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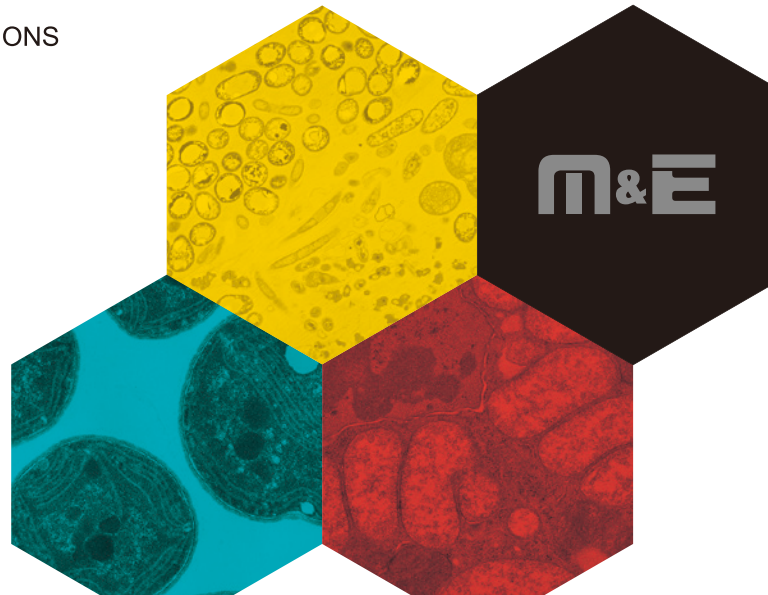

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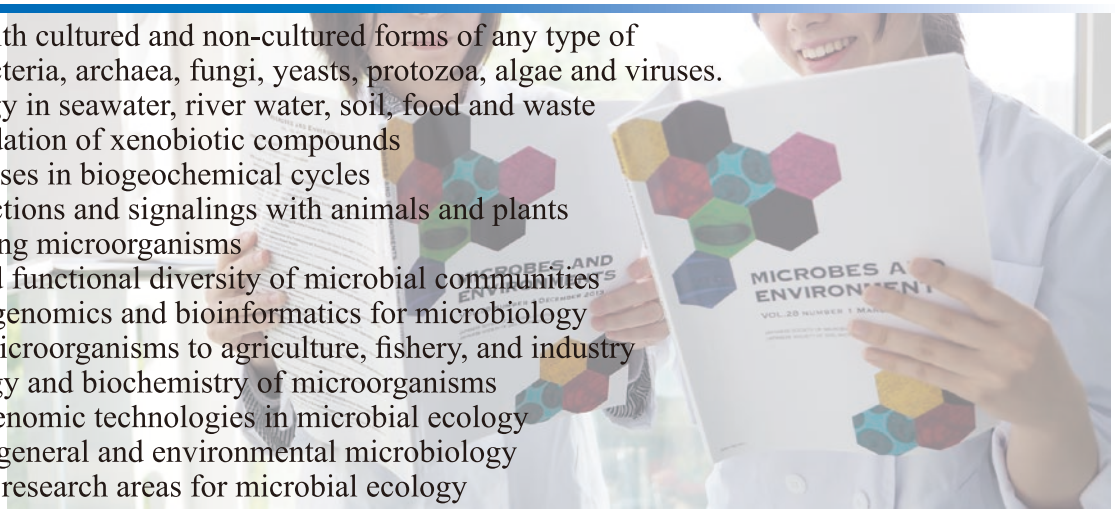

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MOST VALUABLE PAPER OF THE YEAR 2016

FLDS: A Comprehensive dsRNA Sequencing Method for Intracellular RNA Virus Surveillance

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Knowledge of the distribution and diversity of RNA viruses is still limited in spite of their possible environmental and epidemiological impacts because RNA virus-specific metagenomic methods have not yet been developed. We herein constructed an effective metagenomic method for RNA viruses by targeting long double-stranded (ds)RNA in cellular organisms, which is a hallmark of infection, or the replication of dsRNA and single-stranded (ss)RNA viruses, except for retroviruses. This novel dsRNA targeting metagenomic method is characterized by an extremely high recovery rate of viral RNA sequences, the retrieval of terminal sequences, and uniform read coverage, which has not previously been reported in other metagenomic methods targeting RNA viruses. This method revealed a previously unidentified viral RNA diversity of more than 20 complete RNA viral genomes including dsRNA and ssRNA viruses associated with an environmental diatom colony. Our approach will be a powerful tool for cataloging RNA viruses associated with organisms of interest.

Key words: RNA virus, viral metagenome, dsRNA

Viruses are the universal genetic elements associated with all three domains of life (22), and virus-host interactions impact on the status of life and surrounding ecosystems (41). Historically, viruses are most often recognized as pathogens (38), and, thus, have been studied in the field of medical and crop science. Recent advances in high-throughput sequencing technologies have enabled us to identify not only viruses associated with diseases, but also those present in natural environments including oceans (41) and soil (12). Although these sequencing technologies have opened a new era in virus identification (24), a limited number of methods have been established for virus enrichment and library construction. The diversity and distribution of viruses in non-viral nucleic acid-dominant environments, such as the intracellular environments in which viruses actually replicate, still remain unclear due to technical difficulties (16). The development of a new procedure for effective virus enrichment and library construction is required in order to understand the full spectrum of diverse viruses.

RNA sequencing (RNA-seq) is a popular method in RNA virus metagenomics and is widely used for RNA virus identification (35). Purification and library construction methods have been established for RNA viruses at the extracellular stage (7, 10, 40). However, the viral read ratio of intracellular RNA viruses (RNA viruses at the intracellular stage) in the RNA-seq library is typically < 1% because mRNA and rRNA are dominant in the total RNA fraction extracted from biological samples (25). Therefore, the enrichment of viral RNA is essential for maximizing sensitivity in the identification of novel viruses. The physical enrichment of viral particles and nuclease digestion of non-viral nucleotides has been employed to increase the viral read ratio; however, a relatively low abundance of viral reads is still observed in most studies (39). These techniques are only applicable to specific RNA viruses because not all RNA viruses form viral particles (21). In addition, difficulties are associated with capturing terminal RNA sequences in an efficient and effective manner (32) and obtaining uniform coverage using the RNA-seq method. Sample preparation methods for effective viral RNA-seq are still inadequate and the sequence information generated is biased and incomplete.

In an attempt to resolve these issues, an environmental viral metagenomic approach targeting intracellular long double-stranded RNA (dsRNA) has recently been examined (2, 6, 9, 37). Intracellular dsRNA consists of the genomes of dsRNA viruses and replicative intermediates of single-stranded RNA (ssRNA) viruses, and, thus, long dsRNA is known as an RNA virus-specific molecule and molecular marker for RNA virus infection and replication (28). Therefore, a metagenomic analysis targeting intracellular long dsRNA theoretically retrieves dsRNA and ssRNA viruses, except for ssRNA retroviruses, which do not form dsRNA in the replicative stage. In addition, it is possible to eliminate non-viral nucleic acids such as mRNA and rRNA, which dominate RNA-seq reads, by DNase I, S1 nuclease, RNase, or column chromatography (44). However, previous studies have reported technical issues with the purification of dsRNA and library construction. Random priming for the reverse transcription of dsRNA does not enable the terminal sequences of the dsRNA molecule to be determined or eliminate significant contamination by non-viral sequences. The heterogeneous sequencing depth in certain viral genome segments is also an issue associated with this method (2, 6, 9, 37). Although the full-length cDNAs of dsRNA viruses may be obtained using loop primers that are ligated to the dsRNA terminal ends for reverse transcription

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(8), this method is only useful for short dsRNA viruses. Therefore, to the best of our knowledge, this method has not yet been applied to a viral metagenomic analysis.

We herein established a novel strategy to obtain full-length RNA virus sequences with extremely high efficiency by applying a short dsRNA full-length cloning method (8) for physically fragmented dsRNAs. The improved method, named FLDS (fragmented and loop primer ligated dsRNA sequencing), was applied to a diatom colony in a tide pool and revealed previously unidentified RNA viruses. Our results indicate that the diversity of environmental RNA viruses has been underestimated due to the technical limitations in identifying entire RNA viromes in cellular organisms, and this technique will be a powerful tool for cataloging RNA viruses associated with organisms of interest.

Materials and Methods

Model and environmental samples

Mycelial plugs of *Magnaporthe oryzae* strain S-0412-II 1a, naturally infected with Magnaporthe oryzae chrysovirus 1 strain A (MoCV1-A) (45) were incubated in 0.5% yeast extract and 2% glucose liquid broth (YG broth) with reciprocal shaking (60 rpm) at 25°C for 2 weeks in the laboratory of Prof. Teraoka (Tokyo University of Agriculture and Technology). Colonies of a diatom on tidal rocks in Tokyo Bay (35.3405° N, 139.6396° E) were sampled in April 2014. After washing with distilled water, the colonies were stored at –80°C.

Purification and fragmentation of dsRNA

DsRNA was purified as described by Okada *et al.* with a few modifications (31, 46). Briefly, the microbial sample was disrupted in liquid nitrogen in a mortar and total nucleic acids were manually extracted. DsRNA was purified twice through a micro-spin column (empty Bio-spin column; Bio-Rad Laboratories, Inc., Hercules, CA, USA) containing cellulose powder (Cellulose D; ADVANTEC, Tokyo, Japan) to obtain pure dsRNA. The dsRNA eluted from cellulose powder in MQ water was treated with DNaseI (amplification grade, Invitrogen, Carlsbad, CA, USA) and S1 nuclease (Invitrogen) in nuclease buffer (57 mM CH_3COONa, 9.5 mM $MgCl_2$, 1.9 mM $ZnSO_4$, and 189 mM NaCl) and was then incubated at 37°C for 2 h. The final concentrations of CH_3COONa, $MgCl_2$, $ZnSO_4$, and NaCl were adjusted to 90 mM, 15 mM, 3 mM, and 300 mM, respectively. DsRNA was purified using an RNeasy Mini Kit (Qiagen, Valencia, CA). A one-tenth volume of 10 × ShortCut buffer and 10 × MnCl provided with ShortCut RNase III (NEB Japan, Tokyo, Japan) was added to the dsRNA solution and fragmented by ultrasound at 4°C in Snap-Cap microTUBEs using a Covaris S220 (Woburn, MA, USA). The fragmentation conditions were as follows; run time 35 s, peak power 140.0 W, duty factor 2.0%, and 200 cycles/burst. Fragmented dsRNA was divided into two equal volumes, and maintained at 37°C with or without ShortCut RNase III (NEB). DsRNAs were then purified using a ZymoClean Gel RNA Recovery Kit (ZymoResearch, Orange, CA). Note that dsRNA purification from *M. oryzae* was carried out in the laboratory of Prof. Teraoka.

cDNA synthesis and amplification for dsRNA

The PC3-T7 loop primer (5′-p-GGA TCC CGG GAA TTC GGT AAT ACG ACT CAC TAT ATT TTT ATA GTG AGT CGT ATT A-OH-3′) was ligated to fragmented dsRNA as described by Potgieter *et al.* (34), and dsRNA was then purified using the MinElute Gel Extraction Kit (Qiagen). After the addition of DMSO at a final concentration of 15% (v/v), dsRNA was denatured at 95°C for 3 min and snap-frozen in ice-water slurry. RNA was reverse transcribed into cDNA from the ligated loop primer region using the Superscript III First-Strand Synthesis System (Invitrogen). After excess and hybrid RNAs were removed (34), cDNA was desalted and concentrated using the MinElute PCR cleanup kit (Qiagen). Primary cDNA strands were re-annealed by lowering the temperature from 95 to 50°C, as described previously (30). Second strand DNA polymerization was performed using KOD-plus Neo (Toyobo, Osaka, Japan) with a primer complementary to the partial sequence of the PC3-T7 loop primer, PC2 (5′-CCG AAT TCC CGG GAT CC-3′) (34). After heat activation of KOD-plus Neo in the reaction mixture provided at 96°C for 2 min, template cDNA was added and incubated at 68°C for 5 min. After the reaction, cDNA was amplified under the following conditions: 96°C for 2 min, 25 (for MoCV1-A) or 18 (for diatom colony) cycles of 98°C for 10 s, and 68°C for 2 min. Small cDNA and primer dimers were removed using the 1.25 × SPRIselect reagent kit (Beckman Coulter, Brea, CA, USA) according to the Left Side Size Selection procedure in the manufacturer's protocol.

Total RNA extraction, cDNA synthesis, and library construction from an environmental sample

Total RNA was isolated from a diatom colony using the TRIzol Plus RNA Purification Kit (Invitrogen) according to the manufacturer's protocol. The RNA fraction was treated with DNase I (Takara, Otsu, Japan). Double-stranded cDNA was synthesized from 2 µg of total RNA with random primers (9-mers) using a PrimeScript Double Strand cDNA Synthesis Kit (Takara). The resultant cDNA was quantified using a Qubit dsDNA HS Kit.

Illumina sequencing

Ultrasound was used to fragment cDNA in Snap-Cap microTUBEs at 4°C using a Covaris S220 (Woburn, MA, USA). The fragmentation conditions were as follows; run time 55 s, peak power 175.0 W, duty factor 5.0% and 200 cycles/burst. The Illumina library was constructed with KAPA Hyper Prep Kit Illumina platforms (Kapa Biosystems, Woburn, MA, USA). The quantity of the library was evaluated using the KAPA library quantification kit (Kapa Biosystems). Each 300 bp of the paired-end sequences of each fragment were determined with the Illumina MiSeq platform (San Diego, CA, USA).

Data assembly and processing

Raw sequence reads were processed with the CLC Genomics Workbench (CLC Bio, Aarhus, Denmark). Adaptor and primer sequences were trimmed, and low quality sequence regions were removed with default parameters. PhiX sequences derived from control libraries and experimentally contaminated sequences (< 0.05% of total reads) were also removed using a mapping tool. The consensus sequences of viral contigs were obtained *de novo* exclusively with the CLC Genomics Workbench (CLC Bio), and assemblies were manually examined and extended using the Tablet viewer (27). Using the mapping tool, each contig was confirmed to be constructed with at least 3 × sequence coverage, 10 × average coverage, and 1,000 bp in length. In cases of dominant reads (more than 10 reads) that stopped in the same position around the ends of contigs, the position was recognized as a terminal end. The predicted terminal ends of the viral genome segments were also confirmed by the presence of adjacent PCR primer sequences next to the predicted terminal sequence, except for cases of contigs with a poly(A) tail. Contigs with 70–90% nucleotide identity with other contigs were classified as the genome types of the same species. Contigs with > 90% nucleotide identity were assigned as the same genome type and only major contigs were used in further analyses. Sequences were compared against the NCBI non-redundant nucleotide and amino acid (aa) databases using BLASTN-plus and BLASTX-plus, respectively (5), and then classified by MEGAN 5.7.1. (18). A sequence analysis was performed using Genetyx-MAC software version 17.0.0 (Genetyx Corp., Tokyo, Japan) and Genetyx software version 9.1.0 (Genetyx). Most full-length small subunit rRNA sequences in the diatom colony were reconstructed from RNA-seq reads with EMIRGE (26).

Phylogenetic analysis

Multiple alignments based on the deduced aa sequences of putative RNA-dependent RNA polymerase (RdRp) genes in dsRNA contigs were obtained using ClustalX 2.0 (23) and MEGA5 software (42). Phylogenetic analyses were conducted using MrBayes 3.2.3 (36) with the model of aa substitution, RtREV+I+G+F, selected by ProtTest2.4 (1), as judged by the Akaike information criterion (33). Bayesian analyses with the covarion parameter were run with one run and four chains for 1,000,000 generations.

Data accession

The data sets supporting the results of this study are available in the GenBank database repository (accession nos. DDBJ: AP014890–AP014920) and Short Read Archive database (accession no. DDBJ: DRA003723 and DRA003724).

Results

Application of FLDS to a segmented dsRNA virus

The novel dsRNA purification and library construction method, named FLDS, consists of cellulose column chromatography, the physical fragmentation of dsRNA, cDNA synthesis using a loop primer, and the PCR amplification of cDNA (Fig. 1). The purification of dsRNA was achieved by the repeated affinity purification of dsRNA using cellulose powder and the enzymatic removal of ssRNA and DNA. Purified dsRNAs were fragmented using ultrasound to retrieve all types of dsRNA viruses in order to apply the previously reported full-length dsRNA cloning method using a loop primer (8). The full-length dsRNA cloning method requires overlapped cDNAs synthesized from both terminal ends for further cDNA amplification, and was only applicable to short

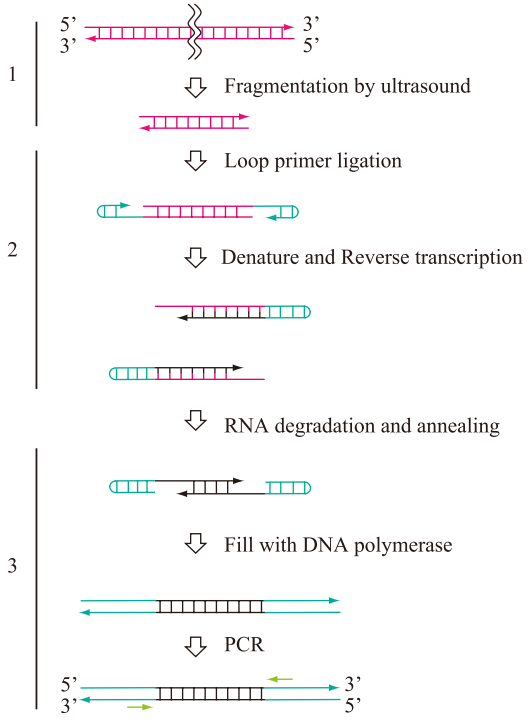

Fig. 1. Schematic work flow of FLDS. 1. Fragmentation of dsRNA by ultrasound. 2. Ligation of a loop primer on 3′-terminal ends and reverse transcription. 3. Selective duplex formation of cDNA from dsRNA, and PCR amplification. Details of the FLDS method are described in the Materials and Methods section.

dsRNA molecules. Reverse transcription was initiated from the ligated loop primer on both ends of the dsRNA fragment. cDNA was then thermally denatured to allow annealing of single-stranded cDNA with the complementary sequence in the 3′ terminal region. The single-stranded regions of annealed cDNA were filled in with DNA polymerase. The double-stranded cDNA derived from dsRNA was amplified by PCR with a single primer (PC2) in order to obtain sufficient cDNA to construct a sequencing library.

Mycelial MoCV1-A was used to test the feasibility of this method. Since PCR amplicons were not observed in the dsRNA-specific RNaseIII-treated sample prior to reverse transcription, most of the amplicons (cDNA) were likely to have been derived from dsRNA (Fig. S1). The results of the sequencing analysis indicated that 99.1% of total reads were derived from the MoCV1-A genome (Table S1). Five contigs obtained by *de novo* assembly were identical to the entire region of the MoCV1-A genome segments attained using a conventional cloning and sequencing method (44, 45) with > 99.9% identity (Table S2). Read mapping on MoCV1-A genomes (Fig. S2) showed that the sequence coverage of terminal regions was generally higher than that of the central regions of each segment with few exceptions. No obvious relationship was observed between read coverage and GC content (Fig. S2). These results indicated that FLDS effectively enriched dsRNA reads, thereby allowing the retrieval of complete genome sequences including terminal regions without the requirement for the additional rapid amplification of cDNA ends (RACE).

FLDS analysis in an environmental diatom colony

Gel electrophoresis showed that the total long dsRNA fraction from the diatom colony contained at least ten dsRNA segments, whereas genomic DNA and rRNA were the predominant in total nucleic acids (Fig. 2). Total dsRNA extracted from 1 g of the diatom colony was analyzed using the FLDS method. PCR amplicons were not observed in the dsRNA-specific RNaseIII-treated sample prior to reverse transcription (Fig. S3). As a result of *de novo* assembly and manual extension, we obtained 42 composite viral contigs (Table 1 and Table S3). More than 98.2% of reads were mapped to these 42 contigs (Table 2) as in the case of the model experiment described above. Both terminal ends of 31 of the viral contigs were identified and recognized as full-length viral genome segments. The terminal sequences of the full-length segments were used to identify segment compositions for some of the viral species because terminal sequences are highly conserved between segments in some dsRNA viral genomes for viral RNA replication and/or encapsidation (19).

Based on aa sequence similarities (E-value $\geq 1 \times 10^{-5}$) in the predicted protein-encoding sequences (CDSs), the number of genome segments in related viruses, and terminal conserved sequences in each segment of a single virus, we identified 22 viral putative composite genomes out of 31 full-length viral segments. Sequence similarities between the 22 putative viral composite genomes were used to classify them into 19 putative viral species, and each of the two genome types was identified in three species (Table 1). Seventeen dsRNA and two ssRNA viral species were identified and named Diatom Colony-Associated dsRNA virus 1–17 (DCADSRV-1–17) and Diatom

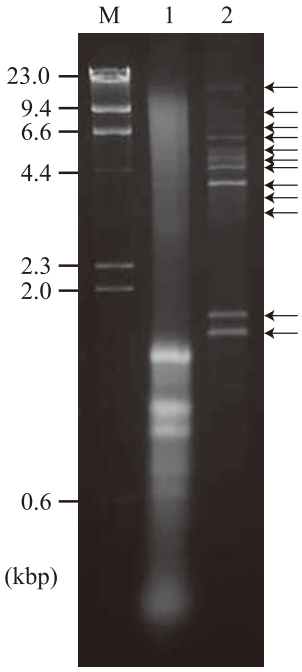

Fig. 2. Agarose gel electrophoresis of purified nucleic acids from a diatom colony. Nucleic acids were stained with ethidium bromide. Lane M, 300 ng of HindIII-digested λ DNA; lane 1, total nucleic acids extracted from 5 mg (wet weight) of the diatom colony; lane 2, purified dsRNA extracted from 1 g (wet weight) of the diatom colony.

Colony-Associated ssRNA virus 1–2 (DCASSRV-1–2) (Table 1). Since ssRNA viruses form an RNA duplex as an intermediate in genome replication, these contigs were most likely derived from replicating ssRNA viruses (11) and not from contaminant ssRNA. An additional seven full-length viral segments with predicted CDSs were also identified; however, we were unable to determine the combination of their segments or reconstruct viral genomes based on information from previously reported viruses. Thus, these viral segments were assigned as Diatom Colony-Associated Virus-Like RNA Segments (DCAVLRS-1–7).

Comparison between FLDS and total RNA-seq

Total RNA from the diatom colony was also investigated using shotgun RNA-seq in order to determine the active organisms of the colony and the abundance of viral RNA genomes in total RNA. Sequence reads derived from rRNA were identified using EMIRGE (26). The results of the analysis revealed that 56% of all trimmed reads were rRNA sequences, while 37.2% of all reads showed more than 99% identity to 18S and 23S rRNA from the diatom *Achnanthes brevipes*. In addition, 4.1 and 6.2% of reads belonged to the other diatom genus *Cylindrotheca* and chlorophyte genus *Cladophora*, respectively. The relative abundance of the rRNA reads was shown in Table S4.

Only 0.3% of reads from total RNA-seq was mapped on the major viral contigs obtained using FLDS with a read mapping algorithm in the CLC workbench (Table 2). Comparisons of the relative read frequencies of each major viral contig between total RNA-seq and FLDS revealed that FLDS achieved 0.8–4372.3-fold enrichment for each viral contig (653.2 mean) (Fig. 3). FLDS also had apparent advantages in uniform read coverage and efficiency for retrieving terminal sequences (Fig. 4). Sequence reads for ssRNA viruses in FLDS were also more abundant than when RNA-seq was used for four out of five ssRNA contigs. In addition, by *de novo* assembly, only six partial viral contigs were obtained using RNA-seq, and no viral contigs specific for total RNA-seq were found. Accordingly, we concluded that FLDS is more efficient than total RNA-seq for the detection and identification of RNA viruses, with the exception of retroviruses, which theoretically cannot be identified using FLDS.

Phylogenetic analysis and characterization of viral RNA genomes

A phylogenetic analysis of viral RNA replicases (RNA-dependent RNA polymerase; RdRp) presented the phylogenetic relationship between viral genomes from a diatom colony and known RNA viruses (Fig. S4). Viruses belonging to the family *Totiviridae* harbor non-segmented dsRNA genomes and form isometric virions that infect either fungi or protozoa (21). Thirteen composite genomes of *Totiviridae*-related viruses were identified and classified into four clades distinct from the five characterized genera of *Totiviridae* (clades a–d in Fig. S4A). Clade c was the sister clade of the proposed genus "*Trichomonasvirus*" and clade d included *Ustilaginoidea virens* RNA virus 1 (UvRV1). In general, –1 ribosomal frameshift signals [the XXXYYYZ motif (4), in which XXX may be any three identical nucleotides, YYY may be either AAA or UUU, and Z may be A, U, or C] or +1 ribosomal frameshift signals [CCCUUUU (14) or UCCUUUCGU (47)] were located in the upstream region of the 2nd CDS, and were used in the expression of overlapping viral genes such as the *pol* (RdRp) of *Totivirus* and *Leishmaniavirus*. These regions were examined in an attempt to better classify the identified viruses. However, as in the case of UvRV1, –1 or +1 ribosomal frameshift signals were not found in any of the *Totiviridae* genomes obtained in this study. CDSs in the predicted *Totiviridae* virus-like segments DCAVLRS-3 and DCAVLRS-4 showed significant similarities with the *gag* (coat protein; CP) and *pol* (RdRp) of known totiviruses, respectively. *Totiviridae* genomes consist of a single genome segment that encodes the two essential CDSs, whereas DCAVLRS-3 and -4 lacked *pol* and *gag*, respectively. These two segments harbored nine identical 5′-terminal nucleotide sequences, which were distinguishable from the other identified terminal viral sequences. Genomic features implied that DCAVLRS-3 and -4 may be parts of a bisegmented viral genome. RdRp in DCADSRV-1 segment 2 showed significant homology with that in fox *Picobirnavirus*, a member of the *Picobirnaviridae*, although DCADSRV-1 was phylogenetically distinct from the known *Picobirnaviridae* viruses (Fig. S4B). Picobirnaviruses are small, non-enveloped, bisegmented dsRNA viruses that infect animals and humans (21). The genome structure of DCADSRV-1 was similar to that of the known *Picobirnaviridae* (21). DCADSRV-14 was classified into the genus *Deltapartitivirus* of the family *Partitiviridae* based on the predicted RdRp sequence (Fig. S4C) (29). To date, all of the alphacryptoviruses have been identified from plants including the angiosperm, gymnosperm, and chlorophytes (29). rRNA sequences belonging to the *Streptophyta*, including land plants, have not yet been

Table 1. List of complete composite genomes of RNA viruses and full-length virus-like RNAs obtained from a diatom colony obtained using FLDS.

