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Genome-centric metagenomic insights into the role of Chloroflexi in anammox, activated sludge and methanogenic reactors

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Abstract

Background The phylum Chloroflexi is highly abundant in a wide variety of wastewater treatment bioreactors. It has been suggested that they play relevant roles in these ecosystems, particularly in degrading carbon compounds and on structuring flocs or granules. Nevertheless, their function is not yet well understood as most species have not been isolated in axenic cultures. Here we used a metagenomic approach to investigate Chloroflexi diversity and their metabolic potential in three environmentally different bioreactors: a methanogenic full-scale reactor, a full-scale activated sludge reactor and a lab scale anammox reactor.

Results Differential coverage binning approach was used to assemble the genomes of 17 new Chloroflexi species, two of which are proposed as new Candidatus genus. In addition, we recovered the first representative genome belonging to the genus 'Ca. Villigracilis'. Even though samples analyzed were collected from bioreactors operating under different environmental conditions, the assembled genomes share several metabolic features: anaerobic metabolism, fermentative pathways and several genes coding for hydrolytic enzymes. Interestingly, genome analysis from the anammox reactor indicated a putative role of Chloroflexi in nitrogen conversion. Genes related to adhesiveness and exopolysaccharides production were also detected. Complementing sequencing analysis, filamentous morphology was detected by Fluorescent in situ hybridization.

Conclusion Our results suggest that Chloroflexi participate in organic matter degradation, nitrogen removal and biofilm aggregation, playing different roles according to the environmental conditions.

Keywords Chloroflexi, Methanogenic reactors, Activated sludge, Anammox, Metagenome assembled genomes

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Background

The phylum Chloroflexi comprises an ecologically and physiologically diverse group of bacteria, which have been detected in high abundance in methanogenic [1, 2], anammox [3–5] and activated sludge reactors [6, 7]. Despite of being highly abundant in wastewater treatment systems (WWTS), the basic ecophysiology of this group is still largely unknown because only few pure cultures have been investigated to date [8–13]. Based on the physiology of isolates, assembled genomes from metagenomes and in situ characterization (e.g., using microautoradiography combined with FISH), it has been proposed

that their role is mainly related to the hydrolysis of complex organic matter, fermentation of carbohydrates and proteins, and degradation of debris from lysed bacterial cells [1, 7, 14–18].

In methanogenic reactors it has been hypothesized that the prevalence of this group is based on a syntrophic association with hydrogenotrophic methanogenic Archaea. This idea is supported by the fact that all Chloroflexi species isolated from methanogenic reactors required co-cultivation with methanogenic archaea for efficient growth [8, 10, 19, 20], the co-localization with filamentous Archaea [17], and the positive correlation between Chloroflexi and methanogens in anaerobic reactors [2].

In Anammox reactors, ammonium oxidation and nitrite reduction are coupled to form nitrogen gas under anoxic conditions [21]. In these reactors, the role of Chloroflexi has been associated to the scavenging of organic matter derived from anammox bacterial cell debris [3], or the utilization of soluble microbial products (SMP) and/or extracellular polymeric substances (EPS) produced by autotrophs [22]. This is supported by the high abundance of Chloroflexi in anammox reactors fed with synthetic wastewater containing NH4+ as the sole electron donor without addition of organic carbon compounds [3, 23–25]. Recent metagenomic-based works also suggested that members of Chloroflexi could facilitate a nitrite loop with anammox bacteria or support complete denitrification due to the expression of the nitric oxide reductase gene (norZ) and nitrite reductase genes (nirK, nirS). Thus, Chloroflexi would enhance the overall nitrogen removal performance in anammox bioreactors [26, 27]. In addition, some Chloroflexi members encode the function of biosynthesizing sticky macromolecular exopolysaccharide for anammox consortium aggregation [27].

On the other hand, it has been widely speculated that Chloroflexi members might play an important role during sludge granulation or flocculation processes. Evidence for this is their growth as filaments and the fact that some members showed cellular adhesiveness. This would enable Chloroflexi to form a backbone for small sludge particles and granules, in anammox, anaerobic and aerobic reactors [22, 28, 29]. Due to their filamentous growth, several authors have related the Chloroflexi overgrowth with bulking episodes, mainly in activated sludge reactors but also in full scale methanogenic reactors and lab-scale anammox reactors [30–34]. The study of Chloroflexi ecophysiology has direct implications in wastewater treatment plants performance, in particular to prevent and control bulking problems.

Here, we hypothesize that even though methanogenic, activated sludge and anammox reactors harbor different Chloroflexi species, their functions are akin. In order to test this hypothesis, we first performed 16S rRNA gene amplicon sequencing to determine the diversity of Chloroflexi in samples from anammox, activated sludge and methanogenic reactors. Then, we applied phylum-specific Fluorescence in situ hybridization (FISH) to determine the morphology of Chloroflexi in the different samples. Finally, we performed genome-centric metagenomics analysis to reveal their phylogenomic diversity and metabolic potential. The combination of different molecular approaches and the study of samples from different reactors allowed us to obtain information on the putative roles of Chloroflexi in different WWTS. Moreover, 17 new Chloroflexi species were identified and two of them are proposed as new Candidatus genus.

Methods

Sampling and DNA extraction

Samples from the following three reactors were analyzed (Table 1): 1) a full-scale upflow anaerobic sludge blanket (UASB) methanogenic reactor treating effluent from a malt industry (MO) ([35, 36], 2) a full-scale activated sludge reactor treating health-care waste treatment wastewater (Inffluent Chemical oxygen demand ($COD_{influent}$) = 8,000 ppm, $COD_{effluent}$ = 500 ppm, pH = 7.5, Hydraulic retention time (HRT) = 4.7 days), the wastes were sterilized by autoclave and the wastewater is generated in this process (RH), 3) a lab scale upflow anammox sludge blanket (UAnSB) reactor (S) fed with sewage after a partial nitrification system [37] (the inoculum used on this reactor was also included in the analysis). This inoculum was sludge from an anammox sequencing batch reactor (AM) fed with synthetic wastewater [38]. The full-scale reactors were located in Montevideo (Uruguay) and the lab-scale reactor was operated in the GENOCOV Research Group in Barcelona (Spain).

All reactors were operated under mesophilic conditions. In order to apply the differential coverage binning approach to assemble genomes [39] several samples were taken from MO, RH and S (including AM used as inoculum of reactor S). Three samples taken at different time points were collected from the methanogenic (MO1, MO2 and MO3) and activated sludge reactors (RH4, RH5 and RH6). Another three samples were collected at different heights of the sludge bed from the UAnSB anammox reactor (S1, S3 and S5) and one from the sludge used as inoculum (AM).

Biomass samples were stored at -20 °C for sequencing workflows and fixed according to the protocol of Amann et al. [40] for FISH. Detailed procedures of DNA

Table 1 Characteristics of the studied reactors

Reactor type	Configuration	Samples name	Sampling day[a]	Scale	Substrate	Condition
Aerobic	AS[b]/floccular biomass	RH4	0	Full-scale	Health-care waste treatment wastewater	Stable conditions for more than 6 years
		RH5	300			
		RH6	390			
Methanogenic	UASB[c]/granular biomass	MO1	0		Brewery-malt processing industry	Stable conditions for more than 12 years
		MO2	210			
		MO3	300			
Anammox	SBR[d]/granular biomass	AM	Inoculum	Lab scale	Synthetic wastewater	Stable conditions for more than 6 years
	UAnSB[e]/granular biomass	S1	81		Sewage after a partial nitrification system	81 days of operation (seeded with sludge from AM)
		S3				
		S5				

[a] For reactors RH and MO day 0 was considered the first day of sampling, for the anammox reactor S day 0 was considered the inoculation day

[b] *AS* Activated sludge

[c] *UASB* Upflow anaerobic sludge blanket

[d] *UAnSB* Upflow anammox sludge blanket

[e] *SBR* Sequencing batch reactor

extraction and FISH are described in the Supplementary Material.

Community profiling using 16S rRNA gene amplicon sequencing

The taxonomic composition of the communities was studied by amplicon sequencing of the 16S rRNA gene with primers for V4 region [41, 42]. The raw data analysis was performed using Quantitative Insights Into Microbial Ecology' pipeline (QIIME2 2020.11 release) [43]. The sequences were classified using MiDAS 3.7 database [44]. Detailed procedures of primers, QIIME2 analyses, data visualization, and phylogenetic analysis of the amplicon sequences are described in the Supplementary Material.

