Article

Cometabolism of the Superphylum *Patescibacteria* with Anammox Bacteria in a Long-Term Freshwater Anammox Column Reactor

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Abstract: Although the anaerobic ammonium oxidation (anammox) process has attracted attention regarding its application in ammonia wastewater treatment based on its efficiency, the physiological characteristics of anammox bacteria remain unclear because of the lack of pure-culture representatives. The coexistence of heterotrophic bacteria has often been observed in anammox reactors, even in those fed with synthetic inorganic nutrient medium. In this study, we recovered 37 draft genome bins from a long-term-operated anammox column reactor and predicted the metabolic pathway of coexisting bacteria, especially *Patescibacteria* (also known as Candidate phyla radiation). Genes related to the nitrogen cycle were not detected in Patescibacterial bins, whereas nitrite, nitrate, and nitrous oxide-related genes were identified in most of the other bacteria. The pathway predicted for *Patescibacteria* suggests the lack of nitrogen marker genes and its ability to utilize poly-*N*-acetylglucosamine produced by dominant anammox bacteria. Coexisting *Patescibacteria* may play an ecological role in providing lactate and formate to other coexisting bacteria, supporting growth in the anammox reactor. *Patescibacteria*-centric coexisting bacteria, which produce anammox substrates and scavenge organic compounds produced within the anammox reactor, might be essential for the anammox ecosystem.

Keywords: anaerobic ammonium oxidation (anammox); *Patescibacteria*; Candidate phyla radiation; *Candidatus* Brocadia sinica; *Candidatus* Jettenia caeni; metagenomic analysis; biological nitrogen removal; wastewater treatment

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1. Introduction

Anaerobic ammonium oxidation (anammox) is a microbial process in which, under anoxic conditions, ammonia is directly oxidized to nitrogen gas with nitrite as the electron acceptor. The anammox process is mediated by a member of the phylum *Planctomycetes* [1]. Six anammox bacteria candidate genera have been proposed: *Candidatus* Brocadia, *Candidatus* Kuenenia, *Candidatus* Anammoxoglobus, *Candidatus* Jettenia, *Candidatus* Scalindua, and *Candidatus* Anammoximicrobium [2,3]. Although the physiological characteristics of several genera have been investigated [4–7], detailed physiologies remain unknown due to the lack of pure cultures [5].

Recently, most of the candidate phyla were renamed, and superphyla predicted by single-cell genomics [8] and metagenomics [9,10] were proposed. The superphylum *Patescibacteria* [8] has been proposed, which is also referred to as Candidate phyla radiation (CPR) [10]. The superphylum *Patescibacteria* has been found in various environments, such

as ground water sediment, lakes, and activated sludge [9,11]. The superphylum *Patescibacteria* has also been found in anammox enrichment cultures fed with ammonia as the sole energy source and lacking an external organic carbon supply [12,13]. Speth et al. [12] reported that candidate phyla OP11 (*Microgenomates*) and WS6 (*Dojkabacteria*) supported fermentative lifestyles, and that candidate phylum OD1 (*Parcubacteria*) could have a parasitic relationship with *Bacteroidetes* in full-scale partial-nitritation/anammox reactors. However, previous studies were mostly focused on the nitrogen cycle in anammox granules; thus, the details of the carbon metabolism of *Patescibacteria* in anammox granules are still largely unknown.

The purpose of the present study was to predict the carbon metabolism of *Patescibacteria* in a freshwater anammox enrichment culture and to investigate the possibility of a cometabolic relationship between anammox bacteria and coexisting heterotrophic bacteria, especially *Patescibacteria*. The anammox culture used in this study was operated for more than 15 years, fed with ammonia as the sole energy source, and lacked an external organic carbon supply [14]; this is a model system used to elucidate cometabolism. In this study, we used metagenomic deep-sequencing analysis to assemble low-abundance members in an anammox enrichment culture, such as *Patescibacteria*. The results of this study provide insights into ecophysiological interactions and substrate/metabolite exchanges in the autotrophic anammox community.