RNA virus species	Accession	Description	Size (nt)	Num. of mapped reads	Average coverage	BlastX analysis		
						Top Hit for each CDS, Virus family	E-value	Protein
DCADSRV-1[a)]	AP014890	segment 1	1,734	1,301,278	191,942	—	—	—
	AP014891	segment 2	1,562	1,717,396	279,580	Fox *Picobirnavirus* *Picobirnaviridae*	1×10^{-33}	RdRp
DCADSRV-2	AP014892		4,026	1,337,570	83,876	*Ustilaginoidea virens* nonsegmented virus 1 Not assigned	5×10^{-15}	RdRp
DCADSRV-3	AP014893		4,911	14,544	703	*Ustilaginoidea virens* RNA virus 1 *Totiviridae*	2×10^{-63}	RdRp
DCADSRV-4	AP014894	Genome type A	4,982	12,325	591	*Aspergillus mycovirus* 178 *Totiviridae*	4×10^{-69}	RdRp
DCADSRV-4	AP014895	Genome type B	4,979	1,074	52	*Ustilaginoidea virens* RNA virus 1 *Totiviridae*	5×10^{-69}	RdRp
DCADSRV-5	AP014896		5,252	7,863	359	*Aspergillus foetidus* slow virus 1 *Totiviridae*	3×10^{-74}	RdRp
DCADSRV-6	AP014897		4,939	2,720	131	*Aspergillus mycovirus* 178 *Totiviridae*	2×10^{-66}	RdRp
DCADSRV-7	AP014898		5,327	1,957	87	*Gremmeniella abietina* RNA virus L1 *Totiviridae*	3×10^{-123}	RdRp
						Ustilaginoidea virens RNA virus 3 *Totiviridae*	2×10^{-56}	CP
DCADSRV-8	AP014899		4,660	1,163	60	*Aspergillus foetidus* slow virus 1 *Totiviridae*	8×10^{-57}	RdRp
DCADSRV-9	AP014900	Genome type A	4,844	1,198	60	*Magnaporthe oryzae* virus 2 *Totiviridae*	1×10^{-65}	RdRp
DCADSRV-9	AP014901	Genome type B	4,845	364	18	*Aspergillus foetidus* slow virus 1 *Totiviridae*	2×10^{-66}	RdRp
DCADSRV-10	AP014902		5,082	1,244	59	*Rosellinia necatrix* victorivirus 1 *Totiviridae*	2×10^{-108}	RdRp
						Ustilaginoidea virens RNA virus 1 *Totiviridae*	6×10^{-50}	CP
DCADSRV-11	AP014903		5,160	1,173	55	*Ustilaginoidea virens* RNA virus 1 *Totiviridae*	4×10^{-128}	RdRp
						Ustilaginoidea virens RNA virus 1 *Totiviridae*	8×10^{-64}	CP
DCADSRV-12	AP014904		5,941	1,219	49	*Beauveria bassiana* RNA virus 1 *Totiviridae*	1×10^{-40}	RdRp
DCADSRV-13	AP014905		4,671	820	42	*Aspergillus foetidus* slow virus 1 *Totiviridae*	4×10^{-58}	RdRp
DCADSRV-14[a)]	AP014906	segment 1	1,576	438	67	Persimmon cryptic virus *Partitiviridae*	3×10^{-97}	RdRp
	AP014907	segment 2	1,490	274	43	—	—	—
DCADSRV-15	AP014908		12,172	1,482	29	*Chalara endornavirus* CeEV1 *Endornaviridae*	1×10^{-115}	Polyprotein
DCASSRV-1	AP014912		11,413	1,011	21	Border disease virus—BD31 *Flaviviridae*	4×10^{-15}	Polyprotein
DCASSRV-2	AP014913		4,586	4,153	224	*Tuber excavatum* mitovirus *Narnaviridae*	5×10^{-20}	RdRp
DCADSRV-16	AP014909		6,635	8,735	310	*Rhizoctonia fumigata* mycovirus Not assigned	4×10^{-10}	RdRp
DCADSRV-17	AP014910	Genome type A	5,907	5,325	218	dsRNA virus environmental sample Not assigned	7×10^{-14}	RdRp
DCADSRV-17	AP014911	Genome type B	5,909	1,564	63	*Botrytis porri* RNA virus 1 Not assigned	1×10^{-13}	RdRp
DCAVLRS-1	AP014914	Interrupted RdRp	4,567	57,802	3,039	*Ustilaginoidea virens* nonsegmented virus 1 Not assigned	3×10^{-11}	RdRp
DCAVLRS-2	AP014915	Interrupted RdRp	4,786	41,181	2,100	*Ustilaginoidea virens* nonsegmented virus 1 Not assigned	2×10^{-11}	RdRp
DCAVLRS-3	AP014916	CP only	3,458	13,140	876	*Ustilaginoidea virens* RNA virus 1 *Totiviridae*	2×10^{-41}	CP
DCAVLRS-4	AP014917	RdRp only	3,190	3,995	294	*Magnaporthe oryzae* virus 2 *Totiviridae*	2×10^{-123}	RdRp
DCAVLRS-5	AP014918	CP only	3,262	1,331	96	*Phomopsis vexans* RNA virus *Totiviridae*	5×10^{-47}	CP
DCAVLRS-6	AP014919	RdRp only	3,325	891	65	*Ustilaginoidea virens* RNA virus 3 *Totiviridae*	6×10^{-102}	RdRp
DCAVLRS-7	AP014920	Interrupted RdRp	1,986	164	20	*Flammulina velutipes* browning virus *Partitiviridae*	4×10^{-63}	RdRp

[a)] The classification was based on the shared 5′ terminal sequences in paired segments, whereas CDSs in the segments that did not show significant similarities with genes in databases.

Table 2. Classification of next-generation sequencing reads obtained by FLDS and total RNA-seq.

	FLDS		total RNA-seq	
	Num. of reads	rate (%)	Num. of reads	rate (%)
Trimmed	4,631,738	100.0	6,979,561	100.0
Major viral reads	4,549,629	98.2	24,036	0.3
Unmapped reads (include minor viral reads)	82,109	1.7	6,955,525	99.6

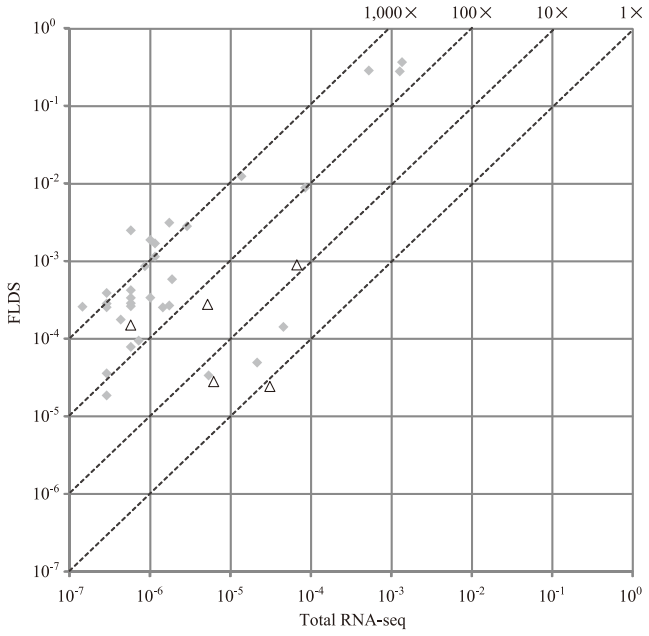

Fig. 3. Comparison of mapped read frequencies for each viral contig between FLDS and total RNA-seq. Plots indicate each viral contig. The rhombus and triangle plots show dsRNA and ssRNA viral contigs, respectively. 10^0–10^{-7} represent the frequencies of reads in each library. Dotted lines with 1×, 10×, 100×, or 1000× show a higher viral read frequency than that with an RNA-seq analysis. Reads mapped with nine contigs found in FLDS were not found in total RNA-seq.

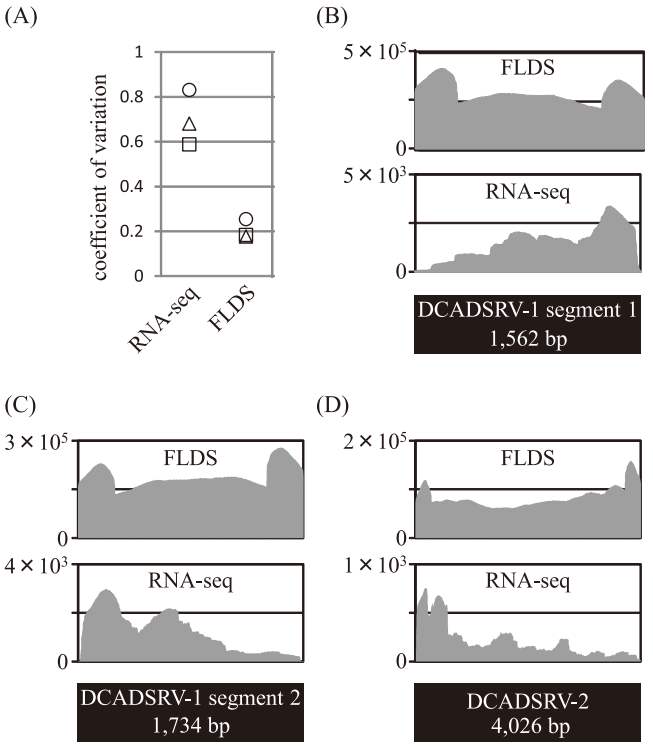

Fig. 4. Comparison of coverage uniformity between FLDS and RNA-seq. DsRNA segments with an average depth of > 200 in RNA-seq were used for the analysis. (A) Coefficient of variation (the ratio of the standard deviation to the mean coverage). Values were plotted on viral dsRNA segments of DCADSRV-1 segment 1 (square), DCADSRV-1 segment 2 (triangle), and DCADSRV-2 (circle), and were plotted on the Y axis. (B–D) Genomic coverage of each viral segment from the FLDS (upper graph) and RNA-seq (lower graph) analysis.

detected by an RNA-seq analysis, whereas *Cladophora* sp. of the *Chlorophyta*, a sister division of *Streptophyta*, were detected (Table S4). The CDSs of DCADSRV-15 and a few viral contigs showed significant homology with viruses belonging to the *Endornaviridae* (dsRNA), *Naranviridae* (ssRNA), or *Hypoviridae* (ssRNA), whose virion formation has not yet been observed.

In the ssRNA viral population, RdRp in DCASSRV-1 presented a close relationship with Border disease virus—BD31 (E-value = 4×10^{-15}), a member of the genus *Pestivirus* of the family *Flaviviridae*, which consists of the arthropod-borne pathogens of humans and other animals. The genome size and CDS structure of DCASSRV-1 (11.4 kb) were similar to those of *Flaviviridae* (9.6–12.3 kb) (21), and the phylogenetic tree of RdRp indicated that DCASSRV-1 was not classified into the three known *Flaviviridae* genera (Fig. S4D). A phylogenetic analysis of RdRp in DCASSRV-2 suggested that the RNA virus was classified into the genus *Mitovirus*, which has a non-segmented ssRNA genome, infects the mitochondria of fungi, and lacks viral particles (Fig. S4E). The presence of multiple UGA codons suggested that the putative coding strand of DCASSRV-2 was likely to be translated in mitochondria. The genome size of DCASSRV-2 (4.5 kb) was larger than those of the known mitoviruses (2.3–3.6 kb) (17).

Discussion

This study revealed the presence of novel RNA viruses associated with a diatom colony and inferred the unexpected evolutionary relationship between environmental viruses and pathogenic animal viruses. Among the RNA viral genomes obtained in this study, some dominant populations showed greater similarities to fungal viruses than to known diatom viruses; however, several ssDNA and ssRNA viruses have already been identified from marine diatoms (20, 43). We cannot exclude the possibility that these viral genomes were derived from fungi associated with a diatom colony, but it is more likely that they came from the major components of a diatom colony because of their high abundance in the RNA viral metagenomic library. Since extracellular viral particles have been a major target of virus surveillance and isolation, information on intracellular viruses in microorganisms is very limited (13, 37). Therefore, the accumulation of knowledge on intracellular RNA viruses infecting diverse host organisms is essential for understanding the evolution and distribution of RNA viruses.

FLDS revealed 22 full-length and some partial composite viral RNA genomes associated with a diatom colony by *de novo* assembly. These were classified into five dsRNA (*Totiviridae*, *Endornaviridae*, *Picobirnaviridae*, *Cystoviridae*, and *Partitiviridae*) and four ssRNA (*Flaviviridae*, *Narnaviridae*, *Virgaviridae*, and *Hypoviridae*) virus families. To the best of our knowledge, this is the largest number of full-length genome sequences of novel RNA viruses identified in one metagenomic library. The viral RNA community successfully detected in this study consisted of dsRNA viruses with or without virion formation and ssRNA viruses detected as replicative intermediates. Our results suggest that FLDS has the potential to detect a wide range of RNA viruses, excluding retroviruses.

Several studies have been performed using metagenomic analyses targeting dsRNA with Next-Generation Sequencing technology. In these studies, viral read abundance reached a maximum of 52.7% (2). In contrast, FLDS provided extremely high viral read abundance. The improvement in viral read rates with FLDS was likely derived from [1] a combination of repeating cellulose powder column chromatography and subsequent enzymatic treatment, [2] the fragmentation and efficient thermal denaturation of dsRNA prior to cDNA synthesis, and [3] the selective duplex formation of dsRNA-derived cDNA prior to PCR amplification. Furthermore, FLDS also presented advantages in reconstructing complete genome sequences including terminal regions, which are difficult to obtain using RNA-seq and random priming methods (3). The complete sequences of viral RNA segments are beneficial for the identification of RNA virus segments, particularly in cases in which coding CDSs did not show significant similarities with viral CDSs in databases. The application of a full-length dsRNA cloning method using a PC3-T7 loop primer (8) to fragmented dsRNA enabled us to determine the terminal regions of long dsRNA genomes. Since T4 RNA ligase requires a 5′ phosphoryl-terminated nucleic acid donor (PC3-T7 loop primer) and 3′ hydroxyl-terminated nucleic acid acceptor for ligation activity, dsRNA fragments with 3′ terminal phosphate were not used as substrates. The terminal structures of dsRNA fragmented by ultrasound have not been reported. However, in the case of dsDNA fragmented by ultrasound, double-strand breaks occur preferentially in 5′-CpG-3′ dinucleotides, and the phosphate group is at the 5′ side of G in the products (15). In this study, fragmented dsRNAs were successfully converted into cDNA and amplified. Taking this into consideration, dsRNA fragmentation using ultrasound with Covaris S220 also produced 3′ hydroxyl-terminated fragments. Furthermore, the lack of any modifications to the 3′ hydroxyl-terminal of viral RNA genomes (21) also allowed us to retrieve the terminal regions of the RNA viral genome.

Total RNA-seq is considered to be a less-biased method for identifying RNA viruses despite the very low abundance of viral reads in general. In the present study, FLDS enriched the viral RNA reads by > 300-fold that with total RNA-seq (Table 2). Notably, FLDS produced significantly more ssRNA viral reads than total RNA-seq; however, FLDS only has the ability to detect ssRNA viruses at the replicative stage. Moreover, FLDS showed more uniform read coverage than RNA-seq. These results indicate that FLDS is more effective than total RNA-seq for revealing all RNA viruses in cellular organisms.

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MOST VALUABLE PAPER OF THE YEAR 2017

Short Communication

Predominant but Previously-overlooked Prokaryotic Drivers of Reductive Nitrogen Transformation in Paddy Soils, Revealed by Metatranscriptomics

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Waterlogged paddy soils possess anoxic zones in which microbes actively induce reductive nitrogen transformation (RNT). In the present study, a shotgun RNA sequencing analysis (metatranscriptomics) of paddy soil samples revealed that most RNT gene transcripts in paddy soils were derived from *Deltaproteobacteria*, particularly the genera *Anaeromyxobacter* and *Geobacter*. Despite the frequent detection of the rRNA of these microbes in paddy soils, their RNT-associated genes have rarely been identified in previous PCR-based studies. This metatranscriptomic analysis provides novel insights into the diversity of RNT microbes present in paddy soils and the ecological function of *Deltaproteobacteria* predominating in these soils.

Key words: paddy soils, metatranscriptomics, denitrification, dissimilatory nitrate reduction to ammonium, nitrogen fixation

Paddy soils are characterized by temporal anaerobic conditions caused by waterlogging, and the active occurrence of anaerobic biogeochemical processes (9). Among these active processes, biological reductive nitrogen transformation (RNT), *i.e.*, denitrification (NO_3^-→NO_2^-→NO→N_2O→N_2), dissimilatory nitrate reduction to ammonium (DNRA; NO_3^-→NO_2^-→NH_4^+), and nitrogen fixation (N_2→NH_4^+) contribute to less leaching of nitrogen pollutants (NO_3^-, NO_2^-, and N_2O) into the environment and the greater retention of nitrogen-based nutrients (NH_4^+) for rice plants in waterlogged paddy soils than in upland soils (8, 22). Therefore, the identification of microbial drivers of RNT in paddy soils is important for successful rice production with minimal environmental nitrogen burden.

However, a comprehensive understanding of the RNT microbial community has not yet been achieved. In order to investigate RNT microbes in paddy soils, genes encoding the enzymes that catalyze each reaction have been assessed via PCR-based culture-independent methods, as represented by a clone library analysis (13, 24). Recent studies based on bacterial genomics reported that the diversity of microbes harboring RNT genes is greater than previously considered; PCR-based methods have underrepresented this diversity because of mismatches in the sequences of the primers used (5, 10, 21), indicating the need for alternative methods without a PCR bias. Furthermore, simultaneous assessments of microbes involved in denitrification, DNRA, and nitrogen fixation in a single paddy field have not yet been performed. Moreover, limited information is available on the transcriptional profiles *in situ* of RNT microbes in paddy soils because of the small number of field studies conducted based on soil RNA, which directly implicates RNT microbial activity. In the present study, we investigated RNT-associated microbial diversity in paddy soils via a shotgun RNA sequencing analysis without any prior PCR preparation (metatranscriptomics).

In order to obtain a more complete understanding of paddy soils with various biogeochemical properties spatially and seasonally (9, 12), soil RNA extracted from paddy soils in shallow (S1, S3) and deep (S2, S4) layers under waterlogged (S1, S2) and drained (S3, S4) conditions (Fig. S1) were subjected to a metatranscriptomic analysis using an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). The sequences of RNT genes were retrieved from the metatranscriptomic libraries obtained and taxonomically annotated through a tandem similarity search with the blat and blast programs (full methods in *Supplementary information*).

Four reactions crucial to denitrification are catalyzed by the following enzymes: NO_3^- reductase (Nar), NO_2^- reductase (Nir), NO reductase (Nor), and N_2O reductase (Nos). The *nar* transcripts detected in all soil samples using metatranscriptomics were related to those of *Deltaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Acidobacteria* (Fig. 1A), suggesting that these bacterial groups are involved in the reduction of NO_3^- to NO_2^-. The *nir* transcripts were mostly derived from *Betaproteobacteria*, *Gammaproteobacteria*, and *Alphaproteobacteria* (Fig. 1A), the members of which include common denitrifiers (5); these were also frequently detected in the same paddy soils in our previous PCR-based survey (24). Furthermore, *nor* and *nos* transcripts were predominantly detected in *Deltaproteobacteria* (Fig. 1A), the transcripts of which were rarely detected via previous PCR assays (2, 24). Successive denitrification steps were considered to be associated with common denitrifiers, such as *Betaproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, and *Actinobacteria*, which harbor *nir*, *nor*, and/or *nos* (18). However, the metatranscriptomic data obtained in the present study suggested that the reduction of NO_2^- into NO was driven by these denitrifiers, and that the reduction of NO and N_2O was mainly progressed by non-denitrifiers such as

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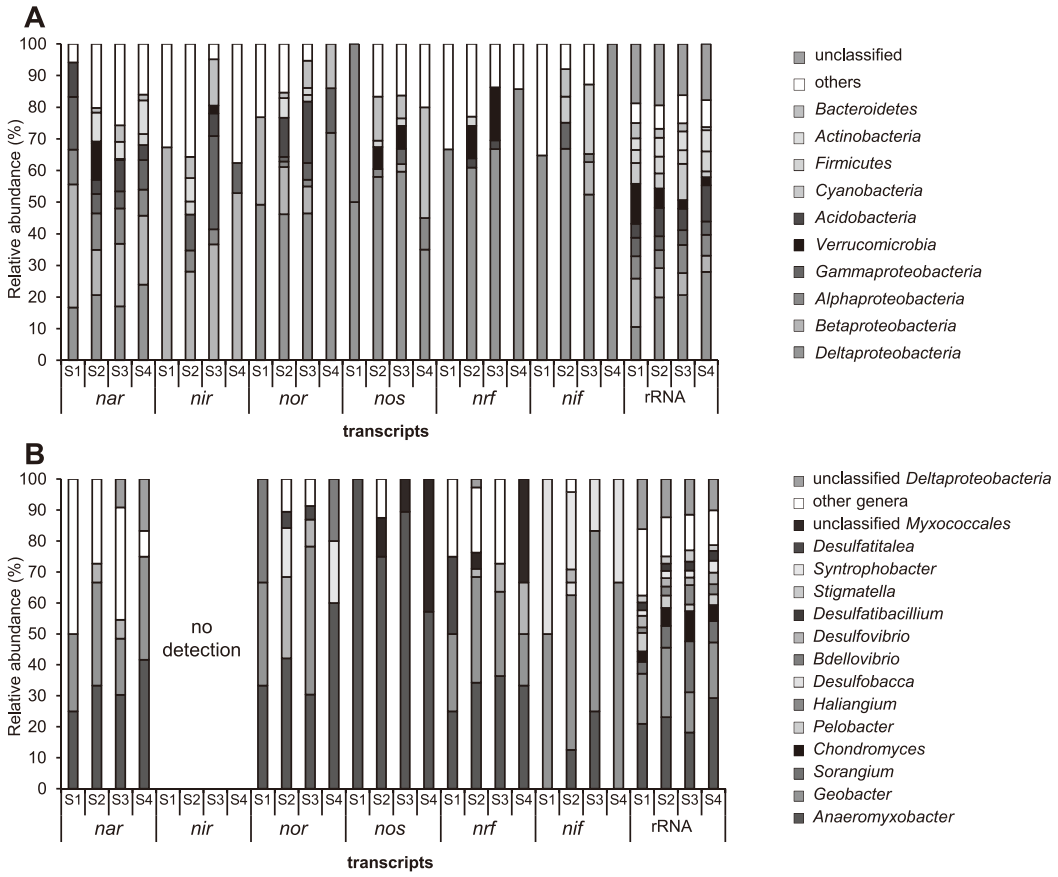

Fig. 1. Microbial diversity of RNT gene transcripts and rRNA. Taxonomic distribution of *nar*, *nir*, *nor*, *nos*, *nrf*, and *nif* transcripts, and rRNA at the phylum and proteobacterial class level (A), and deltaproteobacterial genus level (B). Sample IDs indicate data derived from paddy soils in shallow (S1, S3) and deep (S2, S4) layers under waterlogged (S1, S2) and drained (S3, S4) conditions. Data represent the mean of triplicates.

Deltaproteobacteria, *Bacteroidetes*, *Acidobacteria*, and *Verrucomicrobia*, which harbor *nor* and/or *nos*, but not *nir* (5). Thus, paddy soil denitrification appears to be a cooperative process by each nitrogen oxide reducer, *i.e.*, NO_2^- reducers (denitrifiers) and NO/N_2O reducers (non-denitrifiers), similar to nitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$) orchestrated by NH_4^+-oxidizing bacteria/archaea and NO_2^--oxidizing bacteria (6). These inferences in the denitrification process may be verified using co-culture experiments on denitrifiers and non-denitrifiers.

DNRA, another NO_3^- reduction process, is catalyzed by Nar and NH_4^+-forming NO_2^- reductase (Nrf). Most of the *nrf* transcripts belong to *Deltaproteobacteria*, while some belong to *Verrucomicrobia* (Fig. 1A). Together with the frequent detection of *nar* transcripts derived from *Deltaproteobacteria* as described above, *Deltaproteobacteria* appear to mainly contribute to DNRA dynamics in paddy soils. Although DNRA has been geochemically detected in paddy soils (1, 23), limited information is available on DNRA microbial diversity. To the best of our knowledge, the present study is the first to attempt to identify the key player groups in DNRA in paddy soils.

Diazotrophs harboring nitrogenase (Nif) drive nitrogen fixation. The taxonomic composition of *nif* transcripts was dominated by *Deltaproteobacteria* (Fig. 1A), indicating that *Deltaproteobacteria* represents a key player group in nitrogen fixation. Rhizospheric *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* and phototrophic *Cyanobacteria* were considered to be key diazotrophs in paddy soils (13, 18). However, we detected more *nif* transcripts in *Deltaproteobacteria* than in these well-known diazotrophs; our results were consistent with a recent metatranscriptomic analysis based on a microcosm study on Italian paddy soils (11).

The abundance of RNT genes derived from *Deltaproteobacteria*, as described above, was also demonstrated in a shotgun DNA sequencing analysis (metagenomics) (Fig. S2A). Additionally, the microbial community structure based on rRNA gene/transcript sequences showed that *Deltaproteobacteria* is a major group in paddy soil microbes (Fig. 1A, S2A). These results support *Deltaproteobacteria* being a key player group driving RNT in paddy soils.

Further analyses on *Deltaproteobacteria* at the genus level revealed the consistent detection of RNT gene transcripts in metatranscriptomic data derived from the genera *Anaeromyxobacter* and *Geobacter* (Fig. 1B), as well as their RNT genes in metagenomic data (Fig. S2B). These genera represent obligate anaerobes and metal reducers predominating in paddy soils (7, 20) and exhibit some RNT activities *in vitro* (summarized in Table S1). Although the nitrogen fixation activity of *Anaeromyxobacter* has yet to be characterized, the genomes of some *Anaeromyxobacter* spp. conserve a similar *nif* cluster to that of *Geobacter* spp. exhibiting nitrogen fixation activity (Fig. S3). Together with the detection of the *nif* transcripts of *Anaeromyxobacter* in this study, it is plausible that *Anaeromyxobacter* spp. perform nitrogen fixation.