Metagenome sequencing

Metagenomes were shotgun sequenced by Illumina HiSeq 4000 platform (Macrogen, Seoul, Korea) using Library Kit TruSeq Nano DNA Kit (350 bp). The yield was approximately 5 Gb of raw short-read sequences per sample (2×100 bp).

Genome assembly, binning and genome annotation

The global quality of the metagenomes reads was checked using FastQC (v0.11.8) [45]. The reads were trimmed to remove adapters and bases below a quality score of 25 using Trimmomatic (v0.39) [46]. The trimmed reads from each reactor (MO and RH, separately) were pooled and assembled using MEGAHIT (v1.1.4–2) [47] with minimum k-mer length 43, maximum k-mer 75, with steps of four. The three samples from the anammox reactor S1, S3 and S5 were pooled and analyzed together with sample AM which was used as inoculum. The contigs obtained shorter than 1000 bp were removed. Quality filtered reads for each metagenome were mapped to the co-assembly contigs (> 1000 bp) using Bowtie2 (v2.3.4.1) [48] with default parameters. Genome bins were recovered using MetaBAT2 (v2.12.1) [49]. The completeness levels and contamination of the bins were assessed using CheckM (v1.0.13) [50]. The bins with an estimated completeness > 50% and contamination < 10% were reclassified using Genome Taxonomy Database GTDB-Tk (version v1.3.0 and GTDB-Tk reference data version r95) [51] to determine if they belonged to the Chloroflexi phylum. Chloroflexi bins were reassembled using SPAdes 3.10.0 [52]. All resulting contigs of > 1,500 bp were clustered using ESOM tools (emergent self-organizing map) [53], on the basis of its tetranucleotide frequency to identify and extract contaminant contigs in each genome. GUNC [54] was used to detect chimerism and to assign the taxonomy of the contigs in MAGs. Contigs assigned to a different phylum of Chloroflexi were removed from the MAGs. The CSS (clade separation score) estimated with gunc was closer to 0 for all genomes. A CSS closer to a value of 0 indicates that a genome is free of contamination and all genes are assigned to the same taxonomy.

Statistics of the reassembled bin were obtained using QUAST v5.0.2 and CheckM lineage wf (v1.0.13). Genes encoding ribosomal RNAs (rRNA) were predicted using the Bacterial rRNA predictor (Barrnap, https://github.com/tseemann/barrnap). The Average Nucleotide Identities (ANI) and the Average Amino Acid Identity (AAI) between MAGs and isolated species were determined using FastANI [55] and EzAAI [56]. The Chloroflexi

genomes retrieved from methanogenic, activated sludge and anammox reactors were named as MO, RH and AMX, respectively, with a number suffix.

Genome phylogenetic analyses were performed with the GTDB-Tk tool. Newick format tree file was uploaded onto iTOL, a web-based tool for annotating and editing trees [57].

Metabolic analysis

Protein coding sequences (CDS) were determined with Prokka (1.14.5) [58, 59]. Predicted amino acid sequences were annotated with KOALA (KEGG Orthology And Links Annotation) for K number assignment of KEGG Genes [60] and Metaerg with KEGG, pfam, swiss-prot and tigrfams databases [61]. Pathways and genes completeness were represented as blocks with different colors in Figs. 4 and 5. Carbohydrate-active enzymes (CAZY) were determined using EnrichM v0.5.0 (https://github.com/geronimp/enrichM) with HMMs from dbCAN [62].

Results and discussion

Applying a series of molecular biology tools, the diversity and metabolic potential of the phylum Chloroflexi was studied in three different wastewater treatment systems: a methanogenic reactor (anaerobic, C-removal), an activated sludge reactor (aerobic, C-removal) and an anammox reactor (anaerobic, N-removal).

Diversity of Chloroflexi members in the different wastewater reactors according to 16S rRNA gene amplicon sequencing

The phylum Chloroflexi was detected in all reactor samples. Regarding the relative abundance higher values were detected in the anammox reactor samples compared to the methanogenic and aerobic reactors samples (26.9—33.5% for anammox, 4.9 -22% for activated sludge and 0.8—10.7% for methanogenic reactors) (Fig. 1).

MiDAS database, specifically designed to analyze microbial communities from activated sludge systems and methanogenic reactors [63], was used to classify sequences at different taxonomic levels (non-described microorganisms are labeled with a number according to the MiDAS taxonomy). The results confirmed that Anaerolineae class widely dominated the Chloroflexi community in all reactors (Fig. 2a), which was in accordance with results previously reported in aerobic, anaerobic and anammox reactors [2, 4, 17, 26, 27, 29, 36,

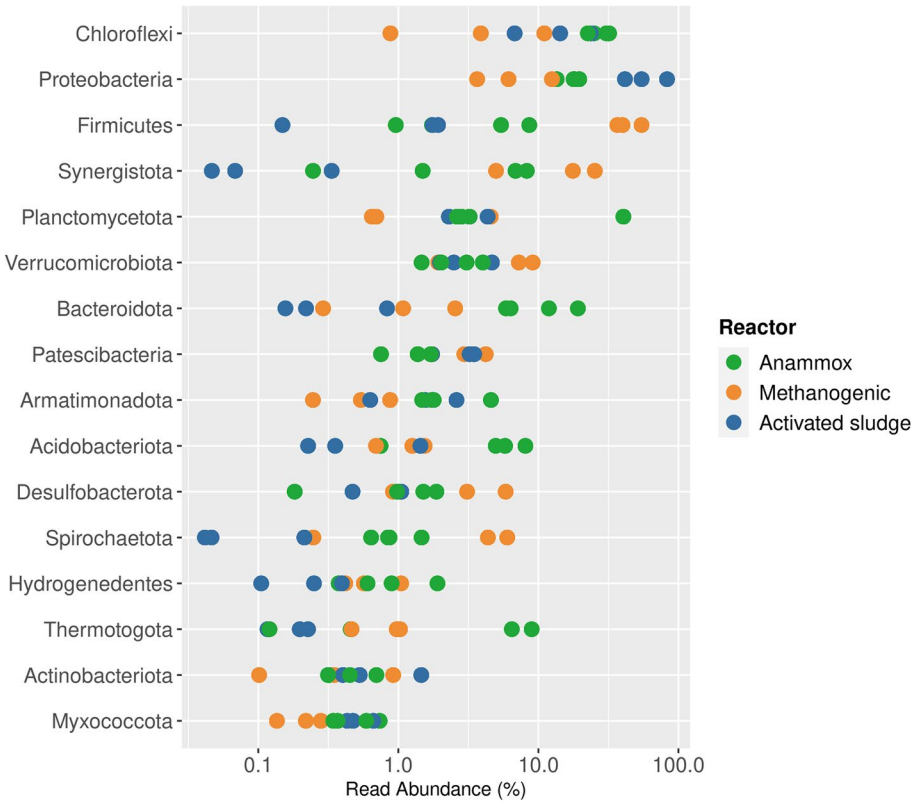

Fig. 1 Boxplots showing the relative abundance of the 16 most abundant phyla in the total community according to the 16S rRNA gene amplicon sequence in each reactor type