2. Materials and Methods

2.1. Reactor Operation and Sampling

Freshwater anammox bacteria-dominated *Candidatus* Brocadia sinica was enriched using activated sludge and cultured in an up-flow column reactor for 15 years. The reactor volume was 300 or 900 mL. The temperature was maintained at 37 °C. The hydraulic retention time was set to 2.5 h. A typical freshwater anammox medium [15] was used: 3.6–5.7 mM NH_4^+, 4.3–7.1 mM NO_2^-, 1000 mg L^{-1} $KHCO_3$, 27 mg L^{-1} KH_2PO_4, 300 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 180 mg L^{-1} $CaCl_2 \cdot 2H_2O$, and trace element solutions. The concentrations of NH_4^+, NO_2^-, and NO_3^- were determined following a previous report [16]. Three biomass samples were collected from the column reactor 4989, 5054, and 5073 days (Figure 1) after the start of operation (Figure S1).
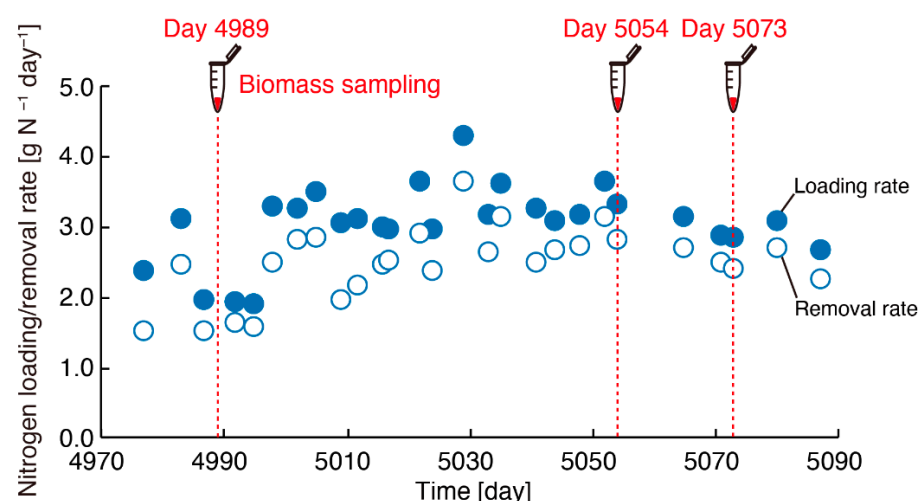
Figure 1. Nitrogen removal performance of the up-flow column reactor. Filled and open circles represent the nitrogen loading and removal rate, respectively. Biomass samples were collected on days 4989, 5054, and 5073.

2.2. DNA Extraction and DNA Sequencing

DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Irvine, CA, USA). The extracted DNA was quantified using a Qubit 2.0 fluorometer (Thermo Fisher

Scientific, Waltham, MA, USA). Three Illumina sequencing libraries were prepared for the three samples using the TruSeq DNA PCR Free (350) kit (Illumina, San Diego, CA, USA) and paired-end-sequenced (2 × 151 bp) using shotgun sequencing on a HiSeq X instrument (Illumina, USA). A PacBio sequencing library was prepared for the sample collected on day 5054 using the SMRTbell Express Template Prep Kit (Pacific Biosciences of California Inc., Menlo Park, CA, USA) after the DNA was purified with Agencourt AMPure XP magnetic beads (Beckman Coulter Life Sciences, Danvers, MA, USA) and sequenced on a PacBio Sequel instrument (Pacific Biosciences of California, Inc., USA). A circular consensus sequence (CCS) read was generated from the Sequel data with a Phred quality score above 20 (Q20, 99%).