However, in contrast to *Anaeromyxobacter* and *Geobacter* rRNA genes, their RNT genes have rarely been detected in paddy soil samples using PCR-based techniques (4, 24). Thus, the putative role of these genera in the RNT process has received little attention despite their predominance in paddy soils. The limited coverage of RNT gene-specific PCR primers used in previous studies may have led to the oversight of these genera (10, 17); additionally, the GC content may be implicated because the *nor*/*nos*/*nrf*/*nif* of *Anaeromyxobacter* spp. showed markedly higher GC contents than the rRNA genes and *nor*/*nos*/*nrf*/*nif* of other bacteria (Table S2). Even improved *nos* universal primers, which have enabled lower rates of sequence mismatches, were unable to amplify *Anaeromyxobacter nos* (10). Therefore, a metatranscriptomic analysis represents a more effective approach to examine the diversity of functional microbes, without any PCR bias arising from the high GC content of target genes as well as primer limitations.

Anaeromyxobacter and *Geobacter*, which have frequently been detected in Japanese, Chinese, and Italian paddy soils (4, 11, rRNA data in Fig. 1B), predominate more in paddy soils than in upland soils, as confirmed by the present study (Fig. 2A, B; Table S3). Their universal distribution and predominance in paddy soils support *Anaeromyxobacter* and *Geobacter* being the key RNT players in paddy soils. Furthermore, the predominance of these genera was found in river sediments (Fig. 2A, B; Table S3); the RNT genes of *Anaeromyxobacter* were frequently and globally detected in upland soil environments in recent shotgun metagenomics studies (14, 15), indicating the contribution of these bacteria to RNT not only in paddy soils, but also in other environments. The further application of metatranscriptomics across different environments will expand our knowledge on the diversity of RNT microbes in nature as well as the ecological function of *Deltaproteobacteria* in soil environments.

Previous studies on paddy soils identified the predominance of *Deltaproteobacteria* and their ecological roles in dissimilatory metal reduction, sulfur/sulfate reduction, and hydrogen production (7, 9, 19). Although genomic studies showed the ubiquitous possession of the RNT genes of *Deltaproteobacteria*, the association of RNT with *Deltaproteobacteria* has not been considered because of the rare detection of their RNT genes in soil environments through PCR-based analyses. The present study revealed the novel ecological functions of *Anaeromyxobacter* and *Geobacter* within *Deltaproteobacteria* dominating in paddy soils, namely, RNT, denitrification support, and NH_4^+ production via DNRA and nitrogen fixation (Fig. 3).

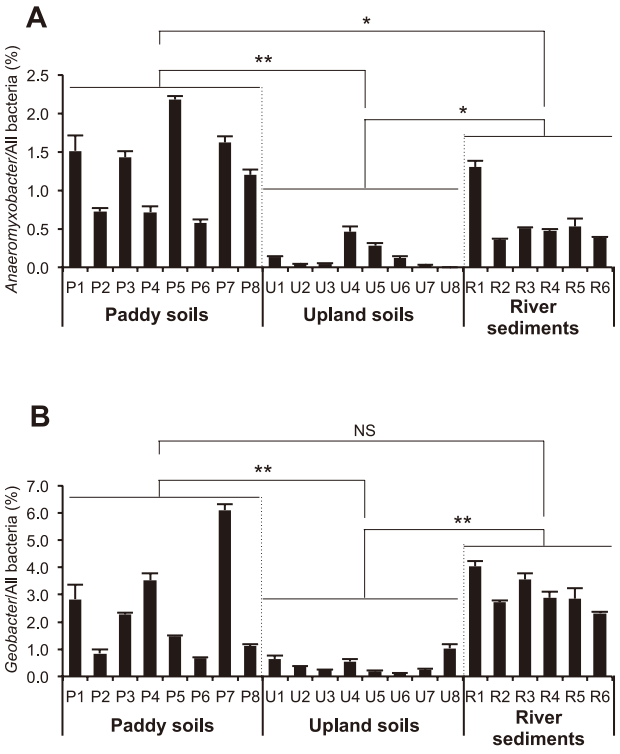

Fig. 2. Distribution of *Anaeromyxobacter* and *Geobacter* in various soil environments. Proportions of *Anaeromyxobacter* (A) and *Geobacter* (B) against all bacteria estimated by a quantitative PCR method. The mean±SD is shown (n=3). The paddy soil sample P3 was collected from the same paddy field used for the metatranscriptomic analysis in this study. Asterisks indicate significant differences (Mann-Whitney U test; *, p<0.01; **, p<0.001); NS, not significant. Details of soil samples and qPCR data are summarized in Table S3.

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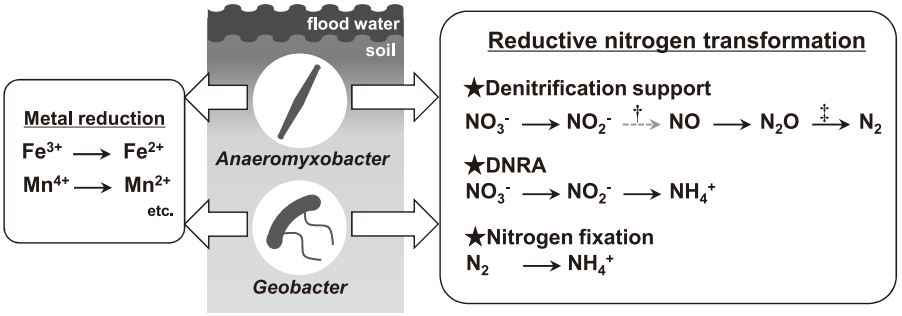

Fig. 3. Ecological functions of *Anaeromyxobacter* and *Geobacter*, belonging to *Deltaproteobacteria*, predominant in paddy soils, expanded by metatranscriptomics in this study. *Anaeromyxobacter* and *Geobacter*, ubiquitously predominant in paddy soils, are key player groups in the reduction of iron and manganese, which actively progresses in paddy soils soon after waterlogging (3, 4, 7, 9, 11, 20, Fig. 1B, S2B). Metatranscriptomics in this study suggested that these genera also associate with reductive nitrogen transformation, *i.e.*, denitrification, DNRA, and nitrogen fixation. Sketches of *Anaeromyxobacter* and *Geobacter* are based on electron microscope photographs published elsewhere (3, 16). †, NO_2^- reduction to NO is driven by common denitrifiers within *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* (5), not by *Anaeromyxobacter* and *Geobacter* within *Deltaproteobacteria*; ‡, *Anaeromyxobacter* reduce N_2O to N_2, whereas *Geobacter* do not (Table S1).

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Bacterial Community Analysis of Drinking Water Biofilms in Southern Sweden

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Next-generation sequencing of the V1-V2 and V3 variable regions of the 16S rRNA gene generated a total of 674,116 reads that described six distinct bacterial biofilm communities from both water meters and pipes. A high degree of reproducibility was demonstrated for the experimental and analytical work-flow by analyzing the communities present in parallel water meters, the rare occurrence of biological replicates within a working drinking water distribution system. The communities observed in water meters from households that did not complain about their drinking water were defined by sequences representing *Proteobacteria* (82–87%), with 22–40% of all sequences being classified as *Sphingomonadaceae*. However, a water meter biofilm community from a household with consumer reports of red water and flowing water containing elevated levels of iron and manganese had fewer sequences representing *Proteobacteria* (44%); only 0.6% of all sequences were classified as *Sphingomonadaceae*; and, in contrast to the other water meter communities, markedly more sequences represented *Nitrospira* and *Pedomicrobium*. The biofilm communities in pipes were distinct from those in water meters, and contained sequences that were identified as *Mycobacterium*, *Nocardia*, *Desulfovibrio*, and *Sulfuricurvum*. The approach employed in the present study resolved the bacterial diversity present in these biofilm communities as well as the differences that occurred in biofilms within a single distribution system, and suggests that next-generation sequencing of 16S rRNA amplicons can show changes in bacterial biofilm communities associated with different water qualities.

Key words: drinking water, biofilm, next-generation sequencing, bacterial communities, 16S rRNA pyrosequencing

Biofilms are populations of microorganisms that are typically concentrated at a solid-liquid interface and surrounded by an extracellular polymeric substance matrix (13). The presence of extracellular polymeric substances within the biofilm protects bacteria by making them more resistant to chemicals such as disinfectants (10). The environment within a drinking water distribution system (DWDS) is oligotrophic and can contain disinfectants, with more than 95% of the bacterial biomass occurring as biofilms on the inner surface of the DWDS and less than 5% existing in the planktonic form (9). Biofilms in the distribution system constitute an ecosystem that can influence the esthetic quality of drinking water by altering taste, color, and odor, and also microbial water quality through the detachment of biomass into the bulk water (22). Bacterial biofilms have also been associated with technical problems within the DWDS such as corrosion (41). Although biofilms are known to have these impacts on drinking water, little is known about the mechanisms involved; therefore, a deeper understanding of the mechanisms by which the microbes in this human-built ecosystem participate in the delivery of drinking water to consumers need to be elucidated in more detail.

Less than 1% of bacteria in fresh and drinking water are currently culturable and bacteria in drinking water biofilms can also be present in a viable but non-culturable (VBNC) state (1, 20, 37, 40). Therefore, culture-independent methods are preferable for providing a more complete picture of the microbial community; however, this type of analysis may not distinguish between dead or living cells (30). While methods using clone libraries, DGGE, and other DNA-based methods have contributed descriptions of the microbes present in drinking water biofilms (8, 33, 42), next-generation sequencing (NGS) is considered to provide the most detailed, high throughput, culture-independent method for the characterization of microbial communities. Previous studies that have examined bacterial communities present in water or biofilms of the DWDS have used the NGS of amplicons from water meter biofilms (16), clear well biofilms (44), faucet biofilms (27), and biofilms in a pilot-scale microfiltration plant that treats drinking water (21). NGS has also been used to examine the impact of changing hydraulic regimes on the bacterial biofilm community structure in an experimental distribution system (5) as well as the influence of chloramination and chlorination on planktonic bacterial communities in water samples (19). However, the reproducibility of amplicon NGS for real DWDS biofilms, the diversity present across biofilms from a single distribution system, and the feasibility of using water meters to investigate changes associated with the consumer perception of water quality have not yet been investigated.

We herein examined bacterial communities in DWDS biofilms using a deep 16S rRNA amplicon NGS analysis. To obtain a representative drinking water biofilm, samples from water meters were analyzed from a single existing distribution network in southern Sweden. The reproducibility of this approach was demonstrated by comparing biofilms from water meters installed in parallel and, thus, experiencing nearly identical hydrological environments. In addition, we

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Table 1. Samples analyzed in this study. Samples were taken in the city of Landskrona, Sweden, in which surface water is used to produce drinking water. All water meters had a rated flow of Qn 2.5 m^3 h^{-1}. A photo showing the parallel water meters (WM1 and WM 2) is included as Supplemental Fig. S3.

Sample	Type	Diameter	Age	Material	DNA extraction	PCR reactions	Sampling date	Description
WM 1 WM 2	water meter	n/a	2007	brass and plastic	Two cotton swabs	2	March 2011	Water meters installed in parallel in an apartment building
WM 3	water meter	n/a	2001	brass and plastic	Two cotton swabs	3	March 2011	Water meter from a family house
WM 4	water meter	n/a	2004	brass and plastic	Two cotton swabs	2	March 2011	Water meter from a family house, in which problems with red water have been reported
P1	pipe	50 cm	1966	cast iron	Two extractions from 200 µL of resuspended biofilm material	3	April 2011	Pipe situated in the same street as WM 1 and WM 2
P2	pipe	15 cm	1908	cast iron	Two extractions from 200 µL of resuspended biofilm material	3	June 2011	Pipe situated in the Landskrona DWDS

Table 2. Water Quality Parameters. Water was obtained from households in which different water meter sampling was conducted and submitted for a routine analysis. Representative water quality data for the city of Landskrona (Sweden) are included for comparisons. The water of WM 4 had a brown color during sampling.

	Landskrona	WM 1 + 2	WM 3	WM 4
pH	8.1	8.1	8	8.1
Conductivity [mS m^{-1}]	40.2	40.2	38.4	39.7
Hardness [°dH]	9.5	11	8.7	8.9
Calcium Ca [mg L^{-1}]	63	70	58	59
Magnesium Mg [mg L^{-1}]	3.1	3.4	2.9	2.9
Sulfate SO_4^{2-} [mg L^{-1}]	—	83	80	85
Turbidity [FNU]	0.21	0.24	<0.1	12
Iron Fe [mg L^{-1}]	<0.05	<0.05	<0.05	1.5
Manganese Mn [mg L^{-1}]	<0.02	<0.02	<0.02	0.04
Total chlorine [mg L^{-1} Cl_2]	—	0.13	0.14	0.05

showed that differences in the biofilm community composition in water meters and pipes as well as perceived water qualities within a single DWDS were resolvable.

Materials and Methods

Sampling

Six biofilm samples were collected from the DWDS of the city of Landskrona (Sweden) in March, April, and June, 2011 (Table 1). Water meter biofilms were sampled using sterile cotton transport swabs. Biofilm samples from the walls of the pipes were taken with a sterile cell spatula (TPP, Trasadingen, Switzerland) and the collected material was resuspended in approximately 5 mL of water from the sampling site. All samples were transported to the laboratory in a cooling box within 8 h after sampling and then stored at –20°C until further analyses. Water samples for water quality testing were taken from households during the sampling of water meter biofilms and the results obtained were reported together with general water quality data representative for drinking water in the city of Landskrona (Sweden) (Table 2). Water quality testing was performed by ALcontrol AB, Malmö, Sweden using standard methods (pH: SS028122-2; conductivity: SS-EN 27888-1; hardness: calculated from magnesium and calcium concentrations; calcium: SS-EN ISO 11885-1; magnesium: SS-EN ISO 11885-1; sulfate: SS-EN ISO 10304-1:2009; turbidity: SS-EN ISO 7027 utg 3, iron: SS-EN ISO 11885-1, manganese: SS-EN ISO 11885-1). Total chlorine was measured with a portable colorimeter (Hach).

Generation of amplicons

DNA was extracted from cotton swabs or 200 µL resuspended biofilm material using the Fast DNA Spin Kit for Soil (MP Biomedicals) and a bead beater. DNA from two cotton swabs was extracted for each sample, pooled together, and diluted 10-fold before PCR amplification. DNA was also extracted from empty cotton swabs and MilliQ water as negative controls for PCR. PCR amplification of a specific bacterial 16S rRNA region was performed using the primers 27F (5' GS FLX Titanium adapter A - TCAG - MID - AGAGTTTGATCCTGGCTCAG 3') and 534R (5' GS FLX Titanium adapter B - TCAG - MID - ATTACCGCGGCTGCTGGC 3') (16) with adapter A representing the forward 454 sequencing primer, adapter B representing the 454 reverse primer, and a 10-bp long sample-specific barcode (MID). Barcodes recommended by Roche were used with the following sequences: ACGAGTGCGT (MID1 for P1), ACGCTCGACA (MID2 for P2), AGACGCACTC (MID3 for WM 4), AGCACTGTAG (MID4 for WM 3), ATCAGACACG (MID5 for WM 1), and ATATCGCGAG (MID6 for WM 2). PCR reactions were carried out in a C1000 thermal cycler (Bio-Rad) and contained: 1 × PicoMaxx Reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.8 µM of each forward and reverse primer, 1.5 U PicoMaxx polymerase, and 5 µL of template DNA (diluted 10-fold) in a total volume of 30 µL. The cycling parameters were: 5 min at 95°C, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final 72°C held for 5 min. Two to three PCR reactions were carried out for each biofilm sample, and pooled together for amplicon purification. The FAST DNA Spin Kit for Soil has been proposed as the method of choice for DWDS DNA extraction, generating representative community information and reproducibility, while PicoMaxx polymerase efficiently amplifies low amounts of DNA in the presence of PCR inhibitors (15, 18). A high concentration of primers was used to outcompete partially extended primers and reduce the formation of chimeras (36).

Amplicon library preparation

Pooled amplicons were purified using the E.Z.N.A® Cycle Pure Kit (OMEGA, Bio-tek) and Cycle-Pure Spin Protocol according to the manufacturer's instructions. Sequencing was conducted at the Lund University Sequencing Facility, Sweden. Pooled amplicons

Table 3. Overview of NGS Results. F represents the forward sequencing direction covering the V1-V2 region of the bacterial 16S rRNA gene and R represents the reverse sequencing direction covering the V3 region.

	chimeras	cleaned sequences	subsampled	OTUs	singletons	doubletons	OTUs[a]
WM 1 - F	218	35,662	26,466	824	289	159	185
WM 2 - F	212	30,346	26,466	669	260	108	177
WM 3 - F	160	45,433	26,466	923	376	171	129
WM 4 - F	247	26,466	26,466	818	218	123	227
P1 - F	189	30,701	26,466	282	93	45	126
P2 - F	495	26,695	26,466	622	198	106	184
WM 1 - R	638	51,553	26,466	622	239	105	154
WM 2 - R	510	42,893	26,466	537	209	106	151
WM 3 - R	271	66,700	26,466	709	275	128	133
WM 4 - R	590	42,174	26,466	543	162	82	165
P1 - R	205	53,023	26,466	216	82	30	105
P2 - R	1,611	42,307	26,466	458	152	66	144

[a] OTUs in which at least one of the six samples contained 20 sequences.

were reduced for short fragments by using Agencourt AMPure XP (Beckman Coulter) and inspected using a DNA 1000 kit on a 2100 Bioanalyzer (Agilent). Amplicons were quantified using the Quant-iT dsDNA assay kit (Invitrogen) and Quantifluor fluorometer (Promega), and pools were diluted to obtain a total of 1×10^7 copies μL^{-1}. Titration and library production (aiming at 10–15% enrichment) were performed using emulsion PCR and the Lib-A kit (Roche). DNA-positive beads were enriched, counted on an Innovatis CASY particle counter (Roche), processed using the XLR70 sequencing kit (Roche), and loaded onto a picotiter plate for pyrosequencing on a 454 Life Sciences Genome Sequencer FLX machine (Roche). DNA sequences were archived at NCBI SRA under the accession number SRP039011.

Data analysis

Amplicons were sequenced from both directions, with reads from forward (V1-V2 region) and reverse (V3 region) directions being treated separately during data analysis following the cleaning step (Supplemental Fig. S1). Reads were sorted by a barcode using sfffile (SFF Tools, Roche). The barcode and TCAG-tags were also removed using sfffile. Reads were quality filtered using PrinSeq lite (v 0.19.3) (34) and custom Perl scripts and discarded if they were shorter than 220 bp, longer than 650 bp, contained ambiguous base pairs, or had a mean quality score below 25. Reads were trimmed after 350 bp or if the mean quality score within a 50 bp sliding window with a step size of 1 was less than 35. Reads with a perfect match to the primer in the 5' region (27F or 534R) were kept and the sequence information corresponding to the primer region was removed. Reads from the reverse direction were converted to their reverse complement using custom Perl scripts. Chimera checking was done using Uchime (7) in the *de novo* mode after de-replication as implemented in Usearch (v 5.2.32) (6). Reads detected as chimeras were removed from the dataset. In order to avoid bias, sequences were randomly subsampled without replacement to the smallest sample size using the Perl script daisychopper.pl (http://www.genomics.ceh.ac.uk/GeneSwytch/Tools.html v0.6) (11). The subsampled sequences were classified using the command line version of the RDP classifier (v. 2.5) (43) and clustered using CROP (v133) with the option -s corresponding to a 97% sequence identity (14). The parameters were 3176 for -b and 480 for -z for both forward and reverse reads. CROP was run on the Lunarc supercomputer at Lund University. Only clusters with at least 20 sequences in one of the six samples were kept to construct a phylogenetic tree. Metaxa (v 1.1) was applied to detect sequences of chloroplasts, mitochondria, archaea, and eukaryotes (2). Sequences were aligned using Greengenes (4) (greengenes.lbl.gov), and a phylogenetic tree was constructed using RAxML (38) and displayed in iTOL (24). OTUs detected by Metaxa as being chloroplasts or having an uncertain origin, or OTUs that did not align to the Greengenes reference dataset were removed before construction of the phylogenetic tree (see Supplemental Fig. S2).

Venn diagrams were constructed using information from the OTU tables and plotted with the Venn Diagram Plotter (http://omics.pnl.gov/software/VennDiagramPlotter.php). A heatmap of the 50 most abundant OTUs was constructed using the pheatmap package in R (http://cran.r-project.org/web/packages/pheatmap/pheatmap.pdf).

Results

16S rRNA gene amplicon NGS of DWDS biofilms

Six biofilm samples from a single DWDS were selected for a detailed community analysis using NGS of the 16S rRNA gene. The aim was to permit comparisons of both dominant and rare members of the communities to determine whether the community changed with location as well as the number of sequences required to resolve these differences between samples.

Sequences obtained from the forward reads encompassed the V1-V2 region of the 16S rRNA gene while reverse reads corresponded to the V3 region. A total of 674,116 reads describing the six bacterial biofilm communities were initially obtained; 174,817 of these reads did not meet the quality requirements and were removed, and random subsampling selected 52,932 sequences (26,466 for each read direction) to represent each biofilm community (Table 3). Sequences used for analyses ranged in length from 200–332 bp, with an average length of 312 bp after trimming and cleaning. Sequences describing either the V1-V2 region or V3 region were classified using the RDP classifier at a confidence level of 80%. Classification of the V1-V2 and V3 regions showed highly similar trends with respect to the community composition for each biofilm sample at the phylum (Fig. 1) and class (Fig. 2) level.

Sequences were clustered into OTUs (phylotypes), applying a threshold corresponding to 97% identity. OTUs relevant for comparisons of the distinct biofilm bacterial communities were restricted to those containing 20 or more sequences from any one of the six biofilm samples in order to avoid including sequences containing errors introduced during sequencing or PCR amplification (17). The removal of rare sequences reduced the number of OTUs from 2,383 to 308. The number of OTUs representing each of the six biofilm communities ranged from 126 to 227 (Table 3, V1-V2 region) with 57 of these 308 OTUs being present in all the biofilm

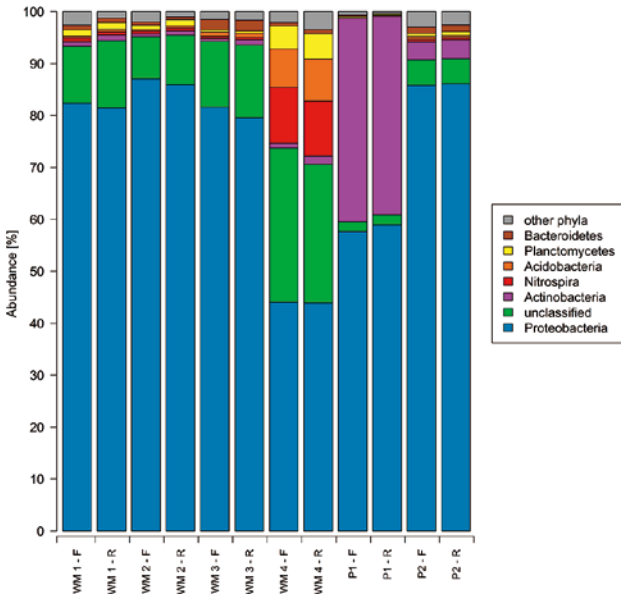

Fig. 1. Relative abundance of bacterial phyla from water meters and pipes. F represents the forward sequencing direction covering the V1-V2 region of the bacterial 16S rRNA gene and R represents the reverse sequencing direction covering the V3 region of the bacterial 16S rRNA gene.

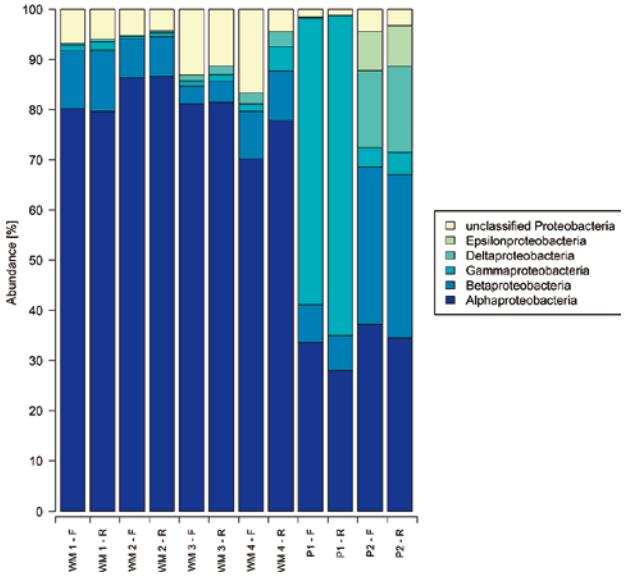

Fig. 2. Relative abundance of *Proteobacteria* from water meters and pipes. 100% corresponds to all sequences classified as *Proteobacteria*. F represents the forward sequencing direction covering the V1-V2 region of the bacterial 16S rRNA gene and R represents the reverse sequencing direction covering the V3 region of the bacterial 16S rRNA gene.

communities examined. Since comparisons of information obtained from the V1-V2 and V3 regions by both RDP classification (Fig. 1 and Fig. 2) and OTU frequencies (Fig. 3, 4, Table 4 and 5) gave similar results, the V1-V2 region was chosen to facilitate comparisons between the different biofilm communities.

Assessment of reproducibility using biofilm communities from parallel water meters

Bacterial biofilm communities in water meters (WM) installed

	OTU based (presence/absence)		OTU and sequence based	
Samples	Venn diagram	Shared OTUs	Venn Diagram	Shared sequences
WM 1 – WM 2		163		19,804
WM 3 – WM 1		114		11,179
WM 3 – WM 2		113		15,818
WM 4 – WM 3		102		2,111
P1 – P2		102		5,600
P1 – WM 1		110		5,752
P1 – WM 2		106		6,009

Fig. 3. Comparison of OTUs and sequences for selected sample combinations. Venn diagrams are shown comparing the presence and absence of OTUs and shared sequences for the V1-V2 region for selected combinations of samples. The left circle represents the first of the two samples listed in the first column. Only OTUs with at least 20 sequences in one of the six samples were considered in the presence/absence comparison (left panel). The number of OTUs in each sample can be found in Table 3. All OTUs and sequences were considered in the sequence-based comparison (right panel). Each sample contained 26,466 sequences. The number of shared sequences between samples was determined for each OTU and then summed to give the total number of all shared sequences for the samples being compared.

in parallel within the DWDS were physically present on distinct surfaces of two water meters (WM 1 and WM 2), and had developed for four years within an apartment building that had experienced identical temperatures, seasons, water flow, and source water (Supplemental Fig. S3). The analysis of these two communities was used to examine the reproducibility of the established high resolution sequencing work-flow for determining community compositions, OTU frequencies, and phylotypes.

