugenholtz and Stackebrandt (34). In particular, the *Caldilinea* cluster did not often form a clade with other members of *Anaerolineae* (93). Thus, it was concluded that 'subphylum I' should be phylogenetically subdivided into at least two class-level taxa (Fig. 1), the class *Anaerolineae* representing the majority of lineages in 'subphylum I' (Fig. 2) and the class *Caldilineae* representing a deeply branched lineage relative to the *Anaerolineae* (Fig. 3). Physiologically, distinct differences, such as aerobic respiration, were found in the cultured members of the two classes (Table 3). Whole-genome analyses of *A. thermophila* and *C. aerophila* are now underway to unveil detailed genetic properties of these organisms (http://www.bio.nite.go.jp/ngac/e/project-e.html), which may also help to further assess the evolutional relationship among the *Chloroflexi* subphyla.

Other cultured subphyla of the *Chloroflexi* phylum. Recently, a novel strain was isolated from a soil and named *Ktedobacter racemifer* (11). The bacterium is a Gram-positive, aerobic, chemolithoorganoheterotrophic organism that produces branched vegetative mycelia, growing well under microaerobic conditions; this finding further expanded the morphological diversity of the phylum. Phylogenetically, the bacterium represents a new subphylum-level clade of the *Chloroflexi* phylum, to which the name *Ktedonobacteria* classis nov. has been given (11, 23). The subphylum (class)-level clade contains a variety of environmental gene clones (>100 sequences in public databases) retrieved mainly from

soil samples (these sequences can be browsed on the recent version of the greengene database (http://greengenes.lbl.gov/).

In addition to the class *Ktedonobacteria*, Davis *et al.* successfully isolated a strain belonging to a formerly uncultured *Chloroflexi* clade at the subphylum level, called the Ellin7237 lineage, from a soil sample using nontraditional aerobic media solidified with gellan (16). The lineage contains a relatively small number of environmental gene sequences mainly from soil environments (these sequences can also be seen on the greengene database (http://greengenes.lbl.gov/). Although detailed physiological properties of the strain have not yet been published, the bacterium will also provide new information on the genetic and phenotypic traits of the newly cultured subphylum. Gellan-based methods were found to be more effective than conventional agar-based techniques for culturing uncultured strains (10, 41, 44), resulting in the isolation of novel bacterial lineages, even at the phylum level, using media solidified with gellan (16, 81). This approach may also be useful for further isolating and characterizing uncultured *Chloroflexi* phylotypes.

Ecophysiology and function of the Chloroflexi *'subphylum I'*

Members of the class *Anaerolineae* in anaerobic sludge. The cultured *Anaerolineae* (the cultured members of the genera *Anaerolinea*, *Levilinea*, *Leptolinea*, *Bellilinea*, and *Longilinea*) share common physiological and morphological traits, such as anaerobic (fermentative) growth on carbohydrates and/or peptides (amino acids) and a multicellular filamentous morphology. Considering that the class *Anaerolineae* contains a number of rRNA gene clone sequences mainly retrieved from anoxic environments, most of which were obtained from anaerobic (methanogenic) sludges (2, 12, 30, 51, 57, 58, 64, 66, 71, 74, 90, 94), the common physiological traits of the cultured *Anaerolineae* are likely to represent those of organisms in the *Anaerolineae*, at least those of the *Anaerolineaceae* lineage (Fig. 2). For example, a layered microbial structure of different trophic groups of anaerobes was often found within granular sludges in upflow anaerobic sludge blanket (UASB) systems treating organic wastewaters (e.g., (36, 70)), with the *Anaerolineae*-type populations often found in the outer most layer of such sludge granules (66, 70, 71, 94). This unique architecture of sludge granules is considered to be a result of substrate profiles formed within the granules (68). That is, because the methanogenic conversion of organic matter is driven by a food web of different trophic groups of anaerobes, i.e., fermentative heterotrophs, proton-reducing syntrophic bacteria, and methanogenic archaea (68), fermentative heterotrophs, that mainly utilize primary substances in wastewaters such as carbohydrates, mainly reside in the outer layer of granules. That *Anaerolineae* populations were found in the outer layers of sludge granules suggests them to be heterotrophic degraders, decomposing carbohydrates, for example. In addition, because yeast extract and peptides are good substrates for cultured *Anaerolineae* (Table 3), and some *Anaerolineaceae*-type filaments were also found inside of sludge granules (94), they may be able to act as degraders of cellular materials (like amino acids) that are present inside sludge granules.