2.3. Bioinformatics

Raw paired-end reads from HiSeq X were trimmed using Trimmomatic v0.39 [17]. The trimmed reads from HiSeq X and CCS reads from PacBio Sequel were co-assembled with SPAdes v3.13.1 [18]. In the assembly, the draft genomes of *Candidatus* Brocadia sinica (GCA_000949635.1) and *Candidatus* Jettenia caeni (GCA_000296795.1) were used as the references (as—trusted-contigs option) because the presence of these anammox bacteria in enrichment cultures was confirmed in previous studies [4,14]. The assemblies were binned using MaxBin2 v2.2.7 [19]. The relative abundance output from MaxBin2 was also used as the abundance of each bin. The completeness and contamination of the bins were assessed using CheckM v1.1.2 [20]. For the Patescibacterial bins, the CPR marker set was used for CheckM [10]. Contamination was manually removed from the contig. Bins with high contamination (>7%) were not used for further analysis. The bins were annotated using PROKKA v1.13 [21]. Predicted amino acid sequences were annotated using KEGG BlastKOALA (KEGG Orthology and Links Annotation) [22]. The metabolic pathways obtained by the BlastKOALA annotation were visualized using KEGG (Kyoto Encyclopedia of Genes and Genomes)-Decoder v1.2 [23]. The taxonomy of each bin was estimated using a BLAST search [24]. A genome tree was constructed using PhyloPhlAn v2.0.3 [25]. The sequence data were deposited in the DDBJ database under the DDBJ/EMBL/GenBank accession number DRA011208.

3. Results and Discussion

3.1. Anammox Reactor Operation

The up-flow column reactor with the freshwater anammox medium was operated for more than 5000 d using varying nitrogen loading rates and reactor volumes (Figure S1). During the sampling period, the average nitrogen loading and removal rates were 3.1 and 2.5 g N L^{-1} d^{-1}, respectively (Figure 1). The average NH_4^+ and NO_2^- removal efficiencies were 90.5% and 93.7%, respectively. The average stoichiometric ratios of consumed NO_2^- to consumed NH_4^+ and produced NO_3^- to consumed NH_4^+ were 1.45 ± 0.18 (standard deviation) and 0.27 ± 0.05, respectively. These values were similar to previously reported ratios of 1:1 and 32:0.26 [7], respectively, indicating a stable reactor operation (stable anammox process) during the sampling period.

3.2. Genome Construction and Basic Information on Bins

In total, 0.76 billion reads were produced by metagenomic sequencing of the three samples (Table S1). After quality trimming and filtering, 0.40 billion high-quality reads (>Q20) were obtained and used for metagenomic analysis. Differences in the guanine-cytosine (GC)-contents of HiSeq X reads indicate that the composition of the microbial community of each sample differed. The combined metagenome assembly generated 5780 contigs (167.2 Mbp contigs), with an N50 value of 169,060 bp. The longest contig length was 1,758,248 bp. In total, 2460 contigs above 1000 bp were extracted from the 5780 contigs and used for binning. The reconstructed contigs were classified into 42 bins. Five of the 42 bins were excluded due to high contamination (>7%; Table 1). Two anammox bacteria, *Candidatus* Brocadia sinica and *Candidatus* Jettenia caeni, were detected. In

addition, *Chloroflexi* (9 bins), *Ignavibacteriae* (2 bins), *Planctomycetes* (3 bins), *Proteobacteria* (11 bins), *Armatimonadetes* (1 bin), *Bacteroidetes* (2 bins), *Actinobacteria* (1 bin), and *Patescibacteria* (6 bins) were detected. Most of the detected bins were comparable to those reported in a previous study [26]. However, in addition, six bins belonging to the superphylum *Patescibacteria* were detected. In the present study, we focused on the metabolic analysis of *Patescibacteria*. A phylogenetic tree of the 37 bins based on protein sequences is shown in Figure 2.

Table 1. Characteristics of the bins obtained in this study.