WM 1 and WM 2 were dominated at the phylum level by *Proteobacteria* (82% for WM 1; 87% for WM 2), and unclassified Bacteria (11% for WM 1; 8% for WM 2, Fig. 1). A total of 185 OTUs were identified in WM 1 and 177 OTUs in WM 2, with the two communities sharing 163 OTUs and 75% of the sequences (Fig. 3). A heatmap illustrating the 50 most abundant OTUs showed highly similar profiles of OTU frequencies for both water meters (Fig. 4).

The phylotype-based analysis showed that the most abundant OTU was classified to the family level as *Sphingomonadaceae* in both WM 1 (22%) and WM 2 (36%). Other abundant OTUs were *Hyphomicrobium* (WM 1: 5% and WM 2: 6%) and unclassified *Proteobacteria* (WM 1: 6% and WM 2: 5%).

To determine whether the bacterial biofilm community changed within a single DWDS or if the water meter biofilms were similar throughout the same DWDS, biofilms were sampled from a third water meter (WM 3) connected to the same distribution system a few kilometers away from the WM 1 and 2 sampling site.

Table 4. Classification of the 20 most abundant OTUs for the V1-V2 region. OTUs are from the bacterial 16S rRNA gene found in the six drinking water biofilm samples from water meters and pipes. Sequences for the OTUs are found in Supplemental Table S1.

Phylum	Class	Order	Family	Genus	WM 1 - F	WM 2 - F	WM 3 - F	WM 4 - F	P1 - F	P2 - F
Actinobacteria	*Actinobacteria*	*Acidimicrobiales*			0	0	0	0	1,875	0
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Mycobacteriaceae*	*Mycobacterium*	118	69	81	4	3,369	859
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Nocardiaceae*	*Nocardia*	2	3	2	3	5,050	2
Gemmatimonadetes	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas*	523	318	6	7	87	141
Nitrospira	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*	196	146	117	2,852	2	137
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Hyphomicrobium*	1,401	1,715	580	751	1,701	664
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Pedomicrobium*	488	363	8	1,949	78	553
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*		1,144	2,332	5,171	102	543	380
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*			3,723	2,263	59	44	321	423
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*			378	336	55	109	146	205
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*			570	312	89	76	99	71
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*		5,952	9,578	10,689	149	581	604
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*		935	617	17	48	160	369
Proteobacteria	*Alphaproteobacteria*				1,084	807	12	715	265	623
Proteobacteria	*Betaproteobacteria*				753	579	10	334	98	1,466
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiales_incertae_sedis*	*Methylibium*	85	27	7	33	24	1,434
Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	1	1	0	0	1	2,470
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Sulfuricurvum*	1	0	0	0	0	1,476
Proteobacteria	*Gammaproteobacteria*				0	0	2	0	8,621	0
Proteobacteria					1,542	1,258	3,490	56	106	13
				Percentage of total sequences	71%	78%	77%	27%	87%	45%

Table 5. Classification of the 20 most abundant OTUs for the V3 region. OTUs are from the bacterial 16S rRNA gene found in the six drinking water biofilm samples from water meters and pipes. Sequences for the OTUs are found in Supplemental Table S1.

Phylum	Class	Order	Family	Genus	WM 1 - R	WM 2 - R	WM 3 - R	WM 4 - R	P1 - R	P2 - R
Actinobacteria	*Actinobacteria*	*Acidimicrobiales*			0	3	0	2	1,630	1
Actinobacteria	*Actinobacteria*	*Actinomycetales*			146	106	103	101	8,445	919
Nitrospira	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*	168	157	119	2,800	5	94
Proteobacteria	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*		998	667	6	677	193	523
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*		7,398	6,937	5,832	4,007	2,645	2,530
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*			365	251	10	145	247	432
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*		6,913	10,667	10,808	254	614	1,166
Proteobacteria	*Alphaproteobacteria*				257	217	75	1,175	55	400
Proteobacteria	*Alphaproteobacteria*				253	206	53	1,204	104	235
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*		619	331	53	46	53	1,015
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Herminiimonas*	562	420	21	38	375	409
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*			131	30	16	62	49	1,800
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*		259	170	52	245	102	629
Proteobacteria	*Betaproteobacteria*				840	639	10	369	92	1,599
Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	2	0	0	0	0	2,779
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Sulfuricurvum*	4	2	0	0	0	1,854
Proteobacteria	*Gammaproteobacteria*				0	2	12	0	9,805	0
Proteobacteria					1,588	1,225	3,596	38	65	2
					439	298	21	905	87	141
					0	0	0	1,820	0	0
				Percentage of total sequences	79%	84%	79%	52%	93%	62%

WM 3 was also dominated by *Proteobacteria* and gave a similar overall picture at the phylum and class levels as WM 1 and WM 2 (Fig. 1 and Fig. 2). Sequences from WM 3 were classified into the 20 most abundant OTUs in largely the same proportions as those classified for WM 1 and WM 2 (Table 4). These included OTUs of *Sphingomonadaceae* (40%), *Hyphomicrobiaceae* (20%), and unclassified *Proteobacteria* (13%). The diversity of WM 3 was represented by slightly fewer OTUs (a total of 129) than WM 1 and WM 2 (185 and 177, respectively) (Table 3). WM 3 shared 114 OTUs and 42% of the sequences with WM 1, and 113 OTUs and 60% of the sequences with WM 2 (Fig. 3).

A bacterial biofilm from an area with unacceptable water quality

A biofilm from a fourth water meter within the same DWDS was sampled from a location in which problems with water quality had been reported by the consumer in order to determine whether changes within the bacterial biofilm community associated with changes in water quality could be resolved.

Differences were observed at the phylum level between WM 4 and WM 1-3. *Proteobacteria* were less abundant than in the other water meters (44% in WM 4 and 82–87% in WM 1-3). However, the composition of classes within the *Proteobacteria* remained the same across all WMs with

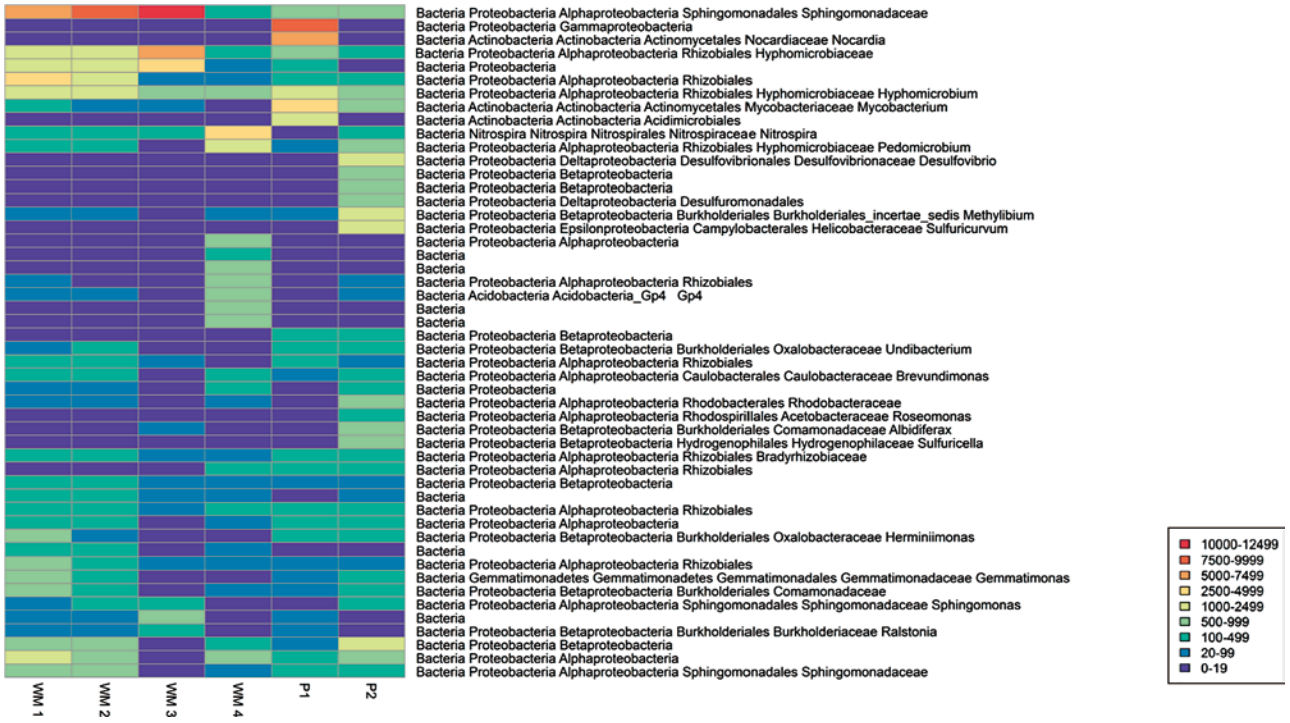

Fig. 4. Heatmap of the 50 most abundant OTUs for the V1-V2 region. High similarity was observed in the number of sequences for the selected OTUs for WM1 and WM2, while other samples were distinguished by the presence or absence of specific OTUs. The legend shows the number of sequences corresponding to the different colors of the heatmap.

Alphaproteobacteria as the most abundant class (Fig. 2). *Planctomycetes* (4%), *Acidobacteria* (7%), and *Nitrospira* (11%) were present in WM 4 and virtually absent in any of the other samples. The amount of unclassified bacteria in WM 4 was the highest at 30%, while these amounts were only 2–13% in the other five samples.

Within the 20 most abundant OTUs, only 1% of the WM 4 sequences belonged to the OTU classified as family *Sphingomonadaceae*.

Certain OTUs contained a large number of sequences from WM 4, but were not represented in other WMs, including sequences classified as genus *Nitrospira* (11%) and genus *Pedomicrobium* (7%) for WM 4.

WM 4 contained 227 OTUs, the highest number of OTUs found in any of the six biofilm samples. WM 4 and WM 3 shared 102 OTUs, similar to the amount of shared OTUs observed between other samples (Fig. 3); however, only 8% of the sequences were shared. Differences in the abundance of the sequences with the OTUs obtained from WM 4 differentiated this bacterial biofilm community from those of WM 1-3: the heatmap profile of OTU frequency (Fig. 4) showed that the distribution of sequences across OTUs was distinct for WM 4.

Bacterial community composition in DWDS pipes

A biofilm was sampled from a pipe (P1) to compare the pipe bacterial biofilm community adjacent to the biofilm communities established on water meters. A second pipe (P2) was selected as a biofilm community from the same DWDS, but with a number of distinct characteristics such as the age of the biofilm (Table 1).

An analysis at the phylum level (Fig. 1) showed that P1 was dominated by *Proteobacteria* (58%) and *Actinobacteria* (39%), with other phyla accounted for less than 3% of the sequences.

The classification identified specific OTUs containing large numbers of sequences that were mainly found in P1. These included class *Gammaproteobacteria* (33%); order *Acidomicrobiales* (7%); and within order *Actinomycetales*, *Mycobacterium* (13%) and *Nocardia* (19%). P1 contained fewer sequences belonging to the family *Sphingomonadaceae* (2%) than those describing WM 1-3.

P1 contained 126 OTUs, the fewest OTUs found in any of the samples examined, sharing 110 OTUs with WM 1 and 106 OTUs with WM 2 (Fig. 3). As observed with WM 4, despite the number of shared OTUs between samples, P1 shared fewer sequences with WM 1 (21%) and WM 2 (23%).

At the phylum level, sequences obtained from P2 consisted of 86% *Proteobacteria* and 5% unclassified Bacteria with other phyla accounting for less than 3%, as observed for P1. Within *Proteobacteria*, both pipes consisted of sequences more widely distributed across the different classes, and were not predominated by *Alphaproteobacteria* as observed for WM 1-3 and WM 4 (Fig. 2). A small overlap in the community structure was observed between P1 and P2. OTU frequencies gave a heatmap profile of P2 that was distinct from all other samples, with the highest OTU frequencies appearing in OTUs that were not predominant in any other sample examined; for example *Desulfovibrio* (9%), and *Sulfuricurvum* (6%) (Fig. 4), and, although a number of the OTUs themselves were shared, only 21% of the sequences were shared between P1 and P2 (Fig. 3).

Discussion

The bacterial communities present in six samples from a single DWDS system in Sweden were analyzed using amplicon NGS of the V1-V2 and V3 regions of the 16S rRNA gene. Sampling sites were chosen to test the resolution and limits of the experimental and analytical protocols, and to determine whether the described work-flow was capable of resolving community changes associated with small variations in a DWDS ecosystem.

Although the most relevant analysis of drinking water biofilms is an examination of those within established drinking water delivery networks, one difficulty associated with working in these systems is obtaining biological replicates to validate the experimental approach. In this study, bacterial biofilm communities of parallel installed water meters (WM 1, WM 2) allowed an approach to be developed and tested for a high resolution community analysis through NGS that included controls for both the experimental approach, by comparing results between WM 1 and WM 2, as well as the influence of changes in the DWDS on the community, by comparing additional drinking water biofilms.

Biofilms from virtually identical locations and sampling regimes associated with WM 1 and WM 2 showed highly similar communities when described by any of the NGS analyses in this study. This degree of similarity between two descriptions of communities has not been reported in previous studies that used NGS to examine the bacterial communities of drinking water biofilms, and may reflect the difficulty in obtaining true biological replicates in working DWDSs. Even small variations in the ecology of the drinking water biofilm can affect the community composition. Hong *et al.* (16) observed numerous differences in communities associated with two water meters that had experienced stable turbidity, pH, and chlorine levels, but differed in the origin of the water meter (two separate households) and the time of year they were sampled (October and December). Douterelo *et al.* (5) examined biofilm communities within a model DWDS, including NGS of material assumed to be biological replicates; however, even samples retrieved from this highly homogenous model system produced biofilm communities that showed a high variability in biological diversities across three biological replicates. The diversity was suggested to have been linked to the short time (28 d) over which the biofilms were grown, and Martiny *et al.* (29) showed that biofilms in a model drinking water system followed a successional formation that only culminated in a stable population after three years. Thus, the high similarity between WM 1 and WM 2 is a reflection of the nearly identical physical and temporal parameters that nurtured the ecology of these two biofilms. This result strongly suggests that the differences observed in the other WM communities arose from ecological changes and not from experimental or analytical artefacts and, in addition to physical parameters defining the ecosystem, the time over which this biofilm developed also contributed to the consistency of the observed community composition.

The results obtained for WM 1 and WM 2 suggest that differences in the WM 3 composition were related to changes in the environment of WM3; small differences in the community, OTU, and phylotype comparisons may be due to changes in the geographical location of WM 3 and/or water consumption associated with this biofilm community relative to WM 1 and WM 2. The biofilms in WM 1 and WM 2 were obtained from a building with high water consumption (436 and 527 m^3 over six months, respectively), whereas that in WM 3 was obtained from a water meter within a family house that had a lower water consumption of 49 m^3 over six months. The communities in WM 4, and P1 and P2 pipe biofilms, may have diverged from those of WM 1–3 to the degree that the surrounding environment of the biofilm differed. Since the environment of WM 4 was the most similar to that of WM 3 (Table 1), differences in this community may be explained by its unique location within the DWDS, and/or the distinct water quality profile associated with this sample. The composition of biofilms was different in pipe biofilm communities and those in water meters, while similar OTUs were present in both types of samples, but only a low percentage of sequences were shared (Fig. 3). The hydrological characteristics (*i.e.* pipe diameter), geographical location (including temperature), sampling season, microbial corrosion, and age of the biofilm differed between P1 and P2, which made it difficult to define any relationship between community composition and any DWDS or sampling parameters.

In addition, sampling of pipe biofilms within a working DWDS is often determined by the water company's activities, which further limits study design. The location of the water meter biofilm at the boundary between the drinking water provider and consumer often represents a change in responsibility for water quality and, together with the results presented here, support and strengthen the application of sampling water meters for biofilm communities in contact with drinking water. In contrast, water meters contain structures and surfaces for the establishment of biofilms that are the same at each sampling location; therefore, the possibility exists for biological replicates within a DWDS if parallel water meters have been installed in some buildings, and installation and removal is simple and monitored so the length of time for the biofilm to have become established is known. Hong *et al.* (16) also proposed that biofilms obtained from water meters were suitable for studying the microbial ecology of DWDS due to the ease of sampling. With uniform physical structures and the possibility for more standardized sampling, comparisons of WM communities can more directly be related to variables such as water quality and/or water usage.

Large numbers of sequences that clustered into OTUs described as *Sphingomonadaceae* were found in the water meter samples WM 1–3, with a reduced number of sequences being detected for this group in WM 4. *Sphingomonadaceae* have previously been detected in drinking water systems (16, 44) and have been related to drinking water quality because they may be responsible for initial biofilm formation (3) and are very resistant to chlorine (39). These phenotypes may promote bacterial growth in the distribution system and, thus, influence drinking water quality. The WM 4 community had a reduced number of sequences that were classified as *Sphingomonadaceae*, and a community composition distinct from the other water meters in this study. This was also observed at phylum level, with *Proteobacteria*, the most abundant phylum in the drinking water biofilms analyzed in

this study and others (16, 25, 33, 44), being markedly reduced in WM 4. A decrease in bacterial biofilm diversity has been associated with a loss of multifunctionality (31) and the larger number of OTUs observed at WM 4 may reflect a more diverse substrate or more active biofilm using the wider spectrum of nutrients present in less-than-ideal drinking water.

Since water consumption was similar between WM 3 and 4 (49 m^3 in six months for WM 3 and 53 m^3 in 6 months for WM 4), other factors may account for the differences observed between these biofilm communities.

Sequences related to *Nitrospira* were more abundant in WM 4 than in WM 1–3. Ling and Liu (26) observed a community shift to *Nitrospirae* in unchloraminated biofilms, suggesting the sensitivity of *Nitrospirae* to the disinfection treatment, and *Nitrospira* was previously detected in a model DWDS using unchlorinated groundwater (29). Even though chlorination has been used for disinfection in the DWDS examined in this study, water samples characterizing flowing water moving past the WM 4 biofilm had a lower total chlorine concentration (0.05 mg L^{-1} Cl_2) than those for the other WM communities (WM 1, WM 2: 0.13 mg L^{-1} Cl_2; WM 3: 0.14 mg L^{-1} Cl_2). Lower chlorine levels may be related to the report of red water for WM 4, as an increase in biofilm activity (due to reduced exposure to a disinfectant) has been associated with an increase in the deposition of iron and manganese into biofilms (12). Cell death within the biofilms or disruption due to hydraulic changes may release deposited iron and manganese, resulting in red water and the elevated levels of iron and manganese previously observed during discolored water events (35). Li *et al.* (25) reported changes in the bacterial community associated with the occurrence of red water and observed a higher percentage of the iron-oxidizing bacteria *Gallionella* together with the extensive precipitation of iron oxides in water samples. Although *Gallionella* was not detected in the present study, *Pedomicrobium* was identified in WM 4, the only community that was exposed to flowing water containing detectable iron and manganese concentrations (1.5 mg L^{-1} Fe and 0.04 mg L^{-1} Mn). The presence of these metals within the water may support the growth of *Pedomicrobium*, which deposits oxidized metals on its cell surface, resulting in the accumulation of metal oxides in biofilms (23, 32).

Conclusion

This study has established a work-flow that has the ability to resolve biofilm communities in sufficient detail to permit their composition to be related to the ecology of real DWDS biofilms; therefore, a definitive relationship between bacterial community compositions may be established by using this approach with a large number of biofilm samples representing diverse properties and qualities of both drinking water and its distribution systems. The high reproducibility observed between the two parallel installed water meters and other water meters from the same city suggests that water meters are an appropriate sampling site if the aim is to compare different locations within a DWDS. This study demonstrated that differences between drinking water biofilms were observable at the phylum level, which indicated that sequencing of the V1-V2 region of the 16S rRNA gene may provide sufficient information regarding bacterial community compositions for high throughput analyses and comparisons. Lundin *et al.* (28) suggested that 5,000 sequences allowed trends in alpha diversity to be estimated; hence, using fewer sequences from a single 16S region and biofilm from easily accessible water meters will facilitate the analysis of many bacterial biofilm communities associated with differing water qualities. The results of the present study support this experimental and analytical approach as a strategy for compiling an accurate and complete knowledge of the ecology of DWDS biofilms and their role in drinking water delivery.

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Minireview

Effect of Probiotics/Prebiotics on Cattle Health and Productivity

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Probiotics/prebiotics have the ability to modulate the balance and activities of the gastrointestinal (GI) microbiota, and are, thus, considered beneficial to the host animal and have been used as functional foods. Numerous factors, such as dietary and management constraints, have been shown to markedly affect the structure and activities of gut microbial communities in livestock animals. Previous studies reported the potential of probiotics and prebiotics in animal nutrition; however, their efficacies often vary and are inconsistent, possibly, in part, because the dynamics of the GI community have not been taken into consideration. Under stressed conditions, direct-fed microbials may be used to reduce the risk or severity of scours caused by disruption of the normal intestinal environment. The observable benefits of prebiotics may also be minimal in generally healthy calves, in which the microbial community is relatively stable. However, probiotic yeast strains have been administered with the aim of improving rumen fermentation efficiency by modulating microbial fermentation pathways. This review mainly focused on the benefits of probiotics/prebiotics on the GI microbial ecosystem in ruminants, which is deeply involved in nutrition and health for the animal.

Key words: rumen, gastrointestinal tract, yeast, oligosaccharide

Overview

The gastrointestinal (GI) microbial community, which consists of at least one thousand different microbial species in human gut (14, 84), has an impact on energy efficiency in the host, including energy intake, transport, conversion, and storage. In ruminants, a large amount of energy recovery from dietary polysaccharides that cannot be digested by the host has been attributed to the function of the microbial community in the rumen; however, this process also depends on the structure of the microbiota inhabiting this organ. Environmental and stochastic factors, such as diet composition, feeding practices, and farm management, have been shown to strongly affect the composition and functions of the microbiota in livestock animals (83).

Most of the GI bacterial community of mammals is affiliated with two phyla, *Bacteroidetes* and *Firmicutes* (1, 39, 77). On the other hand, other phyla have niches in each community, depending on the animal species. Therefore, the GI tract community is unique among species, which require owning different systems to efficiently convert their diet into their energy. The main GI bacterial groups in cattle have been identified as defined groups (mainly genera) for up to 90% of the total community (78, 79). However, a certain proportion of the gut bacterial community has yet to be identified due to an incomplete understanding of the bacterial community structure in GI ecosystems because many of the 16S rRNA gene sequences recovered from fecal samples are derived from unknown (*i.e.*, not previously identified in the intestinal microbiota) species (21). While GI tract ecosystems (especially in humans) are known that a higher proportion of bacteria has been cultivated (18), further research is required to uncover a larger proportion of the unknown microorganisms abundantly present in the GI microbiota.

The term "probiotics" has been amended by the FAO/WHO to "Live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" (24). Several lactic acid bacteria (LAB) strains, species belonging to the genera *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*, are considered beneficial to the host and have, thus, been used as probiotics and included in several functional foods. Probiotics have the ability to enhance intestinal health by stimulating the development of a healthy microbiota (predominated by beneficial bacteria), preventing enteric pathogens from colonizing the intestine, increasing digestive capacity, lowering the pH, and improving mucosal immunity. It is important for the introduced microbes not to disturb the indigenous population, which has already been adapted to the environment of the GI tract to work both for and with the host. Additionally, there are a number of requirements for allochthonous probiotic strains to adapt to the intestinal environment of an animal species, *e.g.*, bile acid tolerance and affinity to the intestinal mucosa and glycoproteins. The situation in the rumen is similar; ingested microbes have to find out a suitable niche to inhabit, such as the rumen epithelium, rumen fluid, or fibrous feed, and exert effects on the health of the host, such as the removal of toxic molecules and digestion of polymeric carbohydrates.

Prebiotics are non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth and/or activity of one or a limited number of microbes in the gut. Impacts of orally administered probiotics (in this case, referred to as symbiotics) and intrinsic beneficial bacteria of the GI tract can be enhanced by the use of prebiotics (28). The most commonly used prebiotics to yield health

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benefits are carbohydrate substrates, such as oligosaccharides or dietary fiber with low digestibility.

Research on probiotics and prebiotics has developed as a collaborative study domain between the fields of food and feed with medicine and pharmaceutics. There are also a number of application studies for cattle; however, few have been discussed in association with the dynamics of the inherent microorganisms. This review explored the better usage of probiotics and prebiotics to improve ruminant performance by discussing the possible impacts of the applications of probiotics and prebiotics on the ruminant-specific GI microbial community.

Gastrointestinal microbial structure of cattle

Neonatal ruminants are unique in that, at birth, they are physically and functionally two different types of animal with respect to their GI system (34). The intestine of a newly born calf is sterile, and colonization of the GI tract begins immediately after birth. Thereafter, a complex and dynamic microbial ecosystem with high densities of living bacteria is established in the large intestine as animals grow to maturity (72). Molecular-based monitoring of the intestinal bacterial communities of calves revealed that the community undergoes dynamic changes during the first 12 weeks of life (78). For example, the main groups detected at a very young age (less than 3 weeks old) were found as major populations in the human fecal bacterial community (*i.e.*, *Bacteroides-Prevotella*, the *Clostridium coccoides-Eubacterium rectale* group, *Faecalibacterium*, and *Atopobium*) (30, 70, 77). *Bacteroides-Prevotella* and the *C. coccoides-E. rectale* group comprised a major fraction of the microbiota (*ca.* 50%–70% of the total) throughout the first 12-week period after birth, whereas the numbers of *Atopobium*, *Faecalibacterium*, and some probiotic bacteria (such as those of the genera *Lactobacillus* and *Bifidobacterium*) decreased as the animal aged. Instead, an uncultivated rumen bacterial group as well as *Ruminococcus flavefaciens* and *Fibrobacter* emerged at detectable levels (1%–2%) in feces sampled after weaning. Changes in the GI microbiota of young calves are in accordance with the metabolic and physiological development of the GI tract (15). As discussed later, this immature and fluctuating gut microbiota has to face an abrupt change in diet, which leads to an increase in the susceptibility of young animals to pathogen colonization and subsequent diarrhea and respiratory disease.

GI microbial communities are involved in the digestion and fermentation of plant polymers, which is of particular importance in mature herbivorous animals. Ruminant animals harbor a complex microbial community consisting of a diverse array of anaerobic microbes in the rumen, which forms a different community structure from aerobic consortia for fiber digestion (20). These microorganisms interact with one another and take part in the systematic digestion of fibrous plant material, which they anaerobically ferment into end products that are, in turn, used as energy sources by the host (66). Microbial characteristics, functions, and current concerns regarding dysfunctions in the two respective sites (*i.e.*, the rumen and lower intestine) in cattle are summarized in Table 1. Even though the major functionalities differ from each other, ruminal community may affect that of large intestine.