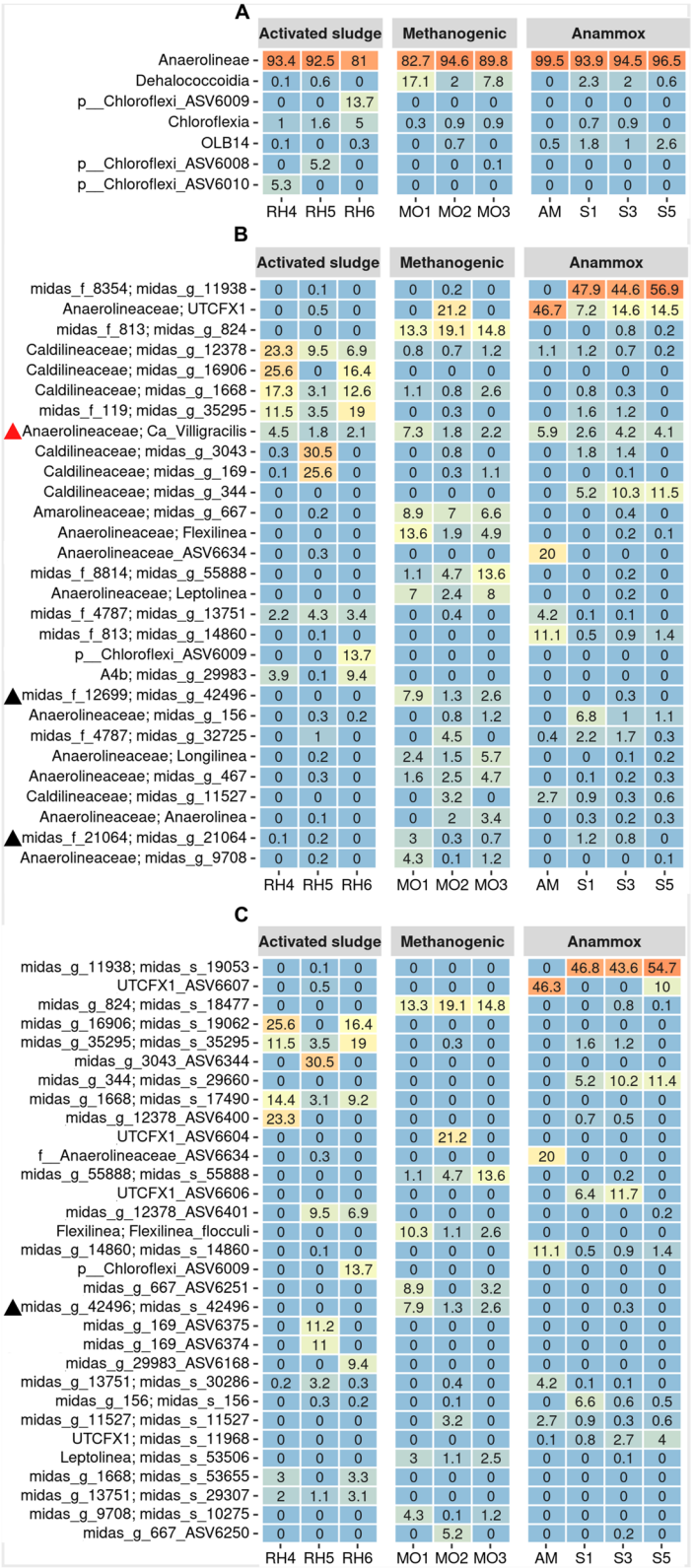

Fig. 2 Heatmaps showing the relative abundance within the phylum Chloroflexi according to the 16S rRNA gene amplicon sequence: **A**) at class level, **B**) at genus level (30 most abundant genus) **C**) at species levels (30 most abundant species). Non-classified microorganisms are labeled with a number according to the MiDAS taxonomy. Black triangles in panels A and B mark genus and species belonging to Dehalococcoidia class, all the remaining microorganisms belonging to the class Anaerolineae. Red triangles in panel A mark '*Ca.* Villigracilis'

64–68]. Most of the genera detected were specifically associated with one type of reactor.

One exception was '*Ca.* Villigracilis', which was shared by all reactors with a similar relative abundance (Fig. 2b). This filamentous bacterium has been reported as widely distributed in activated sludge reactors, where it probably contributes to the matrix supporting floc formation [69]. Although '*Ca.* Villigracilis' has been metabolically characterized by FISH-MAR and reported as the only facultative aerobic member described within Anaerolineae class so far, the genome is not yet available [69].

Several genera classified according to the MiDAS taxonomy, and therefore with no cultured representative, presented high abundance within the Anaerolineaceae and Caldilineaceae families in all reactors. Specifically, genera belonging to the family Caldilineaceae were the most abundant in the activated sludge reactor (Fig. 2b). Meanwhile, genera belonging to the family Anaerolineaceae were the most abundant in anammox and methanogenic reactors.

From all genera detected in our samples, only five harbor cultured representatives: *Flexilinea*, *Bellilinea*, *Longilinea*, *Leptolinea* and *Anaerolinea*. All of them have strains isolated from anaerobic reactors which were described as strictly anaerobic chemoorganotroph involved in carbohydrates fermentation [9, 10, 12]. In pure culture, their growth was enhanced in co-cultivation with a hydrogenotrophic methanogens. This could explain why sequences belonging to these genera were almost exclusively detected in the methanogenic reactor.

Moreover, all genera were represented by few dominant species which in most cases were not shared between reactors (Fig. 2c). In the samples studied, all Chloroflexi members presented filamentous morphology except for the anammox reactor samples (these results are described in the Supplementary material, section Morphology determined by FISH).

Several ASVs determined using the 16S rRNA gene sequences, could not be classified at class (e.g., ASV6009, ASV6008, ASV6010), genera (ASV6634) or species level (e.g., ASV6007, e.g., ASV6634, e.g., ASV6400, etc.). Even though MiDAS database represents a great advance in understanding the microbiomes of wastewater treatment systems, there are still undescribed microorganisms not included and more effort is needed to expand this database.

General genomic features of Chloroflexi metagenomic assembled genomes

We used differential coverage binning [39] to obtain metagenomic assembled genomes (MAGs) from the three different reactors. A total of 75, 48 and 56 MAGs with > 50 completeness and < 10% contamination were successfully retrieved from MO, AMX and RH reactors samples, respectively. Seventeen MAGs belonging to the phylum Chloroflexi (9 AMX, 4 MO and 4 RH) were analyzed further. Recovered genomes completeness ranged from 85.59% and 96.79%, and contamination after the reassembly and polishing (manually curated) ranged between 0% and 3.36% (Table 2).

Three of these MAGs (AMX47, AMX55 and AMX56) were > 90% complete, had less than 5% contamination and importantly, included the full-length 16S, 23S, and 5S rRNA genes, and > 18 tRNA genes (Table 2), satisfying the criteria for high-quality (HQ) draft MAGs, according to the minimum information about a MAG (MIMAG) standard [70].

Phylogenomic analysis of Chloroflexi MAGs

To determine the phylogenetic position of the MAGs, a phylogenomic tree based on concatenated alignments of 120 single copy marker genes was constructed using 105 reference Chloroflexi genomes retrieved from NCBI (February 2021) and the 17 Chloroflexi MAGs obtained in our work. Of the 17 MAGs, six had sufficiently high degree of similarity (> 95% ANI to a representative genome [71]) to be classified at species level. The remaining MAGs presented ANI values below 95% with any reported genome and could not be classified at species level. According to the phylogenomic analysis, 7 were classified to genus level and 4 to family level (Table 2). The results showed that most of the MAGs (16 MAGs) were positioned into the Anaerolineae class while only one genome was positioned within the Dehalococcoidia class (Fig. 3a).

At the order level, MAGs were distributed within the orders Anaerolineales, SBR1031, B4-G1, Caldilineales, UBA3071, UCB3 and UBA2991 (Table 2).

In addition, a phylogenetic tree was constructed based on the 16S rRNA gene sequences retrieved from 10 MAGs and the ASV sequences retrieved from the amplicon sequencing analysis (Fig. 3b, Fig. S2). Because of the few genomes available in the databases, it was not possible to construct both phylogenetic trees (based on 16S rRNA gene sequences and based on genomes sequences) with the exact same members of Chloroflexi. For example, '*Ca.* Villigracilis' and '*Ca.* Sarcinatrix', do not have representative genomes in databases so far, and were therefore not added to the genome-based phylogenetic tree. Moreover, although frequently used for phylogenetic identification, 16S rRNA gene sequences are notoriously difficult to assemble from metagenomes [72]. Thus, 16S rRNA genes were present only in 10 of the 17 MAGs (Table 2). Taking all this into consideration, the phylogenetic placement of 10 MAGs based on the 16S rRNA