Bin ID	Taxonomy	Complete-ness	Contamination	Bin Size (Mbp)	Number of Contigs	Relative Abundance (%)		
						Day 4989	Day 5054	Day 5073
BroJett025	*Patescibacteria, Candidatus Pacebacteria*	97.67 *	0 *	1.17	1	0.2	0.3	0.2
BroJett032	*Patescibacteria, Candidatus Pacebacteria*	100 *	0 *	1.18	5	0.1	0.2	0.1
BroJett037	*Patescibacteria, Candidate division WS6*	95.35 *	0 *	1.18	3	0.1	0.1	0.1
BroJett019	*Patescibacteria, Candidate division WS6*	95.35 *	0*	1.06	5	0.3	0.5	0.5
BroJett008	*Patescibacteria*	97.67*	2.33 *	0.57	8	2.0	0.1	0.0
BroJett034	*Patescibacteria, Berkelbacteria*	93.02 *	0 *	0.68	2	0.1	0.1	0.2
BroJett039	*Chloroflexi*	23.2	0	1.71	48	0.1	0.0	0.1
BroJett021	*Chloroflexi*	90.91	0.91	6.39	43	0.3	0.2	0.1
BroJett001	*Chloroflexi*	93.64	3.09	3.71	153	23.8	8.1	7.1
BroJett015	*Chloroflexi*	75.64	3.82	5.45	63	0.6	0.7	0.2
BroJett018	*Chloroflexi*	91.82	0.91	6.27	45	0.4	0.4	0.1
BroJett007	*Chloroflexi*	98.18	0	4.26	35	5.3	2.0	1.5
BroJett038	*Chloroflexi*	77.27	0.91	4.12	49	0.1	0.1	0.1
BroJett033	*Chloroflexi*	28.8	0	1.12	41	0.1	0.0	0.0
BroJett011	*Chloroflexi*	84.85	3.64	9.27	84	1.1	0.4	0.2
BroJett009	*Armatimonadetes*	91.76	0	2.69	23	1.4	0.4	0.4
BroJett022	*Actinobacteria*	96.98	0	2.70	5	0.3	0.2	0.0
BroJett024	*Alphaproteobacteria*	87.29	6.96	4.67	34	0.2	0.1	0.0
BroJett030	*Alphaproteobacteria*	87.95	1.2	3.49	37	0.1	0.1	0.0
BroJett013	*Alphaproteobacteria*	95.02	0.6	3.78	19	0.9	0.3	0.0
BroJett029	*Alphaproteobacteria*	75.86	6.03	4.62	51	0.2	0.1	0.0
BroJett010	*Gammaproteobacteria*	90.31	1.5	2.94	10	1.3	0.5	0.5
BroJett026	*Betaproteobacteria*	87.56	2.68	4.49	28	0.2	0.1	0.0
BroJett012	*Betaproteobacteria*	91.11	0.52	3.22	101	1.0	2.6	5.5
BroJett006	*Betaproteobacteria*	95.56	0.45	3.30	135	5.8	17.7	10.7
BroJett031	*Betaproteobacteria*	88.27	0.62	4.03	13	0.1	0.1	0.0
BroJett040	*Oligoflexia*	92.86	0	2.76	21	0.0	0.1	0.1
BroJett028	*Deltaproteobacteria*	75.91	5.38	8.63	98	0.2	0.2	0.0
BroJett017	*Ignavibacteriae*	94.97	0	3.60	22	0.5	0.1	0.1
BroJett005	*Ignavibacteriae*	96.65	0.56	3.85	120	9.9	3.7	3.2
BroJett020	*Bacteroidetes*	89.25	0	2.70	21	0.3	0.2	0.5
BroJett042	*Bacteroidetes*	93.99	0	3.74	27	0.0	0.0	0.2
BroJett014	*Planctomycetes*	81.82	4.55	4.02	32	0.9	1.0	0.6
BroJett041	*Planctomycetes (Jettenia)*	84.62	1.1	3.02	23	0.0	0.2	0.1
BroJett002	*Planctomycetes (Brocadia)*	97.8	1.65	4.10	23	14.6	39.0	58.5
BroJett003	*Planctomycetes*	95.01	2.94	3.92	437	13.1	18.0	7.6
BroJett004	*Planctomycetes*	97.66	0	3.22	20	13.0	1.8	1.0