Numerous factors, such as dietary and management constraints, can strongly affect the structure and activities of these microbial communities, sometimes leading to impaired health and performance in livestock animals (16). For example, sub-acute ruminal acidosis (SARA) is a well-recognized digestive dysfunction that is increasingly becoming a health problem. Microbial community changes associated with SARA of lactating dairy cattle have been monitored using terminal-restriction fragment length polymorphisms (T-RFLP) of 16S rRNA genes and real-time PCR (40). Different rumen microbial population structures between grain- and forage-induced SARA were observed even though rumen fermentation conditions were similar. The findings of a T-RFLP analysis indicated that the most predominant shift during SARA was a decline in Gram-negative *Bacteroidetes*. Since inflammatory responses may be attributed, in part, to lipopolysaccharides released by the dead cells of Gram-negative bacteria (44, 58), the change observed in the number of *Bacteroidetes* in response to SARA appears to be reasonable. The potential microbial and physiological factors that increase the incidence of SARA by enhancing the epithelial permeability of lipopolysaccharides have not yet been identified. Furthermore, the overconditioning (excess body fat deposition) of dairy cows is a major risk factor for metabolic, infectious, diges-

Table 1. Cattle GI microbial characteristics and relationships with host health and performance

	Rumen	Large intestine
Major groups in microflora	*Bacteroidetes* *Firmicutes* *Fibrobacter* *Archaea* Protozoan species	in preweaned calves: *Bacteroidetes* *Firmicutes* *Atopobium* *Bifidobacteria* in weaned calves or older cattle: *Bacteroidetes* *Firmicutes* (including uncultured groups) *Fibrobacter*
Major microbial functions	Involved in host nutrition (digestion of fibrous plant material and anaerobic fermentation to short chain fatty acids, which can be used as an energy source by the host; microbial protein synthesis)	Immunological responses Digestion of polymers
Microbial dysfunctions	Overgrowth of lactate-producing bacteria, leading to a decrease in rumen pH and subsequent rumen acidosis Decrease in microbial activity by unbalanced nutrition, leading a decrease in feed protein efficacy	Pathogenesis by harmful bacteria, such as *E. coli* and *Salmonella*.

tive, and reproductive disorders (68). However, the efficient fermentation system in the rumen of dairy cows is primarily responsible for the conversion and accumulation of energy, while the colonial microbiota is majorly contributed to energy harvesting and metabolism in human (14, 38, 45). This implies the importance of controlling rumen microbial fermentation, although rumen function is not directly related to body fat deposition.

Current applications of probiotics in calves

In young pre-ruminants, probiotics such as LAB or *Bacillus* species generally target the lower intestine and represent an interesting means to stabilize the gut microbiota and decrease the risk of pathogen colonization. LAB are well-known probiotic supplement for young calves, and are regarded as applicable to regular feeding practices. Previous findings support the beneficial effects of these products in balancing the GI tract microbiota as well as in animal nutrition and health (Table 2). Diarrhea is the main cause of morbidity and mortality in calves during their early life (13, 36); therefore, its prevention is important to promote the growth of calves (69, 76). Antibiotic therapy has been applied to maintain the performance of calves and reduce scours. However, due to increasing safety concerns regarding the risks of antibiotic resistance due to the release of antibiotics into the environment and persistence of chemical residues in animal products (49, 82), probiotic additives have been developed as alternatives to improve animal health and productivity (4, 8). Although the administration of probiotics to animals has been linked with efficacy on specific groups (pathogens) in the gut microbiota (25, 69, 76), how they interact with the whole gut community currently remains unclear. As discussed above, lactobacilli and bifidobacteria numbers have been shown to decrease in the community in the early stages of life in cattle (78). Optimizing the enteric flora is considered effective for healthy calf rearing because it increases the numbers of such beneficial microorganisms. The supply of microorganisms together with feed from birth in a preventive manner allows the incorporation and establishment of these probiotic strains together with the microbiota of calves. In addition, early colonization by LAB in the intestinal ecosystem may decrease the adherence of pathogens to the intestinal mucosa (37). A stable microbial load of *Lactobacillus* species has been shown to improve weight gain and immunocompetence in young calves (3); however, previous findings regarding the use of probiotics in calf feeding have generally been equivocal, as shown in Table 2. The efficacy of probiotic strains may vary depending on whether calves are raised under healthy conditions because, in previous studies, the effects of probiotics were often significant when control (untreated) calves were less healthy, as determined from fecal scores or rectal temperatures (5, 76). Under stressed conditions, direct-fed microbials may be used to reduce the risk or severity of scours caused by disruption of the normal intestinal environment. A better understanding of how the selected lactobacilli and bifidobacteria strains overcome the effects of pathogens, by antagonizing the pathogenicity, and/or modulating the immune responses to infections is needed (3, 69).

Current applications of prebiotics in calves

Several types of oligosaccharides have been suggested to have specific functionalities in calves. Mannan oligosaccharides (MOS) are complex mannose sugars that are believed to block colonization of pathogens in the digestive tract. A previous study demonstrated that deeding fructooligosaccharides

Table 2. Recent probiotic/prebiotic trials applied for young cattle

Targets and materials applied[a]	Positive effects in respect to			Remarks	Reference
	Weight gain	Feed efficiency	Health		
Probiotics (for heifers):					
Yeast culture	Not assessed	Yes	No		(42)
Yeast culture	No	No	Yes		(53)
Probiotics (for calves):					
Yeast culture	Yes	No	No		(43)
MSPB or CSPB	Yes	Yes	Yes	Effects were determined when the results of four experiments were pooled.	(76)
MSPB	Yes	No	Not assessed	Two mixtures were tested, a commercial probiotic and laboratory-produced probiotic that was made under laboratory conditions.	(5)
Lactobacillus casei ssp. *casei*	Yes	No	Yes	Synbiotic trial	(32)
MSPB[b]	Yes	No	Yes		(22)
MSPB[b]	No	No	No		(23)
Prebiotics (for calves):					
FOS	No	No	Yes		(62)
FOS (short chain)	No	Yes	Not assessed		(29)
MOS	No	No	Yes		(33)
MOS	No	No	No		(74)
MOS	Yes	Yes	Yes	Used crossbred calves	(27)
Cellooligosaccharide	Yes	No	Yes	Synbiotic trial	(32)
A commercial product[c]	No	No	No		(35)
A commercial product[c]	No	No	No	The lactobacilli count in feces was higher and that of bifidobacteria was slightly higher in the prebiotic group.	(60)

[a] MSPB, multi-species probiotic; CSPB, calve-specific probiotic; MOS, mannan-oligosaccharides.
[b] A mixture of *Lactobacillus casei* subsp. *casei*, *Lactobacillus salivarius*, and *Pediococcus acidilactici*.
[c] Derived from a cell-free culture of a *Propionibacterium freudenreichii* strain.

(FOS) in combination with spray-dried bovine serum to calves reduced the incidence and severity of enteric disease (62). It has been suggested that this sugar prevents the adhesion of *Enterobacteriaceae*, most notably *Escherichia coli* and *Salmonella*, to the intestinal epithelium (7, 31). Galactosyl-lactose (GL) is a trisaccharide (galactose plus lactose) that is produced by the enzymatic treatment of whey with beta-galactosidase. The addition of GL to milk replacer (MR) was previously found to have beneficial effects on the growth and health of dairy calves (61). Supplementation with MOS, FOS, and GL may improve the growth performance of calves in either the pre- or postweaning stage; however, modifications to the activities of microbial fermentation by these sugars have not yet been examined in detail. In addition, similar to the case of probiotics, the observable benefits of prebiotics are likely to be minimal when calves are generally healthy (35). As shown in Table 2, most prebiotics may not have any apparent beneficial effects (body weight gain, feed efficiency, or health measures) over probiotics.

We previously evaluated the effects of feeding cellooligosaccharide (CE), which is a commercially available oligosaccharide that consists of glucose with beta-1-4 linkages, on performance and intestinal ecology in Holstein calves fed MR or whole milk (80). No significant differences were observed in fecal bacterial community compositions or organic acid profiles in the MR group. However, this supplementation appeared to effectively modulate the intestinal bacterial community of calves when administered with whole milk because the proportion of the *C. coccoides–E. rectale* group was higher in the prebiotic group in the whole milk-feeding trial. From these results, type of liquid feed (MR or whole milk) to preweaned calves may be responsible for the different responces to feeding CE. Overall, CE supplementation had no effect on the maintenance of *Lactobacillus* and *Bifidobacterium* species levels in the large intestine of preweaning calves. CE is considered to be utilized by specific microbes inhabiting the calf intestine, resulting in increases in the number of butyric acid-producing bacteria belonging to *C. coccoides–E. rectale* (19, 47). Fecal butyrate concentrations were also higher at that time. Along with its value as an energy source, butyrate is also involved in the growth and differentiation of intestinal cells in the large intestine, thereby improving its epithelial structure (59) and enhancing digestion and absorption efficiencies, which may also contribute to a superior ability for nutrition capture (54). CE fed with liquid feed (milk or reconstituted MR) may reach the lower digestive tract vis the esophageal groove reflex (34), and exert prebiotic effects using a similar mechanism to that of monogastric animals.

An *in vivo* study indicated that CE feeding improved daily gain and feed efficiency in calves during the postweaning period, but not the pre-weaning period (32). This may have been mainly due to the enhancement in ruminal fermentation as propionate and total short chain fatty acid (SCFA) levels were increased, which suggests that CE affected the fermentation pattern by providing carbon and energy sources (46). After weaning, solid feeds directly reach the rumen and are then microbially processed. Ruminal CE may eventually be a source of nutrition for various types of indigenous microbes. With the exception of very young ruminants, prebiotics orally administered to ruminants are consumed by ruminal microbes and fail to reach the lower intestine unless protected from ruminal digestion. The administration of oligosaccharides to weaned calves still appears to be advantageous because the formation of a desirable intestinal (rumen and/or lower intestine) community in calves through prebiotic supplementation may contribute to further improvements in growth performance at an older age.

Effects of supplementation with probiotics/prebiotics on the performance of heifers, lactating cows, and beef cattle

Probiotics for adult ruminants have mainly been selected to improve fiber digestion by rumen microorganisms. Such probiotics have positive effects on various digestive processes, especially cellulolysis and the synthesis of microbial proteins. The main form of probiotic commonly used in dairy cows is various strains of yeast (mostly *Saccharomyces cerevisiae*). Regarding bacterial probiotics for adult ruminants, lactate-producing bacteria (*Enterococcus*, *Lactobacillus*), which sustain lactic acids are a more constant level than *Streptococcus bovis*, may represent a possible means of limiting acidosis in high-concentrate-fed animals (55, 56), especially feedlot cattle. *Megasphaella elsdenii* or *Propionibacterium* species, which utilize lactate, have also been administered as direct-fed microbials to avoid the accumulation of ruminal lactate (26, 41, 71).

The most consistent effects following the addition of yeast cultures to the diet include improved productivity in both lactating and growing animals. The mode of action of yeast products has not yet been elucidated in detail, but is generally considered to involve changes in rumen fermentation rates and patterns. Certain strains of active dry yeast are particularly effective at raising and stabilizing ruminal pH by stimulating certain populations of ciliate protozoa, which rapidly engulf starch and, thus, effectively compete with amylolytic lactate-producing bacteria (2, 9, 41, 56, 73, 75). A less acidic ruminal environment has been shown to benefit the growth and fiber-degrading activities of cellulolytic microorganisms (6, 10, 12, 52). Yeast also has the potential to alter the fermentation process in the rumen in a manner that reduces the formation of methane (CH_4) gas (12). In a previous study, commercial yeast product slightly decreased CH_4 in growing beef cattle, while neither the SCFA amount nor the profiles changed (51). The cells of *S. cerevisiae* provide growth factors for rumen microbes, including organic acids and oligosaccharides, B vitamins, and amino acids, which stimulate microbial growth in the rumen, thereby indirectly stabilizing ruminal pH (50). Another function of yeast in the rumen is the scavenging of oxygen, which creates the more anaerobic environment required by ruminal microorganisms (16). In this context, yeast itself functions not only as a probiotic, but also helps other rumen community members grow, and, thus, acts as a type of prebiotic. The effects of active dry yeast on the rumen microbial community structure was recently determined by 16S rRNA gene-based clustering using a pyrosequencing technique (57). An evaluation of the effects of yeast on the microbiota revealed that some bacterial groups were more affected than others. The relative abundance of lactate-utilizing bacteria such as *Megasphaera* and *Selenomonas* as

well as fibrolytic groups (*Fibrobacter* and *Ruminococcus*) increased with yeast supplementation, confirming improvements in cellulolytic activity as a supposed mode of action of yeast.

Intervention studies on the application of commercial yeast cultures to young cattle (heifers and calves) are summarized in Table 2. A large number of studies that evaluated the effects of yeast on dairy production (milking and body mass deposition) were summarized in two studies published concurrently several years ago, one of which was a meta-analysis and the other was a review (17, 63). Desnoyers *et al.* evaluated the effects of yeast supplementation on intake, milk production, and rumen fermentation characteristics using a quantitative meta-analysis. The positive effects of yeast supplementation were an increase in rumen pH and a decrease in lactic acid with the increases in concentrate in the diet and with the intake level. Controversially, the positive effects of yeast supplementation on organic matter digestibility increased with the percentage of fiber in the diet, suggesting an improvement in rumen fermentation by yeast supplementation.

In beef cattle, the stabilization of ruminal pH may also be effective when they are fed a high readily fermentable diet that increases the risk of acidosis. Growth parameters (average daily gain, final weight, intake, and feed to gain ratio) were previously reported to be improved by continuous live yeast supplementation (11), whereas no or little effect on performance was observed in other studies (6, 81). This difference in result may be attributed to a primal difference in the rumen microbial composition, in which respective members have different pH tolerances. For example, fibrolytic bacteria are generally less pH tolerant than saccharolytic bacteria (65).

Although previous findings have supported the efficacy of yeast supplementation, conclusive evidence has not yet been obtained to show that supplementation is beneficial at all times (11). It should be noted that this potential varies markedly with products (51). Increases in profitability are generally variable, especially when taking the rise in feeding costs for these products into account. Some of these differences may be attributed to the type and strain of yeast used as well as whether the cells are alive or dead (48). Furthermore, in some commercial products, the data available have been generated under *in vitro* conditions and in monogastric animals or small ruminants, which do not necessarily correspond to actual dairy and beef production.

Concluding remarks

The cattle GI microbial composition was shown to be altered by various factors, including diet, age, and stress, as an adaptive response of the community to the environment (66). Therefore, GI health may be defined as the ability to maintain a balance of GI ecosystem. Desirable community shift may be attributed to the effect of probiotics and prebiotics, rather than autonomic change. Probiotics and prebiotics both have great potential in livestock productivity as well as human health. Cellooligosaccharide is one example because many rumen bacteria may be able to use it, but, when administered to preweaned calves, the proportion of the *C. coccoides–E. rectale* group specifically increases in the lower intestine. Like CE, it may become possible to use powerful materials that work either or both on the rumen and on the lower intestine.

Although controlled studies demonstrated that probiotics and prebiotics achieved a positive balance in the GI microbiota of cattle, the dynamics and functions of the rumen community need to be examined in more detail. Further studies on the structure and activities of the gut microbiota, functional interactions between gut microbes, and relationships between microbes and host cells are warranted to determine the fundamental aspects of future probiotic/prebiotic research. "Meta-omic" approaches (metagenomic, metatranscriptomic, metaproteomic, and meta-metabolomic analyses) are powerful tools for analyzing the relationships between the GI microbial community and host metabolism (64, 67, 84). Future meta-omic-based studies together with the knowledge obtained to date will provide deeper insights into the effects of "health-improving" diet for animals by better characterizing and understanding the functionalities of probiotics on the balance of the GI microbiota.

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Minireview

Recent Trends in Control Methods for Bacterial Wilt Diseases Caused by *Ralstonia solanacearum*

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Previous studies have described the development of control methods against bacterial wilt diseases caused by *Ralstonia solanacearum*. This review focused on recent advances in control measures, such as biological, physical, chemical, cultural, and integral measures, as well as biocontrol efficacy and suppression mechanisms. Biological control agents (BCAs) have been dominated by bacteria (90%) and fungi (10%). Avirulent strains of *R. solanacearum*, *Pseudomonas* spp., *Bacillus* spp., and *Streptomyces* spp. are well-known BCAs. New or uncommon BCAs have also been identified such as *Acinetobacter* sp., *Burkholderia* sp., and *Paenibacillus* sp. Inoculation methods for BCAs affect biocontrol efficacy, such as pouring or drenching soil, dipping of roots, and seed coatings. The amendment of different organic matter, such as plant residue, animal waste, and simple organic compounds, have frequently been reported to suppress bacterial wilt diseases. The combined application of BCAs and their substrates was shown to more effectively suppress bacterial wilt in the tomato. Suppression mechanisms are typically attributed to the antibacterial metabolites produced by BCAs or those present in natural products; however, the number of studies related to host resistance to the pathogen is increasing. Enhanced/modified soil microbial communities are also indirectly involved in disease suppression. New promising types of control measures include biological soil disinfection using substrates that release volatile compounds. This review described recent advances in different control measures. We focused on the importance of integrated pest management (IPM) for bacterial wilt diseases.

Key words: antibiosis, biological control agent, competition, induced systemic resistance, organic amendment

The world's population is increasing every year. In order to meet the demands of an ever expanding human population, global crop production needs to double by 2050; however, current estimates are far below what is needed (104). Plant diseases, insects, and weeds decrease the production of crops worldwide by 36%, and diseases alone have been shown to reduce crop yields by 14% (5). Thus, the control of plant diseases contributes to increased crop production. Among plant diseases, soil-borne diseases are considered to be more limiting than seed-borne or air-borne diseases in the production of many crops and account for 10–20% of yield losses annually (120).

The top ten bacterial species have been listed based on their scientific and economic importance in plant diseases: i) *Pseudomonas syringae* pathovars, ii) *Ralstonia solanacearum*, iii) *Agrobacterium tumifaciens*, iv) *Xanthomonas oryzae* pv. *oryzae*, v) *X. campestris* pathovars, vi) *X. axonopodis* pathovars, vii) *Erwinia amylovora*, viii) *Xylella fastidiosa*, ix) *Dickeya* (former *Erwinia*) (*dadantanii* and *solani*), and x) *Pectobacterium* (former *Erwinia*) *carotovorum* (and *Pectobacterium atrosepticum*) (79).

R. solanacearum (Smith) Yabuuchi *et al.* (132) (syn. *Pseudomonas solanacearum* [Smith] Smith, *Burkholderia solanacearum* [Smith]) causes a vascular wilt disease and has been ranked as the second most important bacterial pathogen. It is one of the most destructive pathogens identified to date because it induces rapid and fatal wilting symptoms in host plants. The host range is extensively wide, over 200 species, and the pathogen is distributed worldwide and induces a destructive economic impact (57). Direct yield losses by *R. solanacearum* vary widely according to the host, cultivar, climate, soil type, cropping pattern, and strain. For example, yield losses vary from 0 to 91% in the tomato, 33 to 90% in the potato, 10 to 30% in tobacco, 80 to 100% in the banana, and up to 20% in the groundnut (28). Difficulties are associated with controlling this pathogen due to its abilities to grow endophytically, survive in soil, especially in the deeper layers, travel along water, and its relationship with weeds (122).

The management of bacterial wilt with physical, chemical, biological, and cultural methods has been investigated for decades. Elphinstone (28) extensively reviewed bacterial wilt in 2005, and many studies have since been conducted on this topic. We herein reviewed the same topic, but mainly based on findings published between 2005 and 2014.

Elphinstone (28) reported that over 450 studies had been published on *R. solanacearum* since the second International Bacterial Wilt Symposium was held in Guadaloupe in 1997. A broad classification on these studies showed that 24% were concerned with breeding and selection for resistance, while the remainder investigated the diversity, distribution, and host range of the pathogen (22%), disease management and control (18%), pathogenicity and host-pathogen interactions (17%), biological control (10%), detection and diagnosis of

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the pathogen (4%), and epidemiology and ecology (3%). Based on our reference search from books and journals between 1984 and 2014 using the Web of Science, LINK (Springer), InterScience (Wiley), SD-Science Direct (Elsevier), and Synergy (Blackwell), studies on methods regarding the biological control of bacterial wilt (54%) were the most common, followed by those on cultural practices (21%), chemical methods (8%), and physical methods (6%). Some studies also focused on integrated pest management (11%). This finding suggested that many researchers were interested in biological control.

We herein discussed the following points, (i) methods used to control bacterial wilt and their limitations, and (ii) how these methods are useful for improving crop production through the suppression of bacterial wilt.

Methods used for crop protection

Chemical methods (pesticides and non-pesticides). World pesticide use exceeded 5.0 billion pounds in 2000 and 2001 (59). Herbicides account for the largest portion of the total use, followed by insecticides and fungicides. Plant disease control has been largely dependent on the use of pesticides (127). Schreinemachers *et al.* (107) reported that pesticide use per hectare, especially herbicides and fungicides/bactericides, had generally increased more than porportionally with crop output per hectare, and revealed that a 1% increase in crop output per hectare was associated with 1.8% increase in pesticide use per hectare.

Pesticides such as algicide (3-[3-indolyl] butanoic acid), fumigants (metam sodium, 1,3-dichloropropene, and chloropicrin), and plant activators generating systemic resistance on the tomato (validamycin A and validoxylamine) have been used to control bacterial wilt. The combination of methyl bromide, 1,3-dichloropropene, or metam sodium with chloropicrin significantly reduced bacterial wilt in the field from 72% to 100% and increased the yield of tobacco and the tomato. The yield of the pesticide-treated tomato was 1.7- to 2.5-fold higher than that of the untreated control (32, 105).

Edwards-Jones (27) reported that pesticides offered greater net benefits than other control methods, but this has not always been the case. For example, if farmers use pesticides carelessly or without proper knowledge, a percentage of the pesticide may remain in the environment for many years (34), become a contaminant in soil and/or groundwater (2), and be poisonous to farmers (25).

Bactericides (triazolothiadiazine [0.5 to 12 mM, in solution] (58), streptomycin sulfate [400 mg kg^{-1} of soil] (72)), other chemicals such as bleaching powders (application rate to the field, 30 kg ha^{-1}) as sterilizers (108), or weak acidic electrolyzed water (40 ppm of available chlorine, in pH 5.6 solution) (137) have also been shown to effectively destroy microorganisms.

Acibenzolar-S-methyl (ASM) has been proposed to induce systemic resistance (38, 100). The combination of ASM and thymol significantly reduced the incidence of disease and increased the yield of the tomato, whereas ASM or thymol alone did not (43). Silicon (24, 65, 129) or Si and chitosan (61) reduced the incidence of bacterial wilt through induced resistance. Wang *et al.* (123) reported that Si-mediated resistance was associated with increases in the amount of microorganisms in the soil as well as soil enzyme activity (urease and acid phosphatase). The soaking of seeds in a low sodium chloride solution was previously found to increase seedling vigor and tolerance to *R. solanacearum* in the tomato (86).

The mechanism of action of non-pesticide chemicals that suppress bacterial wilt is considered to involve either induced systemic resistance or antibacterial activity. Some new methods have been reported to suppress bacterial wilt. Live microbial cells of the pathogen were captured with 10 g kg^{-1} of coated sawdust with 1% of an equimolar polymer of N-benzyl-4-vinylpyridinium chloride with styrene (PBVP-co-ST) (55) or coagulated in the soil with 10 mg kg^{-1} of a co-polymer of methyl methacrylate with N-benzyl-4-vinylpyridinium chloride at a molar ratio 3:1 (PMMA-co-BVP) (56). Infection by the bacterial wilt pathogen was prevented through bacteriostatic actions with a phosphoric acid solution (89).

Various non-pesticide chemicals have the potential to be applied in the field in order to control bacterial wilt disease because they have less damaging effects on the environment; however, economic considerations often influence the chemicals selected. Expensive chemicals and repeated applications are only possible for valuable crops that may incur substantial economic losses in the absence of treatments. Since crop yield and quality are not damaged when disease severity is low or in the absence of pathogens, a diagnosis based on economic thresholds is essential for determining whether chemical treatments are needed.

Biological method. *1) Biological control agents (BCAs)*

Interest in biological control has increased due to concerns over the general use of chemicals (126). The benefits of BCAs are 1) potentially self-sustaining, 2) spread on their own after initial establishment, 3) reduced input of non-renewable resources, and 4) long-term disease suppression in an environmentally friendly manner (102, 127).

The mechanisms employed by BCAs are sustained by various interactions such as competition for nutrients and space, antibiosis, parasitism, and induced systemic resistance (5, 22). Our reference survey revealed that BCAs have been dominated by bacteria (90%) and fungi (10%). Montesinos (84) found that most patented BCAs are made of bacteria. Topics regarding biocontrol agents for bacterial wilt have been separated into the following categories: isolation, screening and identification of BCAs, application methods of BCAs, improved BCAs, suppression mechanisms of BCAs, and effects of BCAs on the environment.

Previous studies showed the potential value of some promising BCAs, which are dominantly avirulent strains of *R. solanacearum* and *Pseudomonas* spp., followed by *Bacillus* spp., *Streptomyces* spp., and other species, in controlling bacterial wilt. A total of 109 strains of endophytic or rhizobacteria were recently screened for their antibacterial activities against *R. solanacearum*, and effective isolates (a total of 22) consisted of *Pseudomonas* spp. (18 isolates) and *Bacillus* sp. (2 isolates) (103). Kurabachew *et al.* (64) screened 13 out of 150 isolates of rhizobacteria based on *in vitro* antibiosis, and they were *Pseudomonas* spp., *Serratia marcescens*, and *Bacillus cereus*. Among *Bacillus* spp., the number of studies being conducted on *B. amyloliquefaciens* is increasing (21,

26, 44, 116, 124, 143). Plant growth-promoting rhizobacteria are commonly isolated from the rhizosphere of healthy plants and an interesting strategy has been reported. Huang *et al.* (45) revealed that isolates from the rhizosphere of diseased plants performed better in reducing disease incidence that those of healthy plants. In their study, the biocontrol efficacies of the antagonists were related to root colonizing capacities, but not with antibiosis *in vitro*, suggesting that root colonizing capacity may play a key role in disease suppression.