Table 2 Statistics summary for the 17 Chloroflexi MAGs recovered in this study

Reactor	Genome	Genome size (bp	CDS[a]	Completeness[b]/ Contamination	Contigs	N50 (bp)	16S/23S/5S[d]	tRNA[a]	%GC	Coverage	Taxonomic annotation[c]	Closest placement ANI %
Activated sludge	RH21	5,657,355	5097	85.59/0	655	12,275	1(922nt)/1(492nt)/1	4 5	56.34	8.2	c_Anaerolineae;o_SBR1031;f_A4b;g_OLB15	76.07
	RH38	5,163,972	4600	87.29/1.73	1167	5326	1(805nt)/1(342nt)/2	32	64.57	8.6	c_Anaerolineae;o_Caldilineales;f_Caldilineaceae	N/A
	RH43	9,980,378	8014	90.86/4.7	129	94,438	1(807nt)/1/1	44	55.88	3.03	c_Anaerolineae;o_Caldilineales;f_Caldilineaceae;g_CFX5	76.3
	RH52	4,922,116	3510	96.79/1.1	201	35,320	1(62nt)/1/1	57	73.22	20.4	c_Anaerolineae;o_UCB3;f_UCB3;g_UCB3;s_UCB3 sp003576755	99.2
Methanogenic	MO16	3,073,917	2871	86.36/3.36	387	11,990	1(1118nt)/0/0	35	58.98	10	c_Anaerolineae;o_Anaerolineales;f_Anaerolineaceae;g_Longilinea	79.35
	MO53	4,940,107	4005	87.27/0.91	227	44,487	1(337nt)/1(370nt)/1	45	58.31	13.5	c_Anaerolineae;o_B4-G1;f_SLSP01	N/A
	MO66	4,384,884	3803	92.73/3.36	478	14,973	0/0/0	44	64.91	14.7	c_Anaerolineae;o_UBA3071;f_CG2-30–64-16	N/A
	MO118	3,352,740	2734	90.18/2.73	86	51,993	1(847nt)/1(337nt)/1	38	41.05	13.2	c_Anaerolineae;o_Anaerolineales;f_Anaerolineaceae;g_Flexilinea	83.18

Table 2 (continued)

Reactor	Genome	Genome size (bp	CDS[a]	Completeness[b]/ Contamination	Contigs	N50 (bp)	16S/23S/5S[d]	tRNA[a]	%GC	Coverage	Taxonomic annotation[c]	Closest placement ANI %
Anammox	AMX9	4,128,696	3450	98.18/0	54	178,058	1(800nt)/1(617nt)/1	48	62.69	32.7	c_Anaerolineae;o_SBR1031;f_A4b;g_OLB13;s_OLB13 sp001567485	99.66
	AMX14	3,390,571	3072	92.73/0.91	124	43,811	1(329nt)/0/0	41	56.87	25.1	c_Anaerolineae;o_Anaerolineales;f_EnvOPS12;g_OLB14;s_OLB14 sp900696595	99.72
	AMX15	3,646,596	3399	90.91/0.18	38	293,047	1(534nt)/2/1	42	54.08	160.3	c_Anaerolineae;o_Anaerolineales;f_EnvOPS12;g_UBA12294;s_UBA12294 sp003577395	95.27
	AMX39	2,675,910	2460	90.91/0.91	18	265,768	1(445nt)/0/0	48	52.31	19.8	c_Anaerolineae;o_Anaerolineales;f_EnvOPS12;g_UBA12294;s_UBA12294 sp002050275	99.2
	AMX47	2,814,255	2926	91.21/0	319	11,859	1(1498nt, 1518nt)/1/1	42	66.7	10.4	c_Dehalococcoidia;o_UBA2991;f_UBA2991;g_UCB2	82.41
	AMX55	2,990,515	2772	92.73/1.27	61	69,781	1(1509nt)/1(317nt)/1	40	60.81	15.1	c_Anaerolineae;o_Anaerolineales;f_EnvOPS12;g_UBA7227;s_UBA7227 sp002473085	98.72
	AMX56	4,499,265	3993	92.42/1.09	96	86,147	3(1218–1493)/2/2	58	63.48	54.1	c_Anaerolineae;o_Caldilineales;f_J102	76.67
	AMX57	4,700,319	4113	92.73/0	236	29,630	1(703nt)/1(461nt)/1	46	60.87	13.6	c_Anaerolineae;o_SBR1031;f_A4b;g_GCA-2702065	75.87
	AMX68	4,329,913	4110	92.73/0.91	70	171,406	1(195nt)/1(219nt)/1	46	53.34	15.8	c_Anaerolineae;o_Anaerolineales;f_EnvOPS12;g_OLB14	79.8

N/A Not assigned

[a] As predicted using Prokka (details in Methods section)

[b] Genome quality estimates from CheckM (details in Methods section)

[c] Taxonomic assignments from GTDB-Tk (details in Methods section)

[d] Sequences obtained from Barrnap (details in Methods section)

gene (Fig. 3b) was consistent with the phylogenomic tree based on 120 marker genes genes (Fig. 3a).

Interestingly, five MAGs from the anammox reactor (AMX55, AMX15, AMX39, AMX14 and AMX68), formed a monophyletic clade separated from other genomes in the phylogenomic tree (Fig. 3a). The 16S rRNA gene from AMX55 clustered with the sequence of *'Ca.* Villigracilis' in the phylogenetic tree, with a 95.68% of identity according to the MiDAS taxonomy (Fig. 3b). Therefore, the retrieved MAG represents the first recovered genome within the '*Ca.* Villigracilis' genus [73].

Within the '*Ca*. Villigracilis' cluster, an ASV was classified within the UTCFX1 genus according to MiDAS. UTCFX1 represents a MAG retrieved from a nitritation-anammox sequencing batch reactor. The authors detected the presence nitrate reduction genes in this genome which were actively transcribed according to the metatranscriptomics analysis suggesting an important interaction with anammox microorganisms [26].

New genera and species candidates

In total, three MAGs (AMX47, AMX55 and AMX56) satisfied the criteria for high-quality recovered genomes [70] and met the minimal suggested required standards to be proposed as *Candidates* of new genera or species [74] (Table 2, Table S1). The full-length 16S rRNA gene sequence from AMX47, AMX55 and AMX56 showed identity values between 90.14% and 96.99% with their closest relatives according to Silva and MiDAS database comparisons (Table S2). The three MAGs presented ANI values < 79.2% with genomes from described species, and the AAI values for AMX47, AMX55 and AMX56 were 63.1, 57.6 and 56.6%, respectively (Table S1). Given the lack of close relatives, AMX47 and AMX56 genomes represent two different novel genera in the phylum, meanwhile, AMX55 represents the first representative genome of the genus '*Ca*. Villigracilis' (based on the taxonomic sequence identity threshold recommendations of [75].

General analysis of metabolic pathways and genes

Different Chloroflexi genera and species predominate in the different wastewater treatment systems studied but, is their function redundant? For the 17 Chloroflexi MAGs, genes were annotated using a variety of protein databases to infer their metabolic potential. Because most of these MAGs were estimated to be between 85 and 93% complete, genes for additional pathways, might not be identified in this study. To overcome this problem, we also annotated genomes from described species closely related to our assembled genomes according to the phylogenomic tree (Fig. 3a). This allowed us to infer if a metabolic pathway of interest was not present in the genome or if its absence might be due to an incomplete genome recovery.

The metabolic pathway analysis was focused on answering the following questions:

1-Are they potentially capable of performing aerobic and/or anaerobic respiration?
2-Do they have the potential capability to hydrolyze different compounds?
3-Which carbon compound degradation pathways do they have?
4-Do they have N-removal potential?
5-Do they have genes related to biomass adhesion properties?

Aerobic and anaerobic respiration pathways

Reactors were operated in anaerobic (anammox and methanogenic reactors) or aerobic (activated sludge reactor) conditions and therefore, genes related to aerobic and anaerobic respiration, or fermentative metabolisms were searched against the assembled MAG sequences.

Regarding respiration pathways, the genomic analysis showed that most MAGs (10 out of 17) had an incomplete oxidative phosphorylation pathway. Five MAGs (RH43, RH52, AMX55, AMX14 and AMX56) harbor the genes to use O_2 and different nitrogenous compounds (N_2O, NO_3^- and/or NO_2^-) as final electron acceptors, and two of them presented only the genes to use NO_3^- or NO_2^- (MO66 and AMX68, respectively) (Fig. 4, Fig. S3, Table S1).