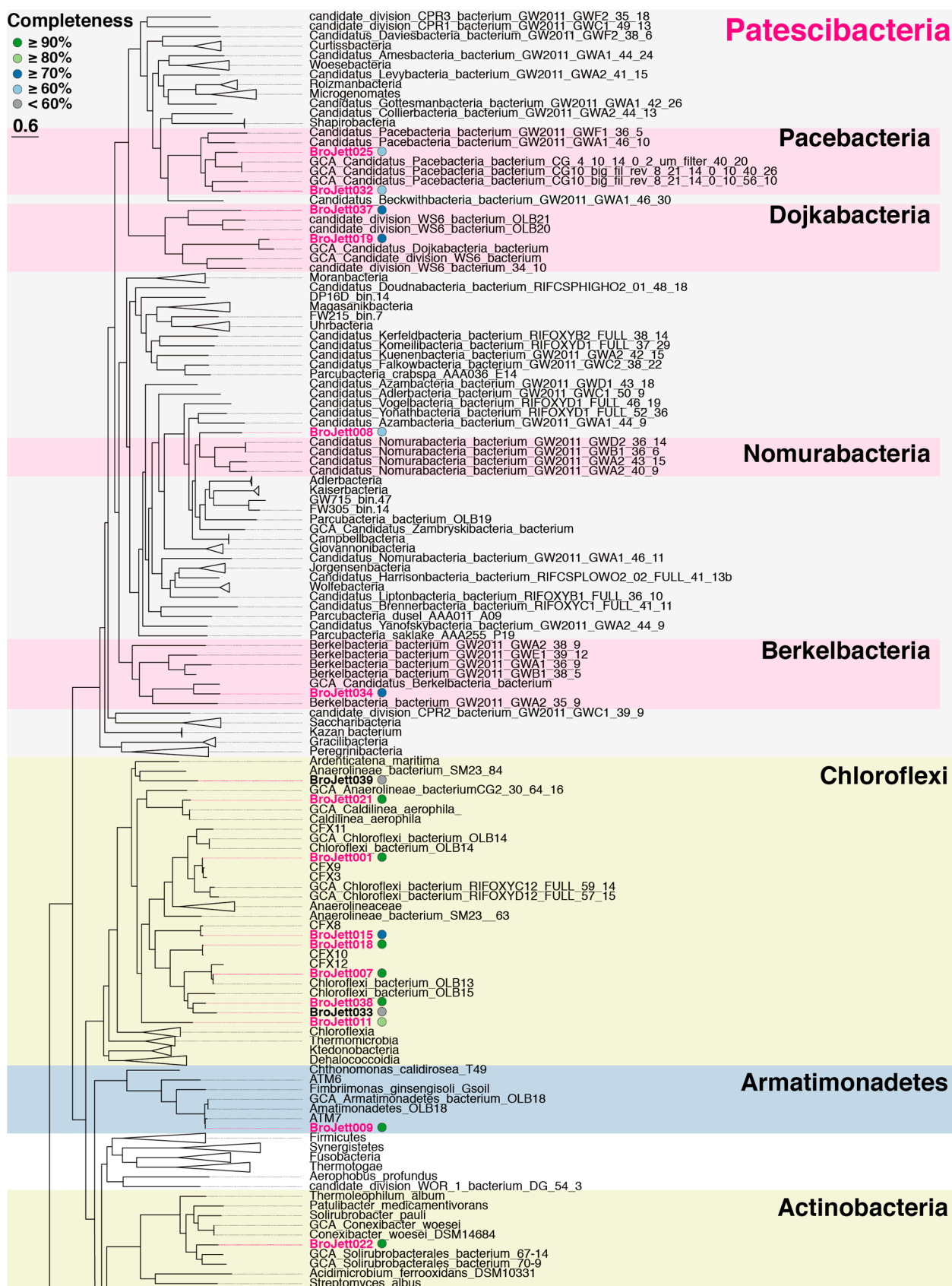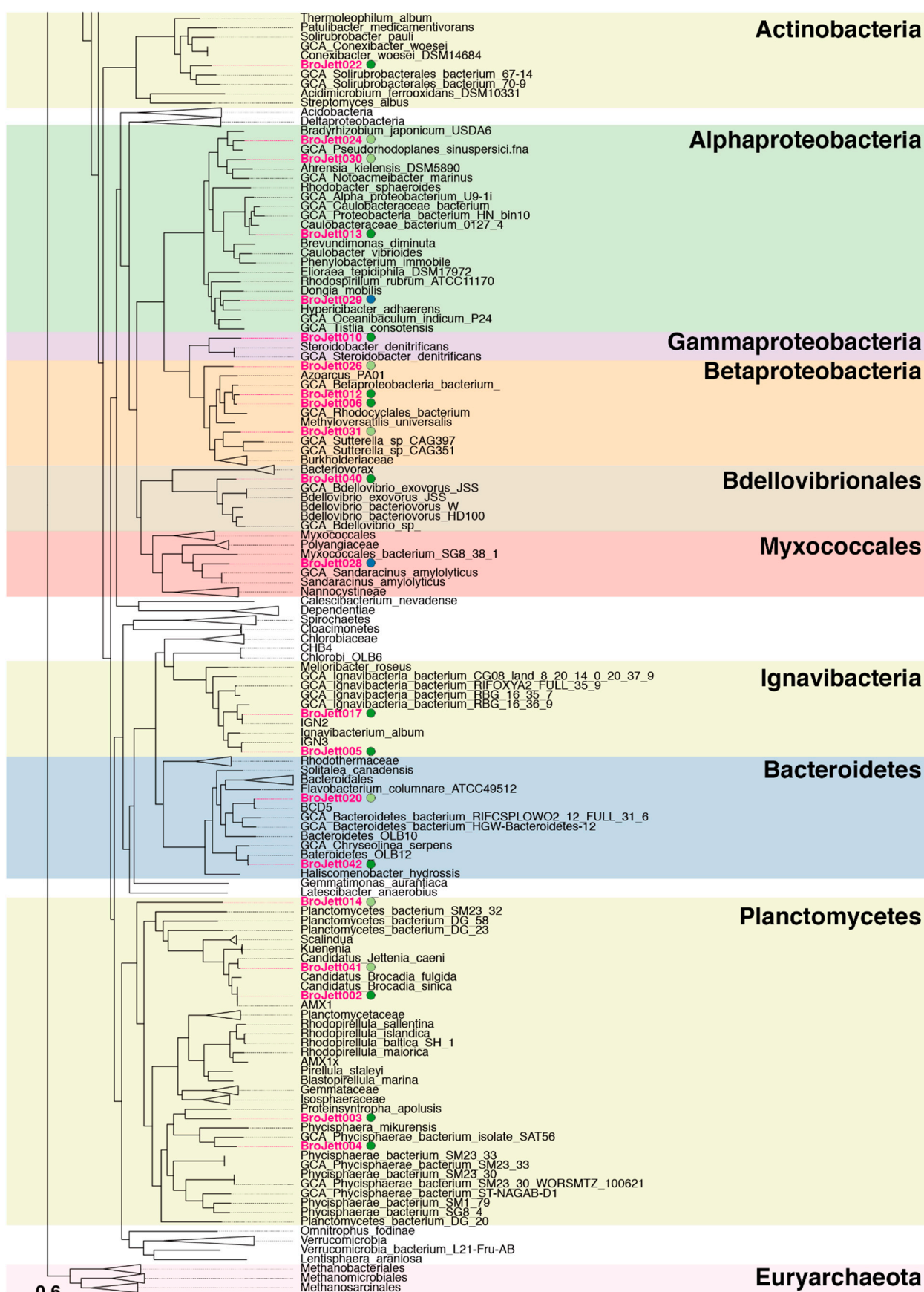