Several new or uncommon BCAs have been reported to control bacterial wilt such as *Acinetobacter* sp. (130), *Burkholderia nodosa*, *B. sacchari*, *B. tericola*, *B. pyrrocinia* (88), bacteriophages (10, 133), *Bacillus thuringiensis* (146), *Chryseobacterium daecheongense* (45), *Chryseobacterium indologenes* (42), *Chryseomonas luteola* (42), *Clostridium* sp. (82), *Delftia acidovorans* (45), *Enterobacter* sp. (130), *Flavobacterium johnsoniae* (45), *Myroides odoratimimus* (138), *Paenibacillus marcerans* (70), *P. polymyxa* (69, 74), *Pseudomonas brassicacearum* (145), *Ralstonia pickettii* (125), *Serratia* sp. (37, 131), *Sphingomonas paucimobilis* (42), *Staphylococcus auricularis* (42), *Stenotrophomonas maltophilia* (81), *Streptomyces rochei* (76), *S. virginiae* (115), and *Xenorhabdus nematophila* (52). The possible suppression mechanisms of these species are competition, induced systemic resistance, antibiosis, and the production of enzymes that degrade the cell wall and siderophores. Successful trials using BCA in the field are introduced in Table 1. Hyakumachi *et al.* (47) recently revealed that *B. thuringiensis*, a famous bioinsecticide-producing bacterium, induced defense-related genes, such as PR-1, acidic chitinase, and beta-1,3-glucanase and showed resistance against a direct inoculation with *R. solanacearum*. The expression of several salicylic acid-responsive defense-related genes was confirmed to be specifically induced (114), and also that suppression by *B. thuringiensis* may differ from the induced systemic resistance (ISR) elicited by many plant growth-promoting rhizobacteria (PGPR), in which jasmonic acid and ethylene-dependent signaling pathways mediate plant resistance to pathogens (47).

Some fungal BCAs have been reported to control bacterial wilt. In pot cultures, populations of *R. solanacearum* in the rhizosphere, on root surfaces, and in the xylem of tomato plants decreased by 26.7, 79.3, and 81.7%, respectively, following the inoculation of *Glomus versiforme*. The coloniza-

Table 1. Various biocontrol agents that have been tested in the field to control bacterial wilt diseases caused by *Ralstonia solanacearum* (2005–2014)

Microorganisms	Inoculation method and application rate	Mechanisms	BE (%)	Yield*	Ref
1. *Bacillus amyloliquefaciens* SQR-7 and SQR-101 and *B. methylotrophicus* SQR-29	Pouring, 6.8×10^{10} cfu plant^{-1} (SQR-7), 7.5×10^{10} cfu plant^{-1} (SQR-101), 8.2×10^{10} cfu plant^{-1} (SQR-7)	Production of indole acetic acid and siderophores	18–60% in tobacco	25–38%	143
2. *Ralstonia pickettii* QL-A6	Stem injection, 10 μL of 10^7 CFU mL^{-1}	Competition	73% in the tomato	NA	125
3. *Pseudomonas monteilii* (A) + *Glomus fasciculatum* (B)	Stem cuttings were dipped in A (9.1×10^8 mL^{-1}), B (53 infective propagules) was added to each cutting, and A was then poured again	Increased plant nutrient uptake (N, P, K) and reduced the pathogen population	56–75% in herbs (*Coleus forskohli*)	54%	111
4. *Brevibacillus brevis* L-25 + *Streptomyces roche* L-9 + organic fertilizer	Mixed with soil at a density of 7.3×10^7 (L-25) and 5.0×10^5 (L-9) cfu g^{-1} of soil	Decreased root colonization by the pathogen	30–95% in tobacco	87–100%	76
5. *Bacillus amyloliquefaciens* + bio-organic fertilizer (BIO23) *B. subtilis* + bio-organic fertilizer (BIO36)	Mixed with soil at a density of 5.5×10^6 (BIO23) and 7.0×10^6 (BIO36) cfu g^{-1} of soil	Plant growth promotion	58–66% in the potato	64–65%	26
6. *Bacillus* sp. (RCh6) *Pseudomonas mallei* (RBG4)	3×10^8 cfu g^{-1} (talc formulation). Seedlings were dipped in antagonist suspension (25 g talc formulation L^{-1}). Leftover suspension was poured around the root zone of the seedling (50 mL plant^{-1})	Production of inhibitory compounds and siderophores	81% in the eggplant	60–90%	103
7. *Trichoderma viride* (A), *B. subtilis* (B), *Azotobacter chroococcum* (C), *Glomus fasciculatum* (D), *P. fluorescens* (E)	D (53 infective propagules) was added to each stem cutting that was dipped in A (1.2×10^6 CFU mL^{-1}), B (1.8×10^8 CFU mL^{-1}), C (2.3×10^7 CFU mL^{-1}), and E (2.5×10^8 CFU mL^{-1}). A total of 5 mL of A, B, C, and E was then poured into 200 g soil.	Competition for nutrient uptake (NPK) and reduced *R. solanacearum* population	7–43% in herbs (*Coleus forskohlii*)	159–227%	110
8. *B. amyloliquefaciens* QL-5, QL-18 + organic fertilizer	Mixed with soil at a density of 1×10^7 (QL-5) or 1×10^7 (QL-18) cfu g^{-1} of soil	Decreased root colonization by the pathogen	17–87% in the tomato	NA	124
9. *B. amyloliquefaciens* Bg-C31	Poured 10 mL of bacterial suspension plant^{-1} (potato dextrose broth culture).	Production of antimicrobial proteins	60–80% in Capsicum	NA	44
10. *Acinetobacter* sp. Xa6, *Enterobacter* sp. Xy3	Poured 20 mL of the bacterial suspension (1×10^9 cells mL^{-1}) plant^{-1} or seedling roots were soaked in the bacterial suspension.	Rhizocompetence and root colonization	57–67% in the tomato	32–41%	130
11. *B. vallismortis* ExTN-1	Bacterial suspension was mixed into an organic fertilizer (10^6 cfu mL^{-1}) and poured onto soil.	Induction of systemic resistance	48–49% in the tomato	17%	119
12. *Glomus mossease*	A total of 30 g of the inoculum (650–700 spores of *G. mossease* 100 g^{-1} soil) was added to a planting hole.	Competition for nutrients and decreased pathogen population	25% in the tomato	16%	113

BE: biological control efficacy, NA: not applicable, Yield*: increase in yield

tion of plants by both *R. solanacearum* and *G. versiforme* increased the contents of soluble phenols and cell-wall bound phenols in the root tissue, which may be related to ISR by the fungus (147). Another fungus, *Pythium oligandrum*, has the potential to control bacterial wilt disease, in which cell wall proteins may play an important role in the induction of resistance to *R. solanacearum*, accompanied by activation of the ethylene-dependent signaling pathway (41). Shiitake mycelia leachate was found to contain an antibiotic ingredient that suppressed the growth of *R. solanacearum in vitro* (93). In addition, three endomycorrhizal fungi (*Gigaspora margarita, Glomus mosseae, and Scutellospora* sp.) (112) and the lichen *Parmotrema tinctorum* (35) have been identified as BCAs against *R. solanacearum*.

In the inoculation methods of BCAs, pouring or drenching soil was more prevalent than other methods, whereas the biocontrol efficacy range appeared to be lower than that of the dipping of roots or seed coating method.

There are some disadvantages to BCAs. The biggest obstacle is their poor performance due to inconsistent colonization. Suppression by BCAs has been observed in a narrow range of host plants or restricted to a single pathogen or disease (127). The degree of suppression is sometimes too low to be commercially acceptable or requires uneconomically high rates of inoculums to be applied (127). Difficulties have also been associated with producing, storing, and subsequently applying BCAs. An option to overcome the storage problem is to select spore formers as BCAs (*e.g.* 21, 47, 74, 116, 124, 146).

2) Organic matter

Organic amendments to soil have direct impacts on plant health and crop productivity. They are advantageous because they improve the physical, chemical, and biological properties of soil, which can have positive effects on plant growth (14).

The degradation of organic matter in soil can directly affect the viability and survival of a pathogen by restricting available nutrients and releasing natural chemical substances with varying inhibitory properties (14). Carbon released during the degradation of organic matter contributes to increasing soil microbial activity and thereby enhances the likelihood of competition effects in the soil (14). Organic amendments to soil have been shown to stimulate the activities of microorganisms that are antagonistic to pathogens (6). In addition, organic amendments often contain biologically-active molecules such as vitamins, growth regulators, and toxins, which can affect soil microorganisms. Youssef and Tartoura (139) recently reported that plant resistance against the bacteial wilt pathogen was enhanced through the augmented activities of ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase following the application of compost.

Organic matter originates from recently living organisms and decays or is the product of decay. It is categorized into plant or animal origins, and simple organic carbons. In the previous references to an *R. solanacearum* study, different organic matter, such as plant residue (80%), animal waste (10%), and simple organic matter (10%), were shown to control bacterial wilt disease. Larkin (67) found that biological amendments were generally effective for delivering microorganisms to natural soil, resulting in a wide variety of effects on soil microbial communities depending on the particular types, numbers, and formulations of organisms added. A new approach is the suppression of bacterial wilt in an organic hydroponic system through a rhizosphere biofilm that only forms on roots in the organic system (33).

2a) Plant residue controlling bacterial wilt

Several previous studies reported that bacterial wilt was suppressed by plant residues derived from, *e.g.* chili (*Capsicum annum*) (117), Chinese gall (*Rhus chinensis*) (142), clove (*Szygyum aromaticum*) (11), cole (*Brassica* sp.) (13, 90, 97), eggplant (*Solanum melongena*), (9), eucalyptus (*Eucalyptus globules*) (94, 95), geranium (*Geranium carolinianum*) (91), guava (*Psidium guajava* and *P. quineense*) (3), hinoki (*Chamaecyparis obtusa*) (141), Japanese cedar (*Cryptomeria japonica*) (46, 80), lemongrass (*Cimbopogon citratus*) (94, 95), marigold (*Tagetes patula*) (118), neem (*Azadirachta indica*) (96), palmarosa (*Cimbopogon martint*) (94, 95), pigeon pea (*Cajanus cajan*), sunn hemp (*Crotalaria juncea*) (20), tamarillo (*Cyphomandra betacea*) (92), thyme (*Thymus* spp.) (53, 99), wood wax tree (*Toxicodendron xylvestre*) (142), and worm killer (*Aristolochia bracteata*) (109). The possible mechanisms of action of the plant residues are mainly considered to be antimicrobial activities, followed by the indirect suppression of the pathogen through improved physical, chemical, and biological soil properties (20). For example, the antimicrobial compounds from *Tagetes patula* that suppressed *R. solanacearum* in an *in vitro* experiment were identified as 5-(3-buten-1-ynyl)-2,2'-bithienyl (BBT) and 5-(4-acetoxy-1-butynyl)-2,2'-bithienyl (BBTOAc) (118). Other plants such as *Cryptomeria japonica* produced sandaracopimarinol and ferruginol (80) while *Cyphomandra betacea* contained a glycosidase inhibitory protein that suppressed *R. solanacearum* in an *in vitro* experiment (92). Lansiumamide B isolated from the seeds of *Clausena lansium* suppressed tobacco bacterial wilt more than an antibiotic streptomycin when applied at a density of 100 mg kg^{-1} (71).

Previous experiments demonstrated the successful application of organic matter against bacterial wilt in greenhouses and in the field. For example, in a greenhouse experiment, when the freshly cut aerial parts of pigeon pea (*Cajanus cajan*) and crotalaria (*Crotalaria juncea*) were incorporated at concentrations of 20–30% and incubated for 30 d, they completely suppressed tomato bacterial wilt 45 d after the inoculation (20); however, the application rate of this organic matter was high and, thus, not feasible for farmers. Thymol oil derived from a thyme plant reduced bacterial wilt by 65% in the fall 2002 tomato cultivation and by 82% in fall 2003 tomato cultivation at an application rate of 0.72% in the field (53). Alfano *et al.* (8) reported that the disease suppressive effects of olive waste compost appeared to be due to the combined effects of suppression phenomena caused by the presence of microorganisms competing for both nutrients and space as well as by the activity of specific antagonistic microorganisms.

2b) Animal waste controlling bacterial wilt

Although many studies have already reported that animal waste controls plant disease, few have shown that animal waste suppresses bacterial wilt disease. For example, the application of pig slurry decreased the population of *R. solanacearum* in the soil (36). The mechanisms underlying

the enhanced decline of the population of this pathogen and disease suppression remains unclear; however, shifts in bacterial community profiles have been proposed. Another study suggested that the suppression of bacterial wilt by poultry and farmyard manure were related to higher microbial activity and higher numbers of cultural bacteria and fungi (50). In that study, a lower disease index was related to the poor survival of the pathogen. However, limitations are associated with the wide use of organic waste. Janvier *et al.* (51) demonstrated that the major key-points for the efficiency of organic matter in suppressing plant pathogens depended on: i) the plant-pathogen combination, ii) the rate of application, iii) the nature/type of amendment, and iv) the degree of maturity of the decomposition stage of crop residues.

2c) Simple organic compounds controlling bacterial wilt

The efficacy of simple organic compounds, including amino acids, sugars, and organic acids, on bacterial wilt in the tomato was evaluated in pot experiments. The application of lysine to a pumice culture medium (0.25 mg g^{-1}) and soil (2.5 mg g^{-1}) reduced bacterial wilt in the tomato by 85–100% (48, 87) and by 58–100% (97), respectively. The suppression mechanism was not attributed to the induction of systemic resistance, but to shifts in the soil microbial community structure that led to the more rapid death of the pathogen (98). In contrast, riboflavin induced a series of defense responses and secondary metabolism in cell suspensions and, thus, protected tobacco against *R. solanacearum* (75). DL,-3-aminobutyric acid (BABA) also increased polyphenol oxidase activity and decreased that of catalase in tomato plants, suggesting the induction of resistance to bacterial wilt in the tomato (40). Another study showed that methyl gallate exhibited strong bactericidal effects on *R. solanacearum* (30).

Physical methods, including biofumigation. A number of physical control methods, *e.g.* solarization and hot water treatments, have proved to be effective against *R. solanacearum*. Vinh *et al.* (121) found that soil solarization using transparent plastic mulches for 60 d prior to the planting of tomatoes reduced the incidence of bacterial wilt. Another study reported that rhizome solarization on ginger seeds for 2 to 4 h reduced bacterial wilt by 90–100% 120 d after planting, and that ginger seeds sterilized with discontinuous microwaving (10-s pulses) at 45°C reduced the incidence of wilt by 100% (63). Baptista *et al.* (16, 17) studied the mechanisms of soil solarization that reduced bacterial wilt in the tomato. Soil solarization reduced soil pH, potassium (K), sodium (Na), boron (B), and zinc contents, microbial biomass, and microbial respiration in soil, but did not significantly affect other soil chemical properties. A heat treatment at either 45°C for 2 d or a minimum temperature of 60°C for 2 h of the infected soil prior to tomato planting reduced the total bacterial population by 60–97%, that of *Ralstonia* sp. from 2 to 7×10^8 cfu g^{-1} to 0 to 115 cfu g^{-1}, and the incidence of bacterial wilt by 50–75% (62). Several parameters need to be carefully considered before the application of soil solarization can be expanded: controlling temperature or the release of volatile compounds and economical and/or practical feasibility in field.

In addition to heat treatments, cold temperatures are also sometimes effective. Bacterial wilt rarely occurs in tobacco crops planted in May or June (winter crop) in north Queensland because of cool weather conditions, whereas the disease developed when crops were planted in spring (September to November), particularly when bacterial wilt had previously occurred and crop rotation was not practiced (7). Lower moisture conditions (20–30% maximum water holding capacity) and pre-incubation at lower temperatures (4°C) reduced bacterial wilt and had a negative impact on the survival of *R. solanacearum* (50). Scherf *et al.* (106) found that *R. solanacearum* survived for 6 months in an infected geranium at a constant temperature, but declined rapidly in repeated winter temperature cycles of 2 d at 5°C followed by 2 d at −10°C. The mechanism of action responsible for the suppression of bacterial wilt by physical methods generally involves killing pathogens with high or low temperatures.

Biofumigation, which refers to the agronomic practice of using volatile chemicals released from plant residues to suppress soil-borne plant pathogens, has recently been attracting attention (61). Biofumigation is called biological soil disinfection (BSD) and the production of organic acids or heavy metal ions is involved in the suppression of pathogens (83).

Another approach is control with a high voltage electrostatic field and radio frequency electromagnetic field, in which ISR is involved in the suppression mechanism (128). Silver-coated non-woven cloth filter and a visible light source (15) or electrostatic spore precipitator ozone-saturated water (144) was developed as a sterilization device and inactivated the pathogen.

Cultural practices. *1) Cultivar resistant*

The growth of cultivars that are resistant to bacterial wilt is considered to be the most economical, environmentally friendly, and effective method of disease control. Breeding for resistance to bacterial wilt has been concentrated on crops of wide economic importance such as the tomato, potato, tobacco, eggplant, pepper, and peanut, and has commonly been influenced by factors such as the availability of resistance sources, their diversity, genetic linkage between resistance, and other agronomic traits, differentiation and variability in pathogenic strains, the mechanism of plant-pathogen interactions, and breeding or selection methodology (19, 28, 39). For example, the *Arabidopsis* NPR1 (non-expresser of *PR* genes) gene was introduced into a tomato cultivar, and enhanced resistance to bacterial wilt and reduced the incidence of wilt by approximately 70% 28 d after the inoculation (73). Potato genotype BP9, which is a somatic hybrid between *Solanum tuberosum* and *S. phureja*, successfully reduced bacterial wilt by 90–100% (31). Somatic hybrids between *S. melongena* cv. Dourga and two groups of *S. aethiopicum* were produced by the electrical fusion of mesophyll protoplasts and were found to be tolerant to *R. solanacearum*. Public acceptance in Japan is needed prior to the commercial use of such genetically modified crops.

Prior *et al.* (101) showed that resistant plants were heavily invaded by *R. solanacearum* without displaying wilt symptoms. Nakaho *et al.* (2004) revealed that bacterial multiplication in the stems of resistant tomato plants was suppressed due to limited pathogen movement from the protoxylem or primary xylem to other xylem tissues (85). A proteomic approach was used to elucidate molecular interactions in the cell walls of resistant and sensitive plants inoculated with *R. solanacearum* (23). Resistance to bacterial wilt in many crops has generally been negatively correlated with yield and

quality. Thus, the release of resistant cultivars may be poor because of other agronomic traits and are not widely accepted by farmers or consumers. The breeding of a good resistant cultivar is expected in the future through stronger efforts in the genetic enhancement of bacterial wilt resistance through biotechnology approaches in order to improve yield crop.

2) Crop rotation, multi-cropping

The benefits of crop rotation are maintenance of the soil structure and organic matter, and a reduction in soil erosion that is often associated with continuous row crops (51). While continuous cropping with the same susceptible host plant will lead to the establishment of specific plant pathogenic populations, crop rotation avoids this detrimental effect and is often associated with a reduction in plant diseases caused by soil-borne pathogens (51, 66). For example, the onset of bacterial wilt was delayed by 1 or 3 weeks and wilt severity was reduced by 20–26% when a susceptible tomato variety was grown after corn, lady's fingers, cowpea, or resistant tomato (4). Potato cultivation rotated with wheat, sweet potato, maize, millet, carrots, sorghum, or phaseolus beans reduced the incidence of wilt by 64 to 94% while the yield of potatoes was 1- to 3-fold higher than that of monocultured potatoes (54). In an example of multi-cropping, Yu *et al.* (140) reported the suppression mechanisms of Chinese chive (*Allium tuberosum*), which reduced the incidence of bacterial wilt in the tomato (approximately 60%) because the root exudates of Chinese chive may prevent *R. solanacearum* from infecting tomato plants.

3) Soil amendment

Previous studies revealed that the application of fertilizers reduced the incidence of bacterial wilt. Calcium (Ca) is the most well-known fertilizer to suppress disease. Increased Ca concentrations in plants reduced the severity of bacterial wilt as well as the population of *R. solanacearum* in the stems of the tomato (134, 136). Furthermore, an increase in Ca uptake by tomato shoots correlated with lower levels of disease severity (135, 136). Lemaga *et al.* (68) reported that the application of nitrogen (N) + phosphorus (P) + K and N + P (application rate of each fertilizer = 100 kg ha^{-1}) reduced bacterial wilt by 29% and 50%, respectively, and increased the yield of potatoes to 18.8 t ha^{-1} and 16.6 t ha^{-1}, respectively, which was higher than that in untreated controls (11.2 t ha^{-1}). Hacisalihoglu *et al.* (38) reported that bacterial wilt induced changes in the distribution of nutrients, especially Ca, B, and P in tomato leaves. Li and Dong (70) showed that the combined amendment of rock dust and commercial organic fertilizer reduced the incidence of bacterial wilt in the tomato. A single amendment with rock dust also effectively reduced the incidence of bacterial wilt in the tomato and higher soil pH and Ca content were key factors in the control of bacterial wilt by the rock dust amendment.

Many elements in the cell walls influence the susceptibility or resistance of plants to infections by pathogens and silicon is considered to be a beneficial element for plants and higher animals (29). Kiirika *et al.* (60) reported that the combined application of silicon and chitosan reduced the incidence of bacterial wilt in the tomato by inducing resistance. Si and chitosan exhibited synergistic effects against the disease.

Integrated Pest Management (IPM)

According to Agrios (5), the main goals of an integrated plant disease control program, regarded as integrated pest management (IPM), are to (i) eliminate or reduce the initial inoculums, (ii) reduce the effectiveness of initial inocula, (iii) increase the resistance of the host, (iv) delay the onset of disease, and (v) slow secondary cycles.

IPM reduced bacterial wilt disease by 20–100% in the field or under laboratory conditions, and typically combines two or three methods among cultural practices and chemical and biological methods. For example, the incidence of bacterial wilt in the tomato was monitored in soil infested with *R. solanacearum* and the addition of an organic mixture consisting of agricultural and industrial waste such as bagasse, rice husks, oyster shell powder, urea, potassium nitrate, calcium superphosphate, and mineral ash or Actigard (active ingredient: acibenzolar-*S*-methyl [ASM]). The addition of the organic mixture decreased the incidence of bacterial wilt in the tomato by 32%, while that of Actigard decreased it by 5%. In contrast, the addition of the organic mixture and Actigard decreased the incidence of bacterial wilt by 53% (12). We previously demonstrated that suppressive effects against bacterial wilt in the tomato were enhanced by combinations of BCAs and their substrates, such as lysine, sucrose, and anaerobically digested slurry, in which the addition of substrates improved the colonization of tomato roots by BCAs (87, 88).

The relative importance of factors accounting for production losses need to be assessed in order to develop IPM. Combinations in cultural practice methods, such as the combination of crop rotation with a resistant cultivar or a soil amendment, or the combination of organic matter with a non-pesticide chemical such as formaldehyde or bleaching powder appear to have effectively reduced the incidence of bacterial wilt and increased crop yield (4, 68, 108, 121). The combined application of ASM and *P. fluorescens* Pf2 resulted in the greatest reduction in the incidence of bacterial wilt in the tomato, while the application of ASM or *P. fluorescens* Pf2 was also effective (1). A previous study reported that the combination of endophytic bacteria (*Bacillus* sp. and *Serratia marcescens*, both of which had no antibiosis) with resistant cultivars of the tomato reduced the incidence of bacterial wilt (18).

Grafting is an important strategy in integrated pest management for soil-borne pathogens. Disease management by grafting has been reported for fungal pathogens (such as *Verticillium*, *Fusarium*, *Pyrenochaeta*, and *Monosporascus*), oomycete pathogens (*Phytophthora*), bacterial pathogens (particularly *Ralstonia*), root knot nematodes, and several soil-borne viruses (78).

We need to select methods that are easy, practical, profitable, and also environmentally healthy to control diseases and improve yields.

Cautions for disease control measures. *1) Keep the environment healthy*

Preventive methods are essential for maintaining fields that are free of bacterial wilt. *R. solanacearum* is a soil-borne bacterium and may survive for prolonged periods in soil, water, and plant materials (77). Thus, to keep environment free of this pathogen, it is important to clean seeds, soil, water, and tools in order to improve crop production by preventing this disease. The use of healthy seeds that are free of

pathogens is the most economical, environmentally friendly, and effective method for disease control. Cultural practices involving soil amendments, including organic matter, crop rotation, and multi-cropping, can be used to maintain soil health. These agricultural practices influence the chemical, biological, and physical properties of soil, which, in turn, influence the viability and distribution of pathogens as well as the availability of nutrients for pathogens in the soil. Researchers are becoming more interested in investigating the effects of such practices on microbial communities, or in assessing their potential to control soil-borne pathogens. Soil health indicators may be very useful for risk prevision and technical advice (53).

The early detection of *R. solanacearum* in irrigation water or soil is essential for preventing its introduction into new areas. A sensitive quantitative assay was recently developed to detect *Ralstonia solanacearum* in soil by the most probable number (MPN) analysis based on PCR results, in which a pre-culture was performed in a buffer containing antibiotics, but no other carbon source in order to allow the pathogen to grow and to suppress the growth of other soil microorganisms (49). This assay enabled pathogens to be detected at levels as low as 9.3 cfu g^{-1} soil.

2) Abiotic and biotic factors to be considered

Plant diseases caused by soil-borne pathogens such as *R. solanacearum* result from the multiple and complex interactions, including both biotic and abiotic factors, they have with plants. Abiotic factors such as nutrient (organic matter and minerals) conditions, soil type, pH, anaerobic conditions, temperature, and moisture content influence the development of *R. solanacearum* in soil, as described above.

Biotic factors are related to microorganisms, flora, fauna in the soil, and plants that can affect *R. solanacearum*. Previous studies investigated the biotic factors controlling *R. solanacearum* such as the microbial community in soil, introduction of BCAs, cultivar resistance, and rotation, as described above. Various suppression mechanisms are considered to be biotic factors for the pathogen, such as enhanced microbial activity, which can suppress *R. solanacearum*, the release of antibiotics, enhanced competition, decrease in colonization, the induction of systemic resistance, and protection against or avoidance of pathogen contact with the host crop.

3) Economic analysis

Many researchers have managed bacterial wilt with biological, physical, and chemical methods and/or with cultural practices; however, few studies have examined the efficiency of these methods to improve crop yield, especially economic analyses. Based on our reference survey, only 10% of the methods reported improved crop yield. In integrated disease management, soil amendments with 300 kg N and 1,500 kg CaO, together with soil solarization using transparent plastic mulches, reduced the incidence of wilt in the tomato by 20% and increased grower profits, equivalent to 369 to 998 US$ per ha (121). Our primary goal is to contribute to safe, sustainable, and high agricultural production. Attention to cost-benefit analyses is indispensable in the short, middle, and long term.