The complete phosphorylation pathway and the presence of nitrate and nitrite reductases in AMX55 was in accordance with the in situ characterization of '*Ca*. Villigracilis', as this genus has the ability to take up substrates under anoxic conditions in presence and absence of nitrate/nitrite [69]. As we mentioned, the metatranscriptomics analysis showed that nitrate reduction genes

(See figure on next page.)

Fig. 3 Phylogenomic and phylogenetic analysis of Chloroflexi based on the metagenomics results and the 16S rRNA gene sequence analysis: **A**) Phylogenomic tree constructed with the 17 genomes from this work and reference genomes retrieved from the NCBI, **B**) Phylogenetic tree constructed with the most abundant ASVs retrieved from the 16S rRNA gene amplicon sequences (with abundance higher than 5% in at least one sample) and the sequences of the 16S rRNA gene from 10 MAGs from this work, and reference sequences of the phylum Chloroflexi retrieved from NCBI. The tree was reconstructed using the ML method and the GTR model. ML bootstrap values greater than or equal to 70% are shown at each node. Bar indicates 0.01 substitutions per site. The ASV and MAGs are colored by reactor: blue for RH, orange for MO and green for AMX. Sequences from the phylum Thermotogota were used as outgroup for rooting trees

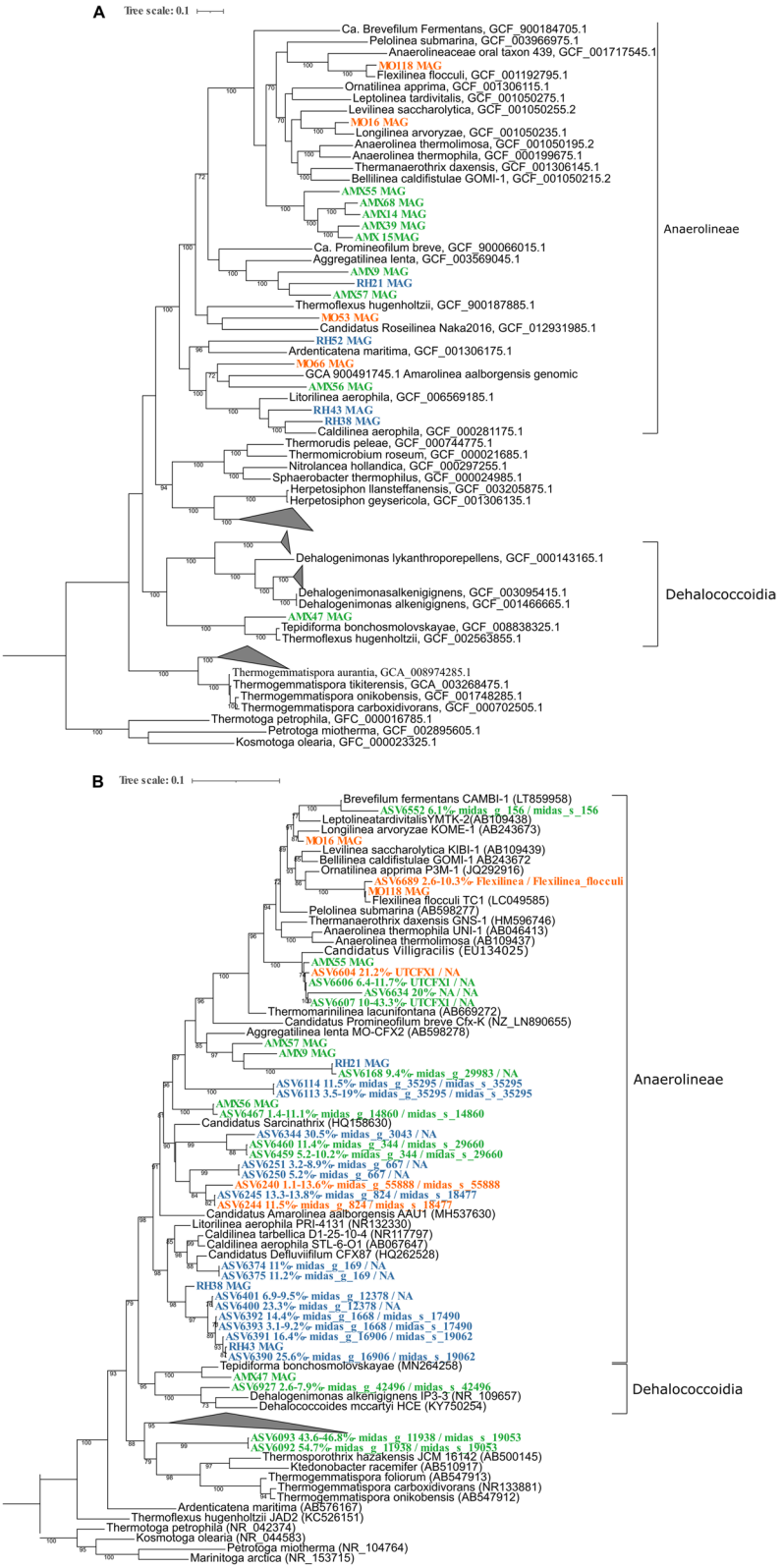

Fig. 3 (See legend on previous page.)

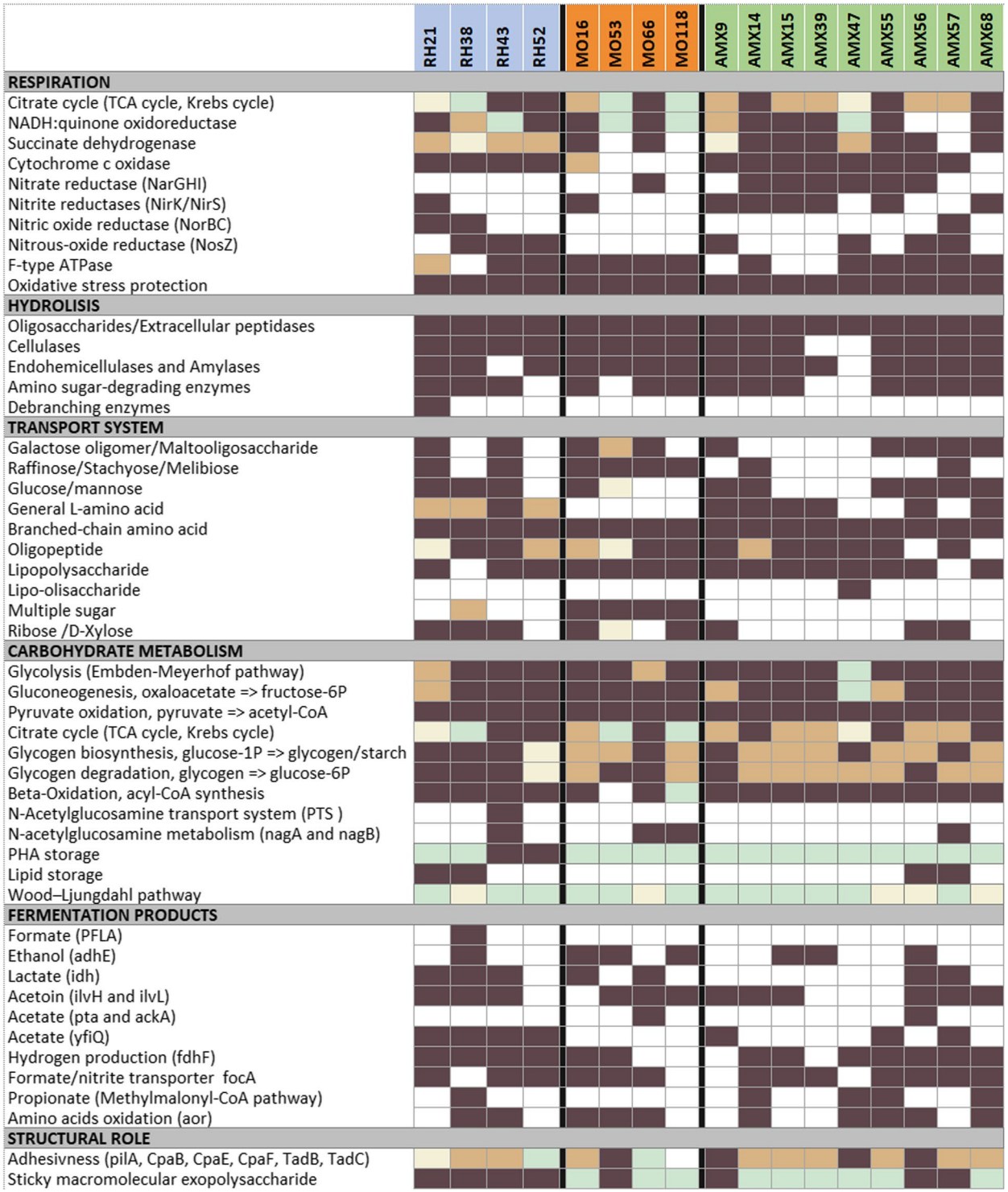

Fig. 4 Heatmap showing the completeness and the incompleteness of each metabolic pathway for the 17 MAG. Colors indicate pathways level completeness: dark brown (complete), light brown (1 block missing), beige (2 block missing), light green (more than 2 blocks missing) and white (not present)

from UTCFX1 (closely related to AMX55 in the phylogenetic tree, Fig. 3b) were expressed in an anammox reactor [26].