Based on FISH using oligonucleotide probes for *Chloroflexi* members (Fig. 2 and Table 2), the *Anaerolineae* in anaerobic sludge were shown to be filamentous. For example, FISH using a probe (GNSB 633) specific for *Anaerolinea thermophila* showed that all of the probe-reactive cells in thermophilic sludges had a thin-filamentous morphotype (70, 71). FISH experiments using GNSB633 or a *Chloroflexi*-specific probe (GNSB941) for various types of anaerobic sludge also indicated that the probe-reactive cells were all filamentous with a wide range of thicknesses (66, 70, 71, 94). Considering that almost all the *Chloroflexi*-related 16S rRNA gene sequences in anaerobic sludges are classified into the class *Anaerolineae*, the filamentous morphology observed with the GNSB941 probe may be a common trait of the *Anaerolineae* lineage.

Interestingly, this filamentous morphotype was found to be important for biotechnological reasons: *Anaerolineae* members are considered important for the granulation of sludge in UASB reactors, as well as the formation of fluffy sludge (bulking) in similar treatment systems. The granulation of sludge (formation of sludge granules with good settleability) is the major premise for the start-up and stable operation of UASB reactors (67, 69). It was reported that the granulation of thermophilic sludges was difficult to achieve when volatile fatty-acid mixtures were used as the sole substrate, while the addition of sucrose or glucose to the influent wastewater resulted in the formation of a granular sludge with good settleability (84, 86). In thermophilic UASB reactors having well-settleable sludge granules, *Anaerolinea*-type filamentous microbes predominated on the surface of the granules (70, 71, 94). Considering these findings together with the physiological properties of the cultured *Anaerolineae*, *Anaerolinea*-type filaments are indispensable organisms to the granulation of thermophilic UASB sludges, forming a web-like coating on the surface of granules (70, 71, 94).

Besides their importance in thermophilic UASB reactors, *Anaerolinea*-type organisms microorganism are a potential causative agent for the bulking of granular sludges (71). Once these filaments outgrew in the system, the sludge became fluffy, resulting in flotation and washout of the sludge from the system (71, 95). Similar observations were made in mesophilic anaerobic systems (71, 91, 95, 96). Therefore it is important to control the growth of these filamentous *Anaerolineae* not only to enhance the formation of granules but also to prevent the bulking of sludge granules.

Members of the class *Anaerolineae* in activated sludge systems. Although *Anaerolineae* strains have not yet been cultivated and isolated from oxic environments, phylotypes of the class have been frequently found in oxic systems like activated sludges (Fig. 2). FISH for the detection of *Anaerolineae*-type cells in activated sludges revealed that all of the probe-reactive cells were filamentous (8, 45, 55, 56, 60, 80). For example, Björnsson *et al.* used a probe (CFX784) specific for part of the class *Anaerolineae* and a probe (CFX109) for the class *Chloroflexi* ('subphylum III') for *in situ* detection of these organisms in various activated sludge systems and found that *Anaerolineae* in sludge samples were generally thin (<1 μm), smooth filaments, although they were less abundant than filamentous *Chloroflexi* ('subphylum III')

cells as detected with CFX109 (8).

To reveal the ecophysiology of these *Anaerolineae*-type organisms in activated sludge systems, functional analyses were conducted using microautoradiography-fluorescent *in situ* hybridization (MAR-FISH) (45, 49). For example, using the MAR-FISH method, substrate uptake patterns of uncultured *Chloroflexi*, particularly those of the class *Anaerolineae*, were studied for autotrophic nitrifying biofilms and revealed that the microbes aerobically utilized N-acetyl glucosamine, a major structural component of bacterial cells, as well as amino acids, implying that they metabolize cellular materials in the biofilms (45, 60). Similarly, Miura *et al.* (55, 56) found using MAR-FISH that filamentous cells of the class *Anaerolineae* in membrane bioreactors (MBR) were metabolically versatile and preferentially utilized N-acetyl glucosamine and glucose under oxic and anoxic conditions. Zang *et al.* observed that *Anaerolineae*-related cells incorporated decayed tritium-labeled bacterial cells in activated sludges (98). Based on these observations, it may be concluded that *Anaerolineae*-type organisms in activated sludge systems are likely to utilize carbohydrates, as well as to scavenge cellular materials formed in the systems, similar to the cultured *Anaerolineae* in anaerobic sludges.