4) Future of fumigants

The use of a fumigant type of agrochemical in Japan has enabled the establishment of a pathogen-free environment and, thus, intensive agriculture, leading to high quality crop production. However, some of these indiscriminating fumigants are prohibited in European countries, *e.g.* chloropicrin has been banned since 2011 due to the risks posed to pesticide operators and aquatic organisms, birds, and bees (EU No 1381/2011). Due to public concerns and environmental impact, it is not advisable to rely only on single controlling methods, such as fumigants, for high yield and quality crop production. Proper control methods need to be adopted based on the density of and crop resistance to pests.

Concluding remarks

The research discussed in this review shows how many different diverse options have been reported on control methods against diseases caused by *R. solanacearum*. This unequivocally indicates the importance of these diseases worldwide. The avoidance of crop losses due to pathogens significantly contributes to increased crop production worldwide. We will be able to identify solutions by integrating a biological control agent and organic matter including simple organic compounds, compost, or plant residue.

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Seasonal Dynamics of Anammox Bacteria in Estuarial Sediment of the Mai Po Nature Reserve Revealed by Analyzing the 16S rRNA and Hydrazine Oxidoreductase (*hzo*) Genes

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The community and population dynamics of anammox bacteria in summer (wet) and winter (dry) seasons in estuarial mudflat sediment of the Mai Po Nature Reserve were investigated by 16S rRNA and hydrazine oxidoreductase (*hzo*) genes. 16S rRNA phylogenetic diversity showed that sequences related to 'Kuenenia' anammox bacteria were presented in summer but not winter while 'Scalindua' anammox bacteria occurred in both seasons and could be divided into six different clusters. Compared to the 16S rRNA genes, the *hzo* genes revealed a relatively uniform seasonal diversity, with sequences relating to 'Scalindua', 'Anammoxoglobus', and planctomycete KSU-1 found in both seasons. The seasonal specific bacterial groups and diversity based on the 16S rRNA and *hzo* genes indicated strong seasonal community structures in estuary sediment of this site. Furthermore, the higher abundance of *hzo* genes in summer than winter indicates clear seasonal population dynamics. Combining the physicochemical characteristics of estuary sediment in the two seasons and their correlations with anammox bacteria community structure, we proposed the strong seasonal dynamics in estuary sediment of Mai Po to be due to the anthropogenic and terrestrial inputs, especially in summer, which brings in freshwater anammox bacteria, such as 'Kuenenia', interacting with the coastal marine anammox bacteria 'Scalindua'.

Key words: anammox bacteria, seasonal dynamics, 16S rRNA, *hzo*, estuary, Mai Po Nature Reserve

Anammox, the conversion of ammonium to dinitrogen gas (N_2) by nitrite under anoxic conditions, has been proposed to play a key role in the global nitrogen cycle (9), which might be responsible for more than half of all oceanic N_2 production (7). The anammox process has been demonstrated in marine ecosystems (15, 19, 20, 31, 43, 45, 47) and freshwater terrestrial ecosystems (29, 41), low-temperature polar regions (32, 33) and high-temperature hot spring and deep sea hydrothermal vents (3, 13), and oil reservoirs (22), even on the surface of marine sponges (12, 26). Certain *Planctomycetes* are monophyletic members linked to this process, and five genera, '*Candidatus* Brocadia', '*Candidatus* Kuenenia', '*Candidatus* Anammoxoglobus', '*Candidatus* Scalindua' and '*Candidatus* Jettenia', have been described (16–18, 30, 37). Interestingly, anammox bacterial diversity and distribution differ among various ecosystems, showing niche specificity (6). The genera 'Brocadia' and 'Kuenenia' are usually found in engineered systems such as wastewater treatment plants though a few reports indicated their presence in freshwater and marine ecosystems (1, 27, 50), while 'Scalindua' genus dominates in natural ecosystems, especially in marine environments (39, 49).

Cape Fear River estuary sediment showed a strong temporal and spatial distribution of anammox bacteria and seasonal variations of anammox bacteria detected by the isotope method and 16S rRNA gene, which extend our understanding of the seasonal distribution of anammox bacteria in estuary ecosystems (6). Hietanen *et al.* suggested that anammox accounted for 10% and 15% of total N_2 production during spring and autumn at a coastal station on the Gulf of Finland in the Baltic Sea (11), but gave no information about the seasonal variation in anammox bacteria community structure. These results indicated anammox bacteria have clear seasonal variations in their activity, as well as community structure, diversity and abundance. However, most information about anammox bacterial diversity and distribution in natural ecosystems is based on 16S rRNA genes. Functional biomarkers that respond to the anammox process are needed to describe anammox bacterial ecology more comprehensively. Hydrazine oxidoreductase (HZO) dehydrogenated the unique anammox intermediate, hydrazine, to form dinitrogen gas, and has been proposed as a functional biomarker for anammox bacteria, especially the two copies of the HZO-encoding gene (*hzo*) within *hzo*-cluster 1 (34, 40), and recent studies also successfully used *hzo* to evaluate the diversity and distribution of anammox bacteria in different natural ecosystems, including marine sediment (23), a wastewater treatment plant (24) and high-temperature petroleum reservoirs (22). On the other hand, knowledge about the temporal community structure of anammox bacteria in natural ecosystems is still limited, especially in natural ecosystems with anthropogenic influences. Thus, elucidating the seasonal variation in bacterial

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community structures and checking whether different genera or species have specific niches or seasonal requirements are obviously important for understanding anammox microbial ecology.

To extend our understanding of the seasonal variation of anammox bacterial communities in natural ecosystems, we investigated the anammox bacterial dynamics in estuarial sediment of the Mai Po Nature Reserve, where rivers bring in wastewater during the wet season in summer. The results of 16S rRNA and *hzo* genes based analyses showed obvious and strong seasonal dynamics of anammox bacteria, controlled by seasonal anthropogenic or terrestrial inputs and regular coastal tides.

Materials and Methods

Site description, sampling and chemical analyses

The Mai Po Nature Reserve of Hong Kong (22°29'N to 22°31'N and 113°59'E to 114°03'E), an intertidal estuary of the Pearl River Delta, China, consists of intertidal mudflats, mangroves, traditionally operated shrimp ponds, fishponds, reedbeds and drainage channels (28). In the Nature Reserve, the Shenzhen River and inland San Pui River especially the former, bring large quantities of domestic sewage and industrial wastewater from adjacent areas where manufacturing activities and residential development have intensified in recent years (21, 28). Furthermore, the Reserve is driven by two seasonal monsoons; the northeast monsoon prevails in winter and the southwest monsoon in summer (4, 42). According to a long-term ecological monitoring project, the Mai Po Nature Reserve varies greatly in physicochemical characteristics between summer and winter, and slightly spring and autumn (unpublished data, Ecological Monitoring Programme for the Mai Po and Inner Deep Bay Ramsar Site from 2003 to 2008). Thus, five sampling sites were selected (Fig. 1) and triplicate sediment samples were collected during summer (May) and winter (November) in 2008. Each sample was labeled according to site (A to E) and season (5 or 11). The temperature, redox potential and pH of the sediment samples were measured *in situ* using an IQ180G Bluetooth Multi-Parameter System (Hach Company, Loveland, USA). The salinity of pore water was measured using a YSI 556 Multiprobe System (YSI, Yellow Springs, USA). The total organic carbon of sediment and concentration of NH_4^+-N, NO_3^--N, and NO_2^--N in pore water of sediment samples, after centrifugation, were measured with an autoanalyzer (QuickChem, Milwaukee, USA) according to standard methods of the American Public Health Association (2). Total ammoniacal nitrogen (T-NH_4^+), total Kjeldahl nitrogen, total nitrogen, total phosphorus and total sulphide concentrations of sediment samples were measured according to the standard methods ASTM D3590-89B, ASTM D51588B and ASTM E200 60–61; while levels of arsenic, cadmium, copper, mercury and lead were determined by USEPA method 6020 (48).

DNA extraction, PCR amplification and clone library construction

Total genomic DNA of each sediment sample was extracted using the SoilMaster DNA Extraction kit (Epicentre Biotechnologies, Madison, USA) according to the manufacturer's instructions, and the DNA concentration of each sample was measured by a Biophotometer (Eppendoff, Hamburg, Germany). PCR amplification of the 16S rRNA gene was carried out using two primer sets: Brod541F (5'-GAGCACGTAGGTGGGTTTGT-3') (29)-Amx820R (5'-AAAACCCCTCTACTTAGTGCCC-3') (36); and AMX368F (5'-TTCGCAATGCCCGAAAGG-3')-Amx820R (5'-AAAACCCCTCTACTTAGTGCCC-3') (36). For amplification of the *hzo* gene, the primer set HZOF1 (5'-GTGCATGGTCAATTGAAAG-3)-HZOR1 (5'-CAACCTCTTCWGCAGGTGCATG-3') was used (23). The reaction conditions were described elsewhere (23). PCR products were checked by electrophoresis on 1% agarose gels and subsequent staining with ethidium bromide (0.5 μg mL^{-1}), purified using the Gel Advance-Gel Extraction System (Viogeme, Taipei, Taiwan), and used to construct the 16S rRNA and *hzo* clone libraries with the pMD-18 T-vector (Takara, Otsu, Japan).

Sequencing and phylogenetic analysis

Clones with the correctly inserted DNA fragment in each library were checked by PCR with M13 primers, and randomly selected for sequencing. Sequencing was performed with the Big Dye Terminate kit (Applied Biosystems, Foster City, USA) and an ABI Prism 3730 DNA analyzer. DNA sequences were examined and edited using MEGA 4.0 software (44) and then checked for chimera using the Check Chimera program of the Ribosomal Database Project (5) or manually. For the 16S rRNA gene, DNA sequences were manually compiled and aligned using Clustal W (46). For the *hzo* gene, nucleic acid sequences were translated into amino acids and the resulting amino acids sequences were aligned using Clustal W. Phylogenetic trees were constructed by MEGA 4.0 with the neighbor-joining method, and bootstrap re-sampling with 1,000

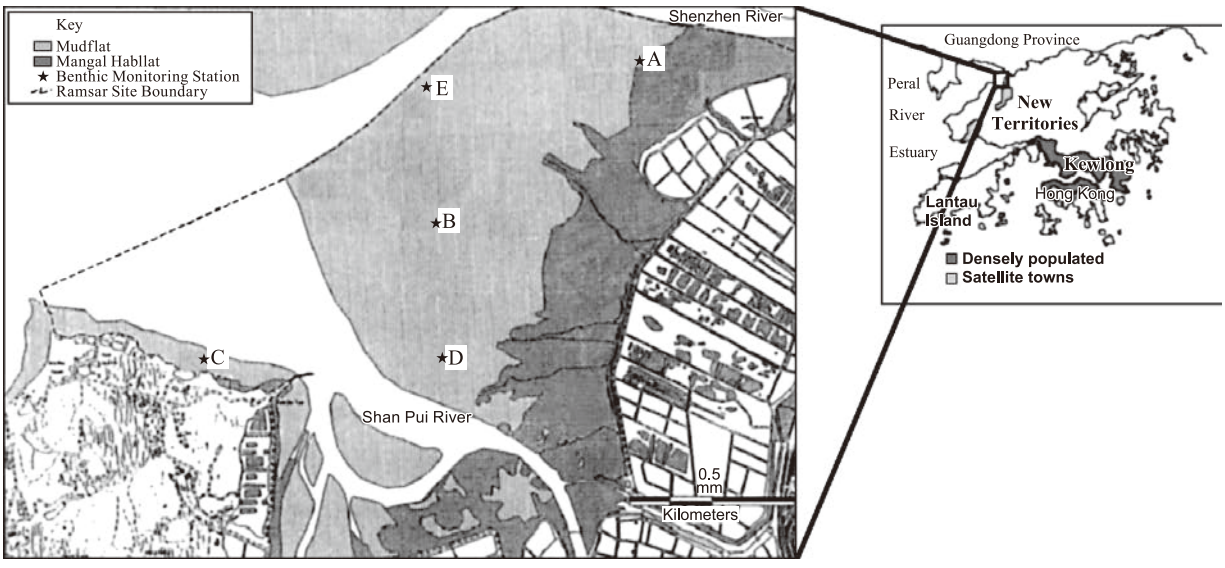

Fig. 1. A map showing the sampling sites at Mai Po Nature Reserve of Hong Kong.

replicates was performed to estimate the confidence values of the tree nodes.

Quantitative PCR

The copy numbers of the *hzo* gene in all samples were determined in triplicate using an ABI 7000 Sequence detection system (Applied Biosystems). The quantification was based on the fluorescent dye SYBR-Green I. Each reaction was performed in a 25 μL volume containing 1 μL of DNA template (30–50 ng μL^{-1}), 0.5 μL BSA (0.1%), 0.5 μL of each primer (20 μM, HZOF1 and HZOR1) and 12.5 μL of Power SYBR Green PCR Master Mix (Applied Biosystems). The PCR profile was 2 min at 50°C and 10 min at 95°C, followed by a total of 45 cycles of 1 min at 95°C, 1 min at 53°C, and 1.5 min at 72°C. A standard plasmid carrying *hzo* was generated by amplifying the gene from extracted DNA of site A and cloning into the pMD 18 T-Vector (Takara). The plasmid DNA concentration was determined and the copy number of *hzo* was calculated directly from the concentration of the extracted plasmid DNA. Ten-fold serial dilutions of a known copy number of the plasmid DNA were subjected to a quantitative PCR assay in triplicate to generate an external standard curve. The amplification efficiency ranged from 0.90 to 0.95 and the correlation coefficient (R^2) of the standard curve was 0.991.

Statistical analysis

Operational taxonomic units (OTUs) for the community analysis were defined by a 3% cut-off in the 16S rRNA gene nucleotide sequences and a 5% cut-off in the *hzo* genes deduced amino acid sequences, as determined using the furthest neighbor algorithm in the DOTUR program (35). DOTUR was also used to analyze diversity including Shannon and Simpson indices for each clone library. To examine the seasonal and geographic distribution of anammox bacteria in estuary sediments of the Mai Po Nature Reserve, 16S rRNA and *hzo* gene sequences were analyzed with the online software UniFrac (http://bmf2.colorado.edu/unifrac/index.psp) using the principal coordinates analysis (PCoA) as suggested previously (25). Pearson moment correlations for bacterial community structures and environmental variables were determined using the Microsoft Excel program.

Nucleotide sequence accession numbers

The GenBank accession numbers for the 16S rRNA gene sequences reported here are GQ427230 to GQ427485 and HM209472 to HM209609. The accession numbers for the *hzo* gene sequences are GQ427486 to GQ427673 and HM209610 to HM209725.

Results

Seasonal variation in environmental variables

Table 1 shows the main physicochemical attributes of the sediment samples and pore water. Many environmental parameters showed strong seasonal variation, for example, pH, the pore water NH_4^+ concentration, salinity and TOC were higher in winter while temperature, and total NH_4^+, total Kjeldahl nitrogen (TKN), Cu and Hg concentrations were lower in winter. However, within the same season, five sampling sites did not show strong spatial variation although some environmental parameters exhibited significant differences among the five sites (Table 1).

Community structures of anammox bacteria within two seasons

From the 16S rRNA gene clone libraries, 1 to 7 OTUs were obtained from each sediment sample, with site E in May (E5) showed the highest number. The Shannon index (0.00–1.19) and Chaol index (1.0–8.5), calculated by the DOTUR program, also showed similar variation (Table 2). However, 343 *hzo* sequences from 10 libraries yielded 52 unique OTUs, with the highest diversity again at E5

Table 1. Environmental characteristic of research sediment samples and pore water

Sampling site	Season	pH	T (°C)	Redox (mV)	NH^{4+}* (μM)	NO_X* (μM)	Salinity* (‰)	T-NH_4^+ (mg kg^{-1})	TKN (mg kg^{-1})	T-N (mg kg^{-1})	T-P (mg kg^{-1})	S^{2-} (mg kg^{-1})	TOC (mg kg^{-1})	As (mg kg^{-1})	Cd (mg kg^{-1})	Cu (mg kg^{-1})	Hg (mg kg^{-1})	Pb (mg kg^{-1})
A	May	6.24	28.9	−59.2	342.5	2.4	5.2	170.1	1160.3	1170.0	1128.0	53.0	13.7	27.0	0.45	103.7	0.22	72.5
	Nov.	7.36	22.8	−96.3	345.2	1.1	13.8	25.1	997.0	1006.0	956.0	552.0	17.9	17.5	0.60	98.3	0.20	66.0
B	May	7.13	30.1	−160.4	20.0	1.1	6.9	172.0	575.9	583.0	1151.0	336.0	10.7	21.0	0.53	100.3	0.23	85.1
	Nov.	7.41	22.2	−90.0	603.9	0.5	15.5	37.1	649.0	857.0	1360.0	400.0	13.5	18.8	0.35	84.0	0.15	75.0
C	May	6.74	29.5	−89.6	43.6	1.5	7.0	148.7	917.8	927.0	1193.0	350.0	10.8	16.0	0.23	61.8	0.12	49.8
	Nov.	7.55	22.4	−126.0	117.1	0.9	25.7	19.6	592.0	600.0	653.0	341.0	13.8	13.9	0.33	55.7	0.09	40.0
D	May	7.16	29.3	−116.0	71.0	2.1	3.5	162.5	872.9	881.0	1608.0	365.0	11.7	31.0	0.45	124.7	0.24	70.2
	Nov.	7.52	22.6	−59.0	683.3	2.7	20.4	31.7	605.0	614.0	836.0	771.0	13.6	18.2	0.55	104.7	0.17	67.3
E	May	7.18	31.2	−154.0	101.6	9.3	6.0	154.2	847.2	864.0	1657.0	101.0	9.2	16.0	0.25	103.7	0.21	72.0
	Nov.	7.49	22.6	−189.0	460.4	0.6	26.3	13.3	711.0	718.0	399.0	132.0	10.0	17.0	0.20	44.7	0.15	43.7

* The concentrations of pore water for each sediment sample.

Table 2. Diversity of anammox bacteria in sediment samples

Sampling site	Season	Clone Number		OTUs		Shannon		Chaol		Coverage	
		16S rRNA	*hzo*	16S rRNA	*hzo*	16S rRNA	*hzo*	16S rRNA	*hzo*	16S rRNA	*hzo*
A	May	45	40	4	8	0.73	1.37	5	14	0.96	0.90
	Nov.	33	28	3	6	0.50	1.40	3	6	0.97	0.96
B	May	31	57	4	3	1.12	0.43	4	3	0.97	0.96
	Nov.	41	26	3	3	0.97	0.78	3	3	1.00	1.00
C	May	34	52	4	5	1.04	1.38	5	5	0.97	0.98
	Nov.	32	20	1	4	0.00	1.19	1	4	1.00	0.95
D	May	59	30	5	4	1.19	0.77	5	4	1.00	0.97
	Nov.	34	23	3	5	0.69	1.12	3	8	0.97	0.87
E	May	38	37	7	8	1.14	1.00	8.5	23	0.92	0.84
	Nov.	47	30	4	6	0.85	1.47	4	6	1.00	0.97

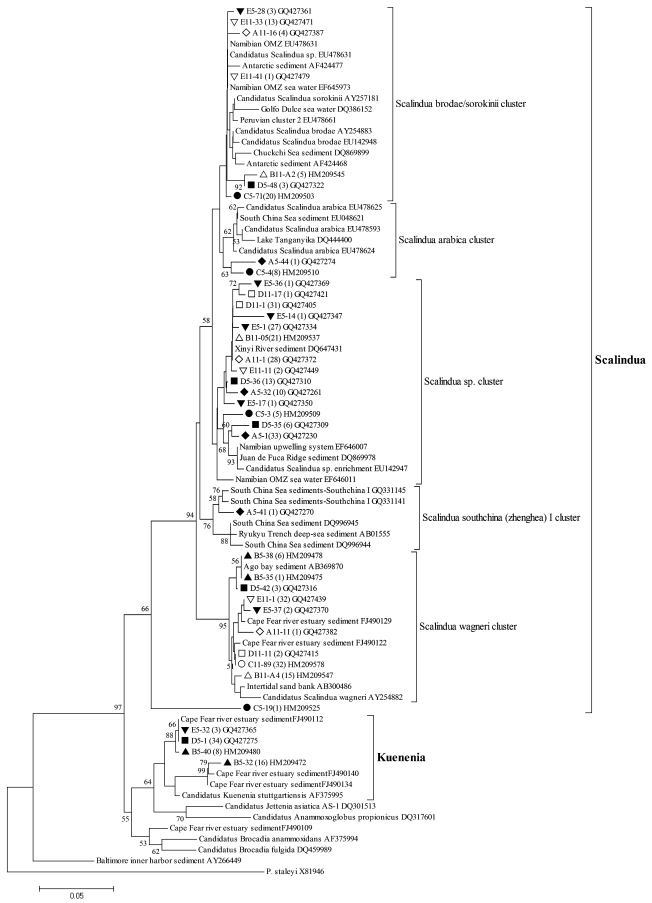

Fig. 2. Neighbor-joining phylogenetic tree of anammox bacterial 16S rRNA gene sequences. Numbers in parentheses refer to how many clones retrieved were assigned to an OTU. The numbers at the nodes are percentages indicating the level of bootstrap support based on 1,000 resampled data sets (only values greater than 50% are shown). Branch lengths correspond to sequence differences as indicated by the scale bar.

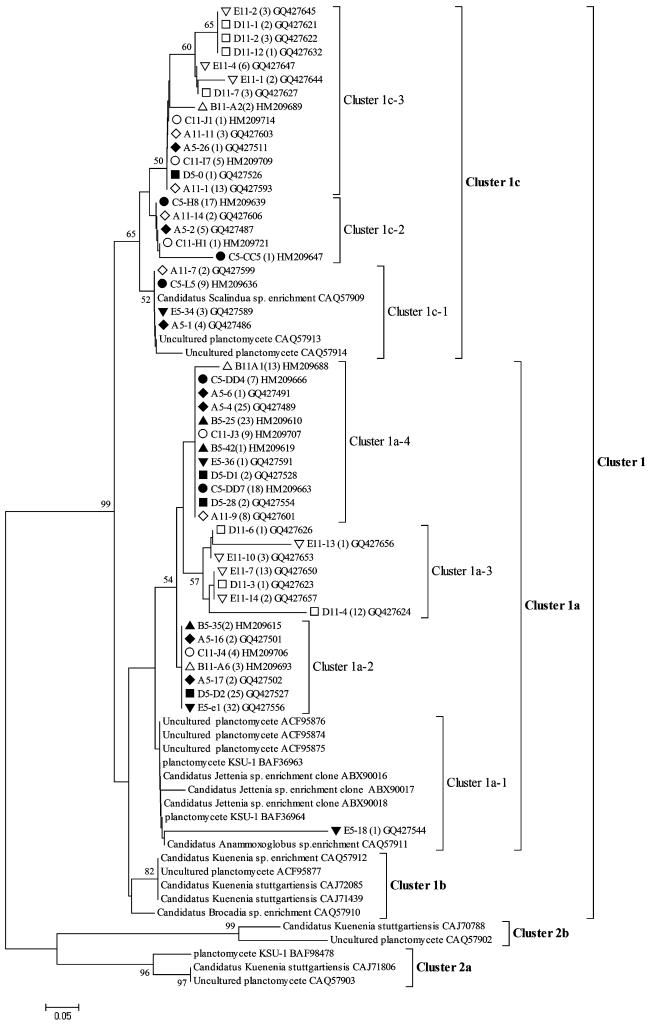

Fig. 3. Neighbor-joining phylogenetic tree of *hzo* gene sequences. Numbers in parentheses refer to how many *hzo* gene clones retrieved were assigned to an OTU. The numbers at the nodes are percentages indicating the levels of bootstrap support based on 1,000 resampled data sets (only values greater than 50% are shown). Branch lengths correspond to sequence differences as indicated by the scale bar.

(Table 2). The coverage ranged from 0.92 to 1.0 (16S rRNA) and 0.84 to 1.0 (*hzo*), indicating that all clone libraries covered the majority of the natural diversity of the 16S rRNA and *hzo* genes targeted by the selected primer sets, as confirmed with the asymptotic rarefaction curves (Fig. S1). Interestingly, diversity among both the 16S rRNA and *hzo* genes was higher in the samples collected in summer than winter though exceptions occurred for *hzo* at sites B and D, indicating a strong seasonal variation of anammox bacteria diversity.

The phylogeny indicated that 394 of the 16S rRNA gene sequences grouped into six clusters, affiliated with the *Kuenenia* and *Scalindua* genera of anammox bacteria (Fig. 2). The 'Kuenenia' cluster consisting of sequences from sites B, D and E in May (B5, D5 and E5) was affiliated with sequences retrieved from Cape Fear River estuary sediment and '*Candidatus* K. stuttgartiensis'. However, the other five clusters were distributed in the 'Scalindua' genus. The "Scalindua wagneri cluster", closely related to '*Candidatus* S. wagneri' and sequences recovered from an intertidal sand bank, Cape Fear River estuary sediment and Ago Bay sediment, was supported by sequences recovered from all samples except A5, C5 and E5. The "Scalindua southchina (zhenghea) I cluster" containing a single sequence retrieved from A5 was affiliated with sequences from sediment of South China Sea and Ryukyu Trench. The "Scalindua sp. cluster" was closely related to '*Candidatus* Scalindua' enrichment and sequences recovered from Namibian OMZ and Xinyi River sediment. The "Scalindua arabica cluster" containing sequences recovered from A5 and C5 was affiliated with the newly described anammox bacterial species '*Candidatus* S. arabica' (49), while the "Scalindua brodae/sorokinii cluster" was composed of sequences recovered from all samples except A5, B5, C11 and D11. C5 further yielded a sequence which did not affiliate with the phylogenetic clusters, but is close to the 'Scalindua' than other genera of anammox bacteria (Fig. 2).