The tolerance to aerobic conditions is determined by the presence of oxidative stress protection genes. The presence of these genes was expected in genomes retrieved from the activated sludge reactor as these microorganisms are continuously exposed to oxygen (RH21 and RH38). Nevertheless, these genes were found in all MAGs (Fig. 4, Table S1). This is in concordance with the results obtained for '*Ca.* Brevefilum fermentans' retrieved from an anaerobic digester [17].

Metabolic pathways involved in polymers hydrolysis, carbon respiration and fermentation

To test the hypothesis that Chloroflexi members are capable of recycling soluble microbial products acting as hydrolytic bacteria, we performed the annotation of genes for glycosyl hydrolases such as cellulases, endohemicellulases, amylases, amino sugar-degrading enzymes, oligosaccharide-degrading enzymes, and also extracellular peptidases. The presence of all these genes indicated that most MAGs have the potential of hydrolyzing cellulose, starch, protein and/or peptides (Fig. 4, Supplementary Data 1). This is in accordance with previous in situ studies, which revealed high level of surface associated hydrolytic enzymes and their involvement in the breakdown of complex organic compounds [14, 76]. This result indicates that the Chloroflexi phylum may play an active role in hydrolyzing complex organic matter in activated sludge, as well as in methanogenic and anammox reactors.

Regarding central carbon metabolism, all MAGs contained multiple transporters for different organic compounds (including sugars, amino acids, proteins and fatty acids) indicating that each species has alternative routes for incorporating and recycling dissolved organic matter (scavenge macronutrients) (Fig. 4, Supplementary Data 1). The Embden-Meyerhof-Parnas (EMP) pathway for glycolysis was complete in all MAGs except in RH21, MO66 and AMX47. RH21 and MO66 are phylogenetically related to *Aggregatilinea lenta* and *Litorilinea aerophila*, respectively, which have the complete glycolysis pathway. Thus, incomplete glycolysis pathways in RH21 and MO66 genomes may be due to incomplete genome recoveries. On the other hand, AMX47 was closely related to *Dehalococcoides mccartyi*, which does not have the complete glycolysis pathway suggesting that AMX47 does not perform glycolysis. The potential of AMX55 to consume glucose was consistent with the results showed for '*Ca*. Villigracilis' [69]. Genes for pyruvate oxidation to acetyl-CoA were annotated in all MAGs (Fig. 4, Supplementary Data 1). Beta-oxidation was annotated in most of the MAGs and might represent an important metabolic route to obtain carbon and reducing equivalents for all Chloroflexi species. Another interesting finding was that genes for N-acetylglucosamine transportation and metabolism (PTS, *nag*AB) were annotated in RH43 (Fig. 4, Supplementary Data 1). These results are in accordance with other reports where Chloroflexi members appear to retrieve N-acetylglucosamine from lysed cells revealed by micro-autoradiography or FISH studies [3, 14, 23, 77–82]. In addition to the N-acetylglucosamine metabolism, we suggest that scavenging occurs through hydrolysis of complex organic compounds outside the cell, which are then transported to the cytoplasm, and are then metabolized via glycolysis or beta-oxidation (degradation of fatty acids and branched-chain amino acids) pathways. These results support the previous hypothesis that Chloroflexi has an important beneficial role in degradation of lysed bacterial cell debris and EPS.

Fermentation pathways including genes for acetate, ethanol, lactate, acetoin, formate and/or propionate production were present in all MAGs (Fig. 4, Supplementary Data 1). These results were in accordance with previous information of the Anaerolineae class (isolates and MAGS) retrieved from wastewater treatment system which were involved in sugar, amino acid or protein fermentation with different end products [8–10, 12].

Metabolic pathways for amino acids degradation and propionate formation (methylmalonyl-CoA pathway and aldehyde:ferredoxin oxidoreductase) was found in five MAGs (RH38, AMX14, AMX47, AMX55 and AMX68). These genes were also annotated in other Chloroflexi members [17, 83–85].

Most of the MAGs (13 MAGs) contained a formate dehydrogenase H (*fdh*F) to convert formic acid decomposition into CO_2. Only AMX55 had the ability to convert formic acid into H_2 and CO_2 by the presence of both *fdh*F and hydrogenases, under anaerobic conditions in the absence of exogenous electron acceptors as was observed in previous studies [82, 86]. In addition, the formate transporter (*foc*A) was annotated in most of these MAGs. Synergistic interaction of Anaerolineae members with the methanogenic archaea, was previously reported in anammox [87] and methanogenic reactors [2, 17, 88]. This could be a common scenario in these systems where the excess of electrons from organic carbon oxidation by Anaerolineae members could be transferred to methanogenic archaea.

Potential for polymers and lipids storage

The potential to store glycogen, polyhydroxyalkanoates and lipids was more common among MAGs retrieved from activated sludge than from methanogenic or anammox reactors. RH21, RH38, RH43, MO66 and AMX9 had

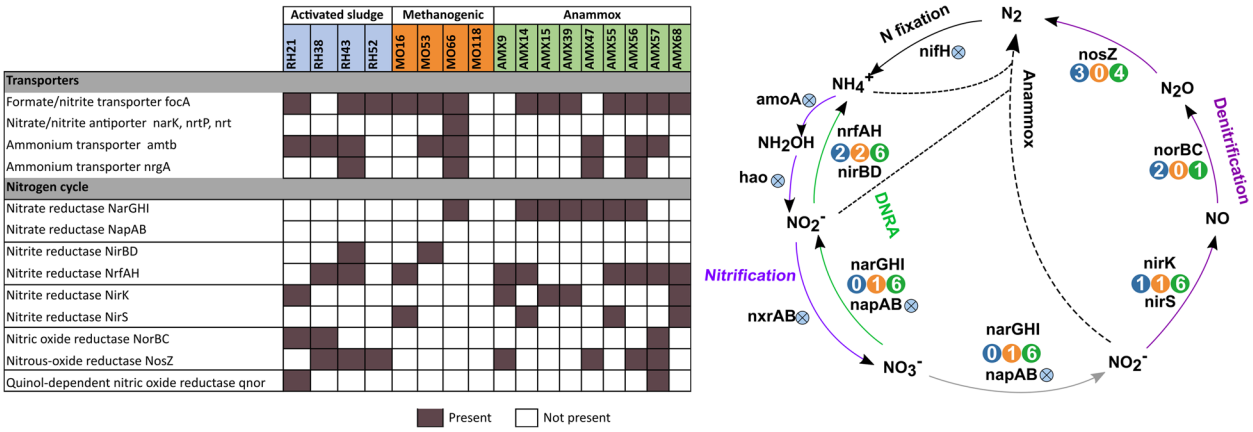

Fig. 5 Heatmap showing the nitrogen cycle annotated genes in the 17 MAGs from activated sludge, methanogenic and anammox reactors, Nitrogen cycle, next to each gene, the number of genomes that contain that gene is shown. Blue: activated sludge. Orange: methanogenic. Green: anammox. Dark brown blocks indicate genes that are present and white blocks indicate genes that are not present

complete glycogen biosynthesis and degradation pathways, suggesting that this polysaccharide may serve as a possible storage compound under unbalanced growth conditions (e.g., when C and/or N is temporally limited) (Fig. 4, Supplementary Data 1) [89, 90]. This result is in accordance with the information reported for '*Ca.* Amarolinea aalborgensis' and '*Ca.* Promineofilum breve' both retrieved from activated sludge [7, 16].