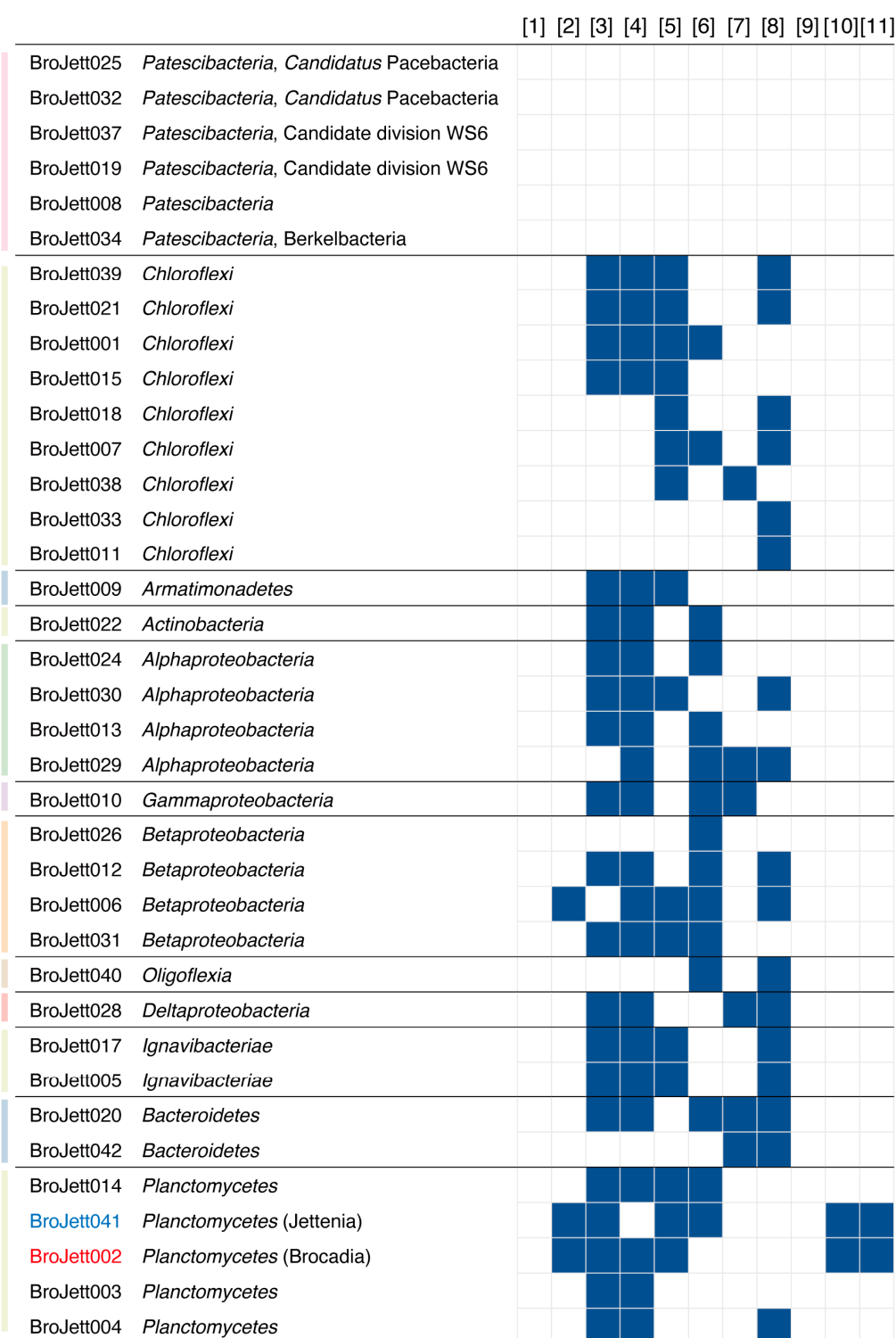* Calculated with the CPR marker set.