For the phylogeny of the *hzo* gene, sequences distributed into two major groups, Cluster 1a and Cluster 1c (Fig. 3). Within each group, 4 and 3 sub-clusters were evident, namely Cluster 1a-1 to Cluster 1a-4, and Cluster 1c-1 to Cluster 1c-3, respectively. One sequence in Cluster 1a-1 recovered from E5 was closely related to the '*Candidatus*

Anammoxoglobus' enrichment culture CAQ57911, grouping with sequences of the HZO proteins BAF36963 and BAF36964 from planctomycete KSU-1, and ABX90016, ABX90017, ABX90018, ACF5874, ACF5875 and ACF5876 from '*Candidatus* J. asiatica' enrichment reactors (Fig. 3); while sequences in cluster 1a-2, cluster 1a-3 and cluster 1a-4 constituted novel sister clades with the sequences of cluster 1a-1, representing niche specific groups in estuary sediment of the Mai Po Nature Reserve. More interestingly, cluster 1a-3 only consisted of sequences from C11 and D11, indicating a seasonal specific group for *hzo* genes (Fig. 3). Within cluster 1c, sequences in cluster 1c-1 were affiliated with those of the HZO proteins CAQ57913 and CAQ57914 of uncultured planctomycetes and CAQ57909 from a '*Candidatus* Scalindua' enrichment culture, and cluster 1c-2 and cluster 1c-3 also constituted novel sister clades with cluster 1c-1, again indicating the site-specificity of HZO sequences in Mai Po. Meanwhile, a branch in cluster 1c-3 further showed a seasonal distribution, being composed of sequences from sites D and E in November only (Fig. 3).

Seasonal dynamics of the community composition and population of anammox bacteria

According to the phylogeny of anammox bacterial 16S rRNA genes, two different genera were confirmed. The proportions of the two genera showed that 'Scalindua' was the only group detected in most clone libraries, except in sediment collected from B, D and E during the summer, where 'Kuenenia' represent 77.4% (B5), 57.6% (D5), and 7.9% (E5), respectively (Fig. 4). However, *hzo* cluster 1-a, related to 'Anammoxoglobus', 'Jettenia' and planctomycetes KSU-1, was the dominant *hzo* group in most sediment samples, representing 28.6% to 100%; while cluster 1-c, affiliated with 'Scalindua', was the second group except at site A in winter. The abundance of anammox bacteria was estimated through a quantitative analysis of the *hzo* genes. Results showed that abundance ranged from $(10.2\pm1.9)\times10^4$ (A5) to $(39.8\pm9.1)\times10^4$ (E5) copies per gram of sediment for samples collected in May, and from $(2.9\pm0.6)\times10^4$ (A11) to $(18.9\pm2.5)\times10^4$ (E11) in November (Fig. 4). The result clearly showed a higher abundance of the *hzo* gene in May than November, indicating strong seasonal population dynamics in mudflat sediment of the Mai Po Nature Reserve.

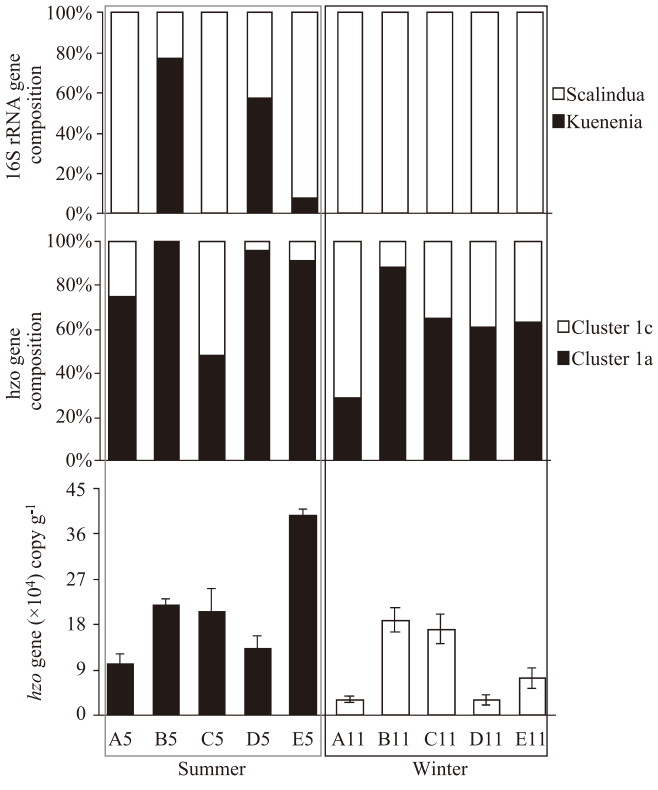

Fig. 4. The summer to winter community composition and population dynamics of anammox bacteria in estuarial mudflat sediment. Error bars represent standard deviations of triplicate experiments.

Classification of anammox bacteria

To better understand the seasonal and spatial distribution of anammox bacteria in sediment in the Mai Po Nature Reserve, PCoA was carried out by Unifrac. Results showed that the 16S rRNA genes had strong seasonal variation, with all the samples collected in winter grouped together, sharing similar community structures. For summer samples, A5 and C5 showed a similar 16S rRNA gene structure while B5, D5 and E5 shared a similar community structure (Fig. 5a). However, the *hzo* genes did not show a strong seasonal distribution though sites A and D in summer had a similar *hzo* gene composition to the winter samples (Fig. 5b).

Correlations of anammox bacteria with environmental factors

The diversity of the 16S rRNA gene was significantly correlated with temperature, the concentration of NOx (NO_2^- +NO_3^-) in pore water, and total NH_4^+ and total phosphorus concentrations, and negatively correlated with salinity. However, *hzo* diversity was significant correlated with the concentration of NOx (NO_2^-+NO_3^-) in pore water, and TKN and Pb levels. In addition, *hzo* gene abundance was correlated positively with temperature and the concentration of NOx (NO_2^-+NO_3^-) in pore water and negatively with sediment TOC (Table 3).

Discussion

Occurrence and seasonal variability of anammox bacteria

In the present study, we investigated the dynamics of the anammox bacterial community based on the 16S rRNA and *hzo* genes. Both genes had higher OUT numbers in summer than winter with some exceptions for *hzo*. The environmental classification also supported that the 16S rRNA and *hzo* genes shared a similar community composition in sediment samples collected in winter. Furthermore, using 16S rRNA and *hzo* as biomarkers provided some unexpected results regarding the variation in anammox bacteria in the Mai Po Nature Reserve within different seasons. From the 16S rRNA gene phylogeny, two major groups of anammox bacteria were found, with 'Scalindua'-like anammox bacteria detected in all samples collected in summer and winter, making them the dominant groups as in previous reports (39, 49), 'Kuenenia'-like 16S rRNA gene sequences were detected in summer not winter, representing season-specific anammox bacteria. However, two major *hzo* gene clades

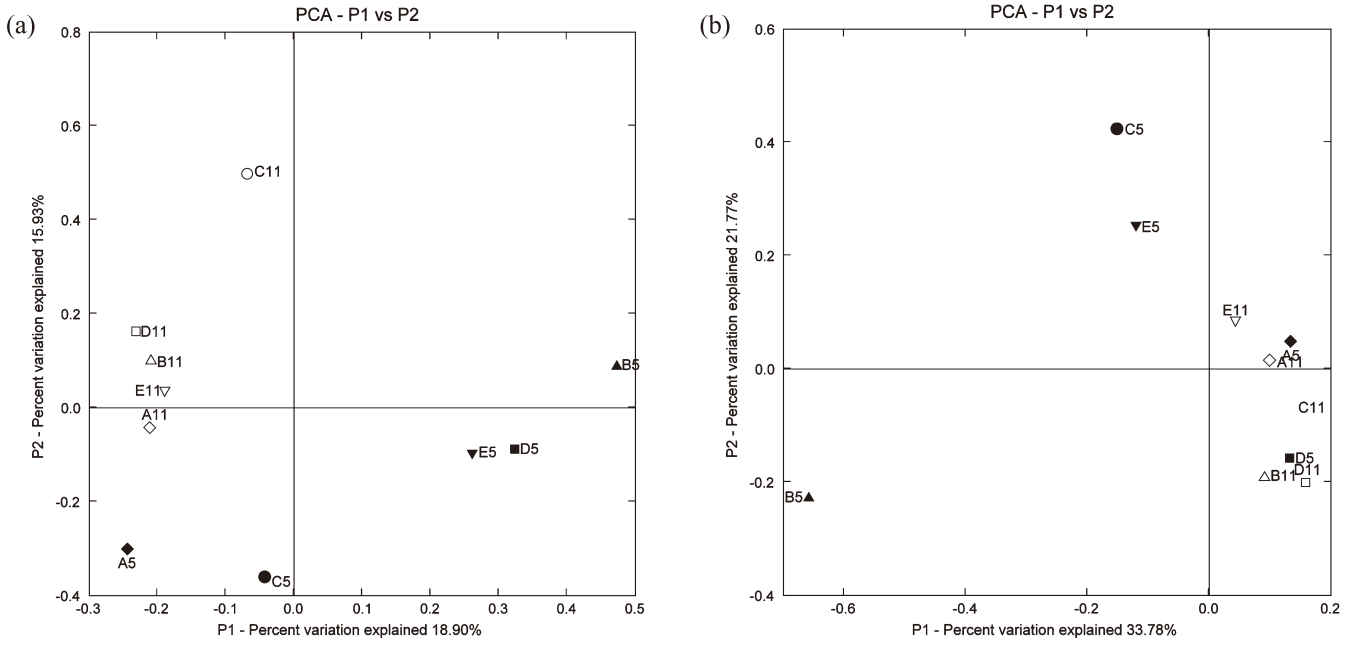

Fig. 5. PCoA based on the UniFrac metric of 16S rRNA (a) and *hzo* (b) sequence diversity of anammox bacteria, including sequence abundance data.

Table 3. Correlation analyses of environmental parameters and anammox bacteria diversity and abundance

Parameter	Pearson moment correlation*						
	OTUs		Shannon Index		Chao index		Abundance
	16S rRNA	*hzo*	16S rRNA	*hzo*	16S rRNA	*hzo*	*hzo*
pH	–0.33	–0.45	–0.30	–0.16	–0.45	–0.33	–0.22
Temp.	**0.75**	0.27	**0.66**	–0.33	**0.78**	0.45	**0.64**
Redox	–0.30	0.06	–0.20	0.20	–0.21	–0.02	–0.39
NH_4^+	–0.31	0.04	–0.20	0.25	–0.34	–0.07	–0.56
NO_X	**0.76**	0.61	0.33	–0.08	**0.82**	**0.91**	**0.68**
NH_4^+/NO_x	–0.29	–0.25	–0.04	0.06	–0.35	–0.31	–0.26
Salinity	**–0.66**	–0.19	**–0.67**	0.37	**–0.68**	–0.32	–0.42
NH_4^+	**0.65**	0.19	**0.65**	–0.38	**0.69**	0.34	0.51
TKN	0.35	**0.69**	0.12	0.49	0.44	0.42	–0.10
T-N	0.31	0.58	0.17	0.40	0.40	0.36	–0.05
T-P	**0.66**	0.04	**0.66**	–0.53	**0.65**	0.36	0.61
S^{2-}	–0.47	–0.49	–0.23	–0.12	–0.53	–0.47	–0.49
TOC	–0.62	–0.05	–0.62	0.27	–0.61	–0.28	**–0.64**
As	0.30	0.00	0.42	–0.32	0.21	–0.05	–0.21
Cd	–0.25	–0.18	–0.15	–0.29	–0.33	–0.22	–0.51
Cu	0.44	0.09	0.40	–0.50	0.37	0.28	0.04
Hg	0.62	0.24	0.57	–0.43	0.53	0.32	0.05
Pb	0.42	–0.06	0.56	**–0.69**	0.35	0.19	0.20

* Pearson moment correlation (r) was determined using the following equation:

$r=\frac{n(\Sigma XY)-(\Sigma X)(\Sigma Y)}{\sqrt{[n\Sigma X^2-(\Sigma X)^2][n\Sigma Y^2-(\Sigma Y)^2]}}$. Boldface denotes a P value of <0.05, which is typically regarded as significant, as determined by Excel function TDIST from the t value given by the following equation:

$t=\times\sqrt{\frac{n-2}{(1-r^2)}}$. The number of samples is given by n.

were also detected, one related to the 'Scalindua' genus and the other affiliated with *hzo* genes recovered from a '*Candidatus* Anammoxoglobus' enrichment culture, planctomycetes KSU-1, and a '*Candidatus* J. asiatica' enrichment culture. Although sequences in the two major *hzo* gene clades could be found in summer and winter sediment samples, season-specific clusters could still be observed (cluster 1a-3 and branch in cluster 1c-3). In addition, the quantitative analysis of the *hzo* gene copy number within the two seasons also clearly indicated that anammox bacteria have strong seasonal variation in not only diversity but also abundance.

Environmental factors influence the seasonal dynamics of anammox bacteria

To further understand the seasonal variation in the bacterial community structure, the influences of sampling location and environmental factors should be considered. According to the physicochemical data from sediment samples, May samples had higher values for temperature, NOx in pore water, total NH_4^+, TKN, TN, TP and several metals in sediment, but lower values for redox potential, reduced sulfide (S^{2-}) and total organic carbon (TOC) compared to November samples, indicating strong anthropogenic and terrestrial input during the summer season. The quite low salinity in May also provides evidence of a large quantity freshwater during the summer, which is consistent with previous study in the area (28). The strong anthropogenic and terrestrial inputs, mainly through the Shenzhen River, would certainly bring a lot of freshwater anammox bacteria, such as 'Kuenenia', into the Mai Po Nature Reserve. This speculation is reflected by the variation in bacterial community structure with numerous 'Kuenenia' anammox bacteria detected from May except at sites A and C. On the other hand, the northeast monsoon prevails and the South China Coastal Current dominates the coastal water of the Nature Reserve during winter (4, 42), and thus marine anammox bacteria, usually 'Scalindua' (39, 49), become the dominant group in this area. Recently, reports also found 'Brocadia' and 'Kuenenia' to be present in marine ecosystems (1, 6, 27); however, these ecosystems are strongly affected by anthropogenic or terrestrial influences, such as estuaries and internal bays. Furthermore, the highest *hzo* abundance of site E in summer further indicates the Shenzhen River to be the source of '*Candidatus* K. stuttgartiensis'. So, the 'Kuenenia' anammox bacteria in summer but not winter in estuary sediment of the Mai Po Reserve may be possibly from the strong anthropogenic or terrestrial inputs, which mostly come from the Shenzhen River. These freshwater or terrestrial bacteria together with anammox bacteria increase the diversity of anammox bacteria in summer, with strong positive correlations among the diversity and abundance of anammox bacteria and the concentration of NOx (NO_2^- +NO_3^-) in pore water, total NH_4^+ content and total phosphorus level of sediment, and strong negative correlations with salinity. The absence of 'Kuenenia' anammox bacteria at site A, close to the Shenzhen River, might be due to mangrove plants that have a strong influence on the distribution of anammox bacteria through interaction for available nitrogen. In addition, its significant positive relationship with bacterial community and abundance further indicates that temperature is an important environmental factor influencing the seasonal variation of anammox bacteria in Mai Po. Previous studies have found that 'Scalindua' anammox bacteria are distributed in low-temperature environments in sea water columns, surface and subsurface of ocean sediment (8, 14, 49), while quite different in high-temperature environments, such as hydrothermal vents (3) and oil reservoirs (22), indicating 'Scalindua' species prefer low-temperature environments. Our results are consistent with these findings, with 'Scalindua' bacteria dominating in winter. Last but not least, the significantly negative correlation between *hzo* gene abundance and the total organic carbon (TOC) concentration might also indicate the un-favorable effect of organic matter on the growth of anammox bacteria though organic matter cannot be excluded as either a carbon or energy source for anammox bacteria in marine environments (10).

In conclusion, anammox bacterial community structure and abundance in estuary sediment of the Mai Po Nature Reserve showed strong seasonal dynamics, which might be affected by anthropogenic and terrestrial inputs within different seasons, and also by mangrove coverage and other environmental factors.

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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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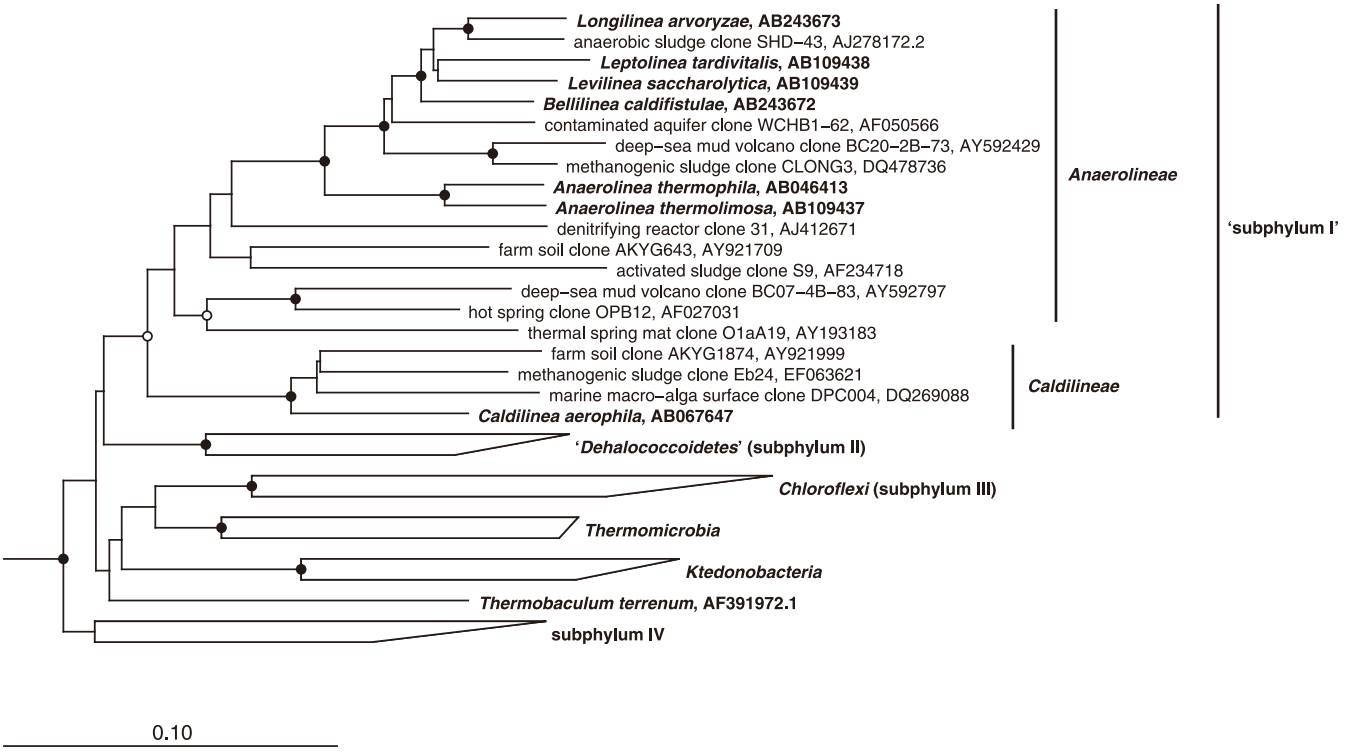

Fig. 1. Evolutionary distance dendrogram of the bacterial phylum *Chloroflexi* derived from a comparative analyses of 16S rRNA gene sequences, showing the phylogenetic relationship of the subphyla (class)-level taxa of the phylum. The sequences were aligned, and the phylogenetic tree was constructed 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%)

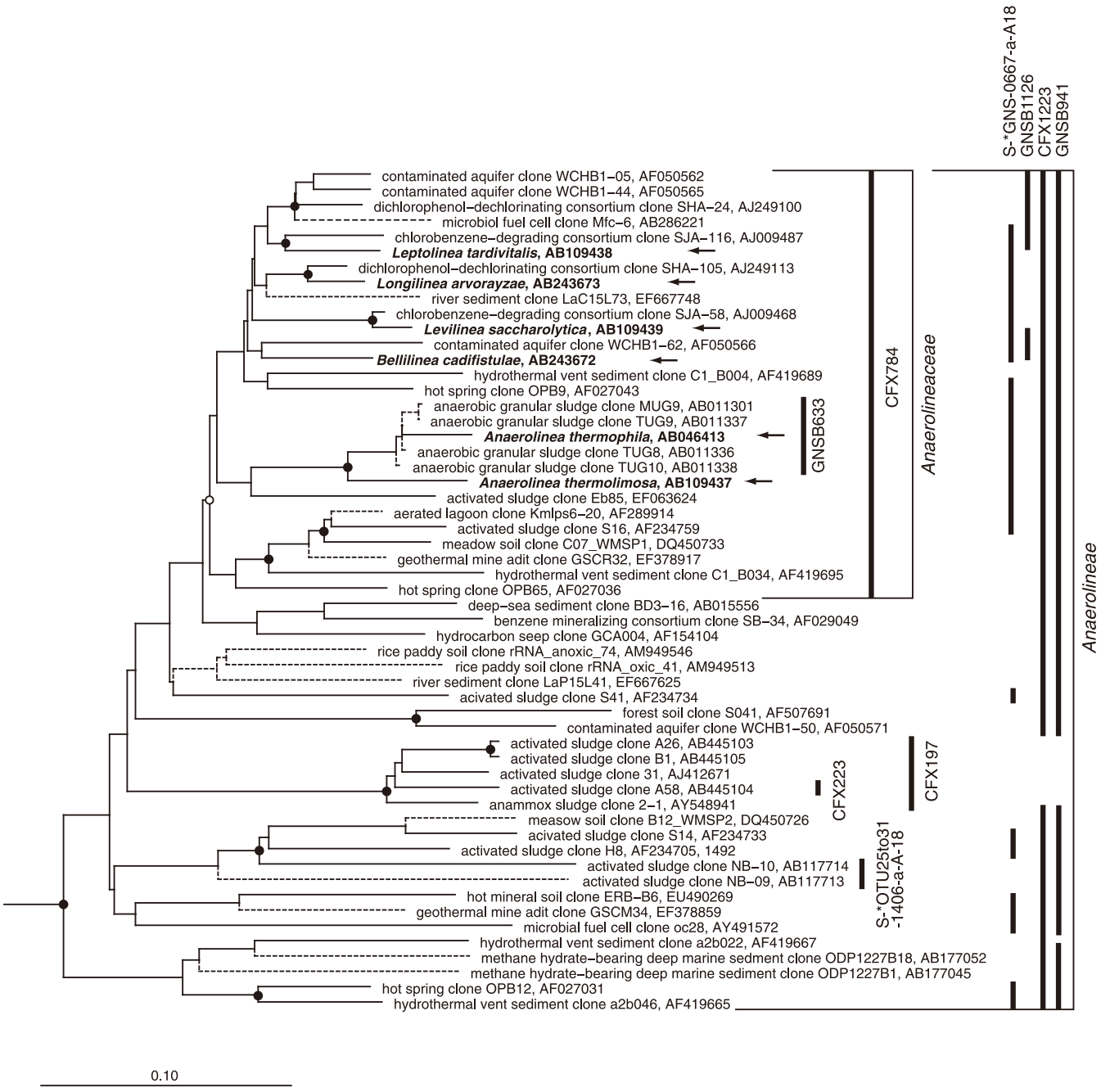

Fig. 2. Evolutionary distance dendrogram of the class *Anaerolineae* derived from a comparative analyses of 16S rRNA gene sequences, showing the phylogenetic positions of cultivated strains belonging to the class (indicated by arrow) and other related gene clones that were retrieved from a variety of 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, chemolitho-organoheterotrophic 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

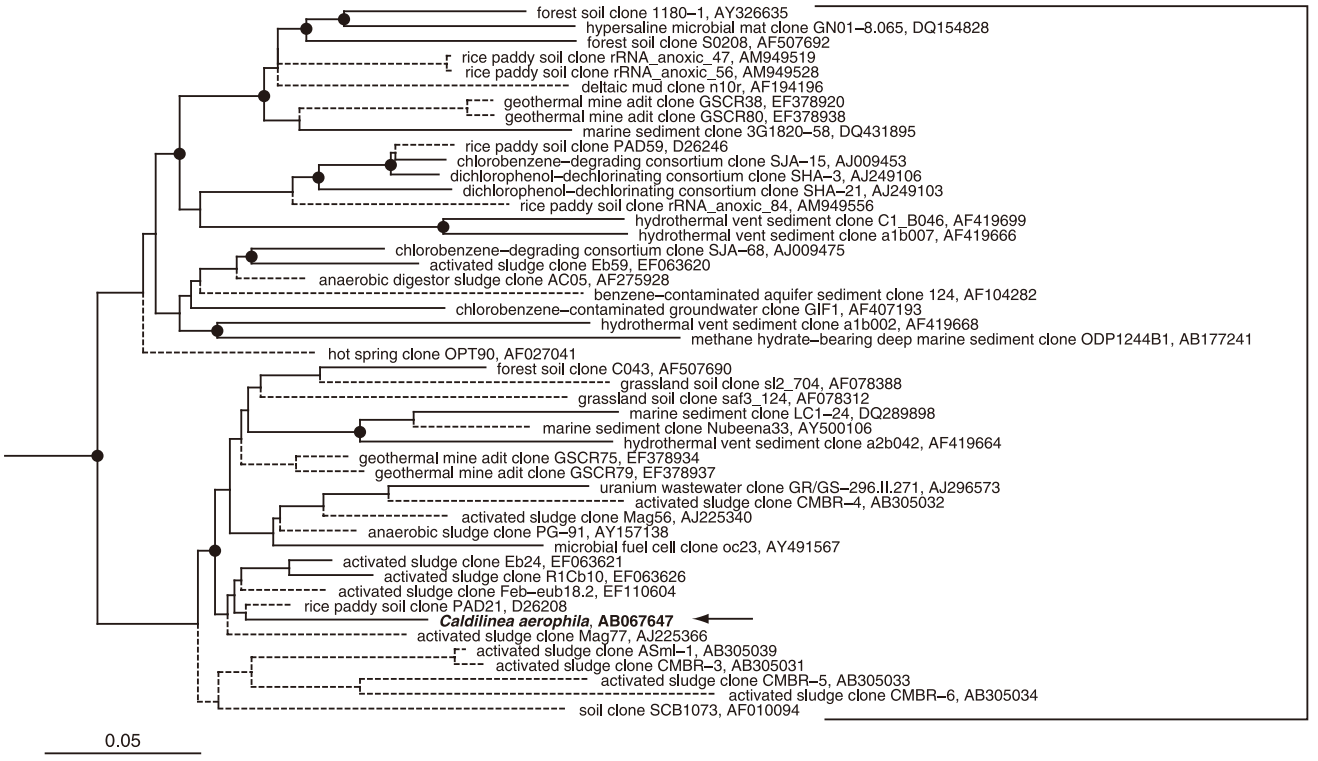

Fig. 3. Evolutionary distance dendrogram of the class *Caldilineae* derived from a comparative analysis of 16S rRNA gene sequences, showing the phylogenetic positions of *Caldilinea aerophila* (indicated by arrow) and other related gene clones that were retrieved from a variety of environments. The tree 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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