In addition to glycogen as storage compound, RH43 and RH52, encodes a potential biosynthesis pathway for polyhydroxyalkanoates (PHA) (Fig. 4, Supplementary Data 1), which are usually formed as carbon and energy storage compounds [91] under conditions of carbon excess and nitrogen or phosphate limitation [92]. Thus, the potential for PHA storage could favor these organisms with intermittent carbon availability present in these systems. Also, the annotation of a putative acyl-CoA:DAG acyltransferases (*atf*A), which catalyzes the final step in the synthesis of triacylglycerols, indicates the potential for lipid storage [93] in RH21, RH38, AMX56 and AMX57. This capability was also reported for '*Ca.* Promineofilum breve' [16].

Presence of Nitrogen metabolism pathways

Different genes related to the nitrogen cycle such as the dissimilatory nitrate reduction to ammonia (DNRA) and partial denitrification were mostly annotated in Chloroflexi MAGs from anammox reactors. DNRA is part of the nitrogen cycle, and it has been annotated in several Chloroflexi genomes [7, 16, 26]. In this work, three Chloroflexi species from anammox reactors (AMX14, AMX55 and AMX56) have the potential capability to perform DNRA due to the presence of both dissimilatory nitrate (*nar*GHI) and nitrite reductases (*nrf*AH) (Fig. 5).

AMX14 and AMX55 genomes were positioned in the '*Ca.* Villigracilis' and UTCFX1 cluster. As mentioned above, UTCFFX1 had nitrate reductase genes activity [26]. Thus, probably both species might perform DNRA. The six remaining MAGs have the potential to reduce nitrate to nitrite (*nar*GHI), or nitrite to ammonium (*nrf*AH). Our results show that all MAGs obtained from the anammox reactor could have a specific role in the nitrogen cycle, supplying nitrite or ammonia to anammox bacteria enhancing the overall nitrogen removal performance.

Two MAGs retrieved from the activated sludge reactor harbor genes to perform the reduction of nitrite to ammonia. These results are in accordance to those reported for '*Ca.* Amarolinea' (Chloroflexi MAG retrieved from activated sludge) [7]. Despite its potential, the in situ characterization did not confirm the use of nitrate or nitrite [18]. Also, putative nitrite reductase (*nrf*AH) has been found in *Caldilinea aerophila* and *Anaerolinea thermophile* genomes, but their ability to utilize nitrite as an electron acceptor to support anaerobic growth is yet to be assessed [8].

A nitrite reductase and nitric oxide reductase were annotated in RH21, suggesting the potential to denitrify (Fig. 5). This ability has only been proved for one Chloroflexi species, *Ardenticatenia maritima* [94]. In the anammox reactor, the presence of *nir*K/*nir*S and *nos*Z genes was common among the assembled MAGs. It was consistent with several studies which report that denitrification is more frequent in Chloroflexi species from anammox reactors than in the ones retrieved from aerobic and anaerobic reactors [26, 27, 68]. A recent

metagenomic study revealed that most of the heterotrophic organisms in anammox granules encode the ability to respire nitrate via partial denitrification, possibly completing a nitrite loop with anammox and nitrite oxidizing bacteria (NOB) by reducing nitrate back to nitrite [68]. This activity could contribute to the removal of excess nitrate produced from the system during anammox growth or nitrite oxidation by NOB.

Filamentous morphology, adhesion capability and exopolysaccharides production

It has been extensively proposed that Chloroflexi plays an important role in granule and floc formation. In order to deepen our knowledge on this subject, three important capabilities were specifically searched in the MAGs: the filamentous morphology (studied by FISH), the adhesiveness and the production of exopolysaccharides. A complete set of genes for the pilus assembly (pilA, CpaB, CpaE, CpaF, TadB, TadC) which favor adhesiveness, was annotated in MO53, AMX9, AMX47 and AMX56 (Fig. 4, Supplementary Data 1). The other MAGs mainly missed only the pilus assembly protein CpaB. As has been already noted, pili are often involved in facilitating adhesion and colonization in a wide variety of scenarios. In a previous study of Anaerolineae members, the adhesiveness by the expression of the tight adherence protein (Tad) on the active type VI pili indicated its function for cellular attachment, which was further testified to be more likely related to cell aggregation other than cellulose surface adhesion [29].

Regarding the production of exopolysaccharides, it has been suggested that some Chloroflexi-affiliated bacteria encode the function of biosynthesizing sticky macromolecular exopolysaccharides such as UDP-GlcNAcA, GDP-Man, and GDP-Rha from partial nucleotide sugars biosynthesized by anammox bacteria (UDP-ManNAc and CDP-Glc) [27]. We found the same set of genes for sticky macromolecular exopolysaccharide synthesis in all MAGs from RH, 3 MAGs from AMX and 1 MAG from MO, including the enzyme lactate dehydrogenase (prerequisite for exopolysaccharide production) (Fig. 4, Supplementary Data 1). The rest of the MAGs lack some of the necessary genes. Therefore, the results obtained showed that some Chloroflexi have the capability to promote cell aggregation and consequently the formation of cores or carriers, which help to form the initial framework of small sludge particles. Zhao et al. [87] reported that Anaerolineae members affected the nitrogen removal performance through affecting the aggregation because of the positive correlation relationship of this group with the abundance of EPS formation genes. The results obtained showed that key genes involved in B-vitamin biosynthesis were missing in all Chloroflexi MAGs. For example, genes for thiamin (vitamin B1) biosynthesis (thiamine-phosphate synthase and thiamine-monophosphate kinase), biotin (vitamin B7) biosynthesis (adenosylmethionine-8-amino-7-oxononanoate aminotransferase and biotin synthase) and adenosylcobalamin (vitamin B12) biosynthesis (cobalamin synthase and adenosylcobinamide-phosphate synthase) were absent in all Chloroflexi MAGs (Supplementary Data 1) as previously reported by Lawson et al. 2017 [95]. These authors postulated that in anammox reactors *Brocadia* sp. could supply B-vitamins to Chloroflexi members. This suggests that other microorganisms may support B-vitamin requirements for Chloroflexi community. This hypothesis could be extended to activated sludge and methanogenic reactors.

Table 3 Role proposed for the Chloroflexi microorganisms in the different reactors according to the metabolic function detected in the MAGs

Reactor	Process	Wastewater composition	Metabolic role	MAGs harvoring this function	Structure role in biomass
MO	Anaerobic treatment for C removal	Polysaccharides, proteins, carbohydrates	Hydrolysis of complex polymers from the wastewater by fermentation, EPS degradation	All MAGs	Granules structure
RH	Aerobic treatment for C-removal	Polysaccharides, proteins, carbohydrates	Hydrolysis of complex polymers from the wastewater by fermentation or aerobic respiration, EPS degradation	All MAGs	Flocs structure
S and AM	N-removal by anammox	Ammonium and nitrite	EPS and cell debris degradation	All MAGs	Anammox granules structure
			Reduction of nitrate	AMX14, AMX15, AMX39, AMX47, AMX55, AMX56	
			Reduction of nitrite	AMX9, AMX14, AMX55, AMX56, AMX57, AMX68	