The *Chloroflexi* filaments in activated sludge may provide a stabilizing backbone for sludge flocs, explaining one important role of these organisms in the systems (8). Miura *et al.* indicated that *Anaerolineae*-type filaments were responsible for the degradation of soluble microbial products, including carbohydrates and cellular materials from cells, consequently reducing membrane fouling potential in membrane bioreactors (55, 56). *Anaerolineae*-type filaments were also shown to be a causative agent for the filamentous bulking in activated sludge systems as well. Recently, Speirs *et al.* reported that *Anaerolineae*-type filamentous cells, as detected with the probes CFX197 and CFX223 (Table 2 and Fig. 2), are causative agents for aerobic filamentous bulking, which had long been recognized as Eikelboom Type 0092 (80). These studies demonstrate the importance of *Anaerolinea* organisms in activated sludge systems, drawing an analogy between their functions in oxic and anoxic (methanogenic) biological waste/wastewater treatment systems.

The class *Caldilineae* in natural and biotechnological systems. No detailed in situ studies have been conducted for the class *Caldilinea*, *i.e.*, no specific oligonucleotide probes have been designed and no substrate-uptake properties have yet been elucidated. Although 16S rRNA gene sequences have been obtained from various ecosystems (Fig. 3), including hot springs (33), anaerobic sludges (76), aerobic sludges (13, 25, 56), geothermal soil (79), marine sediment (35), hydrothermal vents (83), rice paddy soils (75) and chlorinated-solvent-contaminated aquifers (88), the ecophysiology and functions of this class remain to be clarified.

Concluding remarks and perspectives

In summary, recent cultivation and molecular-based studies suggest that microbes in the formerly uncultured *Chloroflexi* subphyla, *Anaerolineae* and *Caldilineae* in particular, may be filamentous, slowly growing, aerobic and anaerobic heterotrophs decomposing carbohydrates and amino acids, and often need to be associated with other microbes (syntrophy) for growth. They are ubiquitous in natural and artificial environments, and likely to play indispensable roles in ecosystems. They are often closely associated with the process performance of biological treatment systems, such as granule and floc formation and/or sludge bulking. Their ecophysiology and function have been well established based on information from cultured representatives, as well as from molecular-based ecological analyses, including an assessment of their substrate uptake patterns with the MAR-FISH technique. This synergism between traditional (or rRNA-directed) cultivation and molecular ecological analyses may be a promising strategy for further elucidating the function of these yet-to-be cultured lineages.

The cultivated *Anaerolineae* only make up part (the family *Anaerolineaceae* in Fig. 2) of the *Anaerolineae* lineage, and the class still contains surprisingly diverse, yet-to-be cultured environmental clades even at the subclass level (Fig. 2). The phylogenetic depth of the class is the highest among the *Chloroflexi* phylum (approximate rRNA gene sequence divergence of the class is 18%), possibly suggesting the presence of more genetically (and phenotypically) diverse *Anaerolineae* organisms than the cultured *Anaerolineae* strains. Similarly, the recently cultured classes, *Caldilineae* and *Ktedonobacteria*, contain only single cultured strains. Other uncultured subphyla of the *Chloroflexi* phylum remain to be characterized (63). To further unveil the function of these lineages that are less represented by cultured organisms, rRNA-directed cultivation and molecular ecological analyses may be useful, and should be applied to environments where targeted populations are abundant. To efficiently isolate and cultivate these organisms, it may be necessary to employ newly developed cultivation approaches for yet-to-be cultured microbes (10). More comprehensive studies of these *Chloroflexi* subphyla, involving cultivation, molecular ecological analyses, (meta-) genomics, and transcriptomics, will allow us to gain deeper insight into their functions, which may answer questions such as why these organisms are so abundant and ubiquitous in the environment.

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