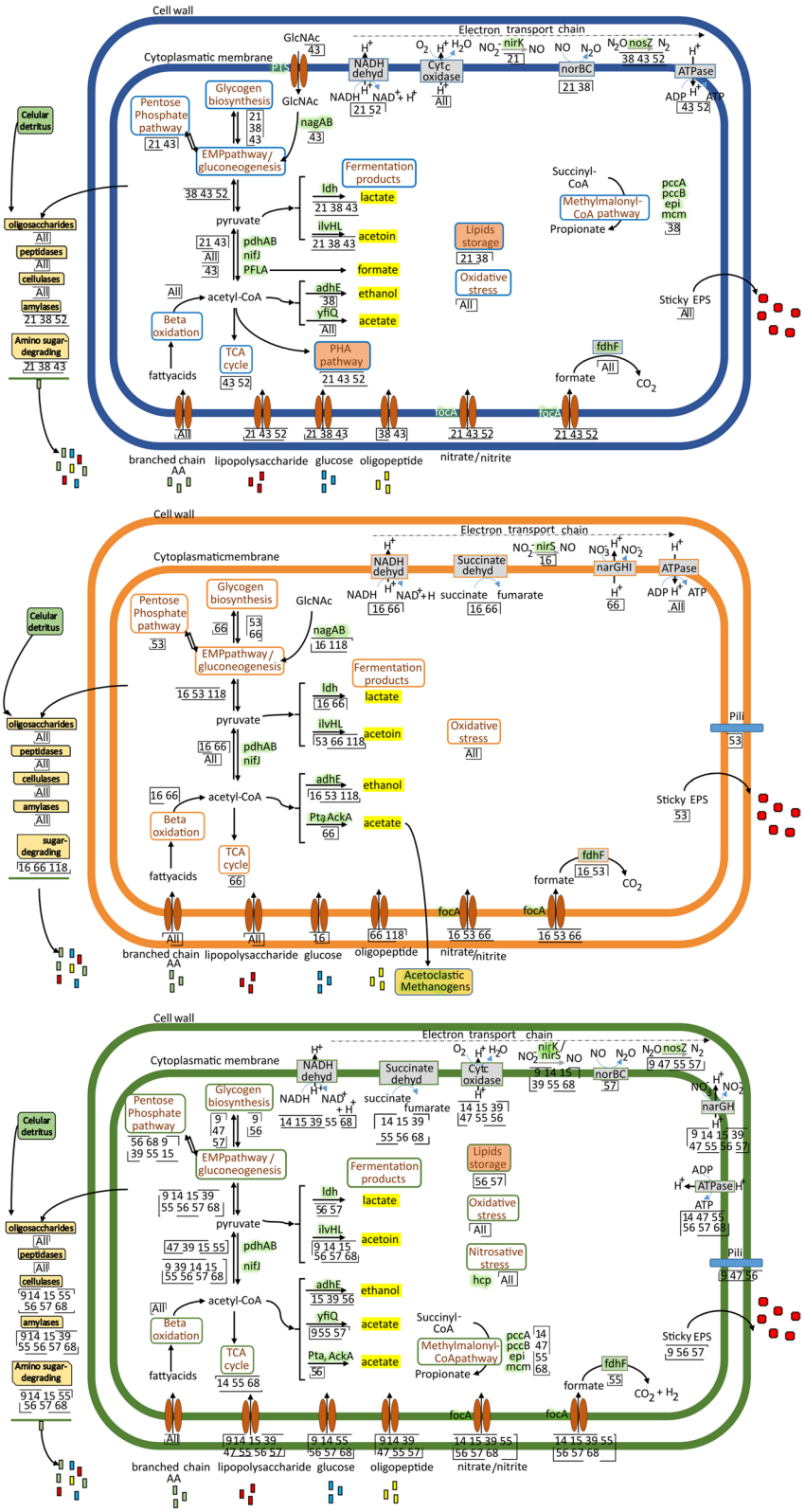

Fig. 6 Metabolic model of the Chloroflexi MAGs by reactor: Blue: MAGs retrieved from the aerobic reactor, Orange: MAGs retrieved from the methanogenic reactor, Green: MAGs retrieved from the anammox reactor and its inoculum. The identifying number of each MAG appears in the boxes when the gene or metabolic pathway is present

The role of Chloroflexi species in each reactor

The role of the different MAGs was postulated according to the genome annotation and the reactor´s characteristics (Table 3).

Although the reactors were fed with very different wastewaters and operated under different conditions (anaerobic, aerobic and anoxic) a common role can be postulated in the degradation of polymers (polysaccharides or proteins) either from the wastewater or from biomass decay or exopolymers secreted by cells. Another important role can be also postulated in the maintenance of cell aggregates as granules or floccules. Yet, Chloroflexi species can grow by using a fermentative way of life (anaerobic), aerobic respiration or nitrate-nitrite reduction, depending on the reactor's operation.

In conclusion, although the information retrieved from the metagenomics analysis showed shared functions in the different reactors, adaptation to each operation condition was observed, indicating high versatility within this group of microorganisms.

General Chloroflexi features and metabolism

A summary of the metabolic and structural functions from the MAGs retrieved from the three ecosystems is shown in Fig. 6 and Table 3.

The genomic annotation of the 17 MAGs revealed the potential for facultative and strict metabolism. All MAGs had the potential of a versatile metabolism related to the hydrolysis and fermentation of complex and simple organic compounds, suggesting that these hydrolytic and fermentative bacteria occupy a niche in recycling microbial dissolved organic matter. In addition to the filamentous morphology, some species could have a crucial structural role providing the structural matrix around which floc and granules material aggregates due to their adhesiveness and EPS production. Also, these characteristics could represent a selective advantage for the retention of these bacteria in the reactors.

Although the MAGs were retrieved from very different ecosystems, there was functional redundancy in the same reactor and analogous functions between reactors related to carbohydrates metabolism, and a more specific role related to the nitrogen removal in anammox reactors (Fig. 6). The functional redundancy of Chloroflexi members enables functional resistance to reactor operation disturbances due to the presence of multiple species that can perform the same metabolic function.

While MAGs analysis expanded our knowledge on the diversity and potential function of the phylum Chloroflexi, further experiments are necessary to confirm the expression of the identified metabolic functions under certain operational conditions. On the other hand, studying gene expression in bulking episodes would deepen the knowledge about their implication in these events. This knowledge would allow us to understand further which Chloroflexi groups are functionally important in these systems and how their disappearance or overgrowth could affect the processes. And as a final goal manage them and for example reduce and control bulking events. The difficulty of isolating organisms of the phylum Chloroflexi are probably related to their slow growth. New tools have been developed that facilitate the isolation of slow-growing microorganisms such as Droplet-based high-throughput cultivation [96]. Therefore, it is expected in the coming years to have a greater quantity of Chloroflexi members isolated in pure culture, in order to reveal and confirm their role in wastewater treatment systems.

Taxonomic proposal for the new Candidates *'Ca. Villigracilis nielsenii' sp. nov.*

As a name for the new candidate species (represented by the AMX55 genome) within the genus '*Ca.* Villigracilis', we suggest '*Ca.* Villigracilis nielsenii'. Niel.se'ni.i. N.L. gen. masc. n. nielsenii, of Nielsen, named in honor of Per Halkjær Nielsen, the Danish environmental microbiologist who made important contributions to research and practices in the field of Microbial Ecology and Water Engineering.

Conclusion

This study provides a first insight into the diversity and metabolic potential of 17 Chloroflexi species in three different wastewater treatment bioreactors, unveiling the ecological role of many species with no representatives in pure culture. The recovered genomes appear in several taxonomic orders, attesting to the broad diversity of Chloroflexi members in WWTS.

Our results suggest that Chloroflexi participate in organic matter degradation, nitrogen removal and biofilm aggregation, playing different roles according to the environmental conditions. Additionally, we proposed two novel genera within the classes Dehalococcoidia and Anaerolineae, and a new species of the genus '*Ca.* Villigracilis' representing the first representative genome within this genus.

Abbreviations

WWTS	Wastewater treatment systems
SMP	Soluble microbial products
EPS	Extracellular polymeric substances
FISH	Fluorescence in situ hybridization
UASB	Upflow anaerobic sludge blanket
UAnSB	Upflow anammox sludge blanket

MAGs	Metagenomic assembled genomes
ANI	Average Nucleotide Identities
AAI	Average Amino Acid Identity
MIMAG	Minimum information about a MAG
ASV	Amplicon sequence variant

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-023-02765-5.

Additional file 1.

Additional file 2.

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Authors' contributions
AC and CE conceived and designed the project. PB-W performed the metagenomic and genomic analysis with assistance of LG and LE. PB-W, AC, and CE discussed the results. PB-W wrote the manuscript. All the authors contributed to manuscript revision and approved the final manuscript.

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Availability of data and materials
The raw amplicon and metagenome sequences and the 17 MAGs were deposited under the NCBI BioProjects: PRJNA728853 and PRJNA780299. Scripts are available in GitHub (https://github.com/PatoUru/Bovio-Winkler_etal_2022/blob/main/Bovio-Winkler_etal_2022).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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