Figure 2. *Cont.*

Figure 2. Phylogenetic tree of bins and related genomes. The bins found in this study are shown in bold. BroJett033 and BroJett039 had low completeness and are represented in a bold black font.

3.3. Relative Abundance

The relative abundance of each bin of the three samples was estimated from the coverage calculated using MaxBin2 (Table 1). After five bins were excluded due to high contamination, the samples collected on days 4989, 5054, and 5073 accounted for 98.6%, 99.7%, and 99.5% of the relative abundance, respectively. *Candidatus* Brocadia sinica (Bin ID: BroJett002) was the most dominant bacterium, except for the sample collected on day 4989. BroJett001, which belongs to *Chloroflexi*, was the most dominant bin of the latter sample. The relative abundance of *Candidatus* Brocadia sinica increased with increasing reactor operation. In addition, the anammox bacterium *Candidatus* Jettenia caeni (BroJett041) was detected in all samples, but its relative abundance was 0.01–0.2%. *Patescibacteria* (BroJett008), *Chloroflexi* (BroJett001 and BroJett007), *Armatimonadetes* (BroJett009), *Gammaproteobacteria* (BroJett010), *Betaproteobacteria* (BroJett006 and BroJett012), and *Planctomycetes* (BroJett002, BroJett003, BroJett004, and BroJett014) accounted for more than 1% of the relative abundance of the three samples. The relative abundance of *Patescibacteria*, except for BroJett008, was lower (0.1–0.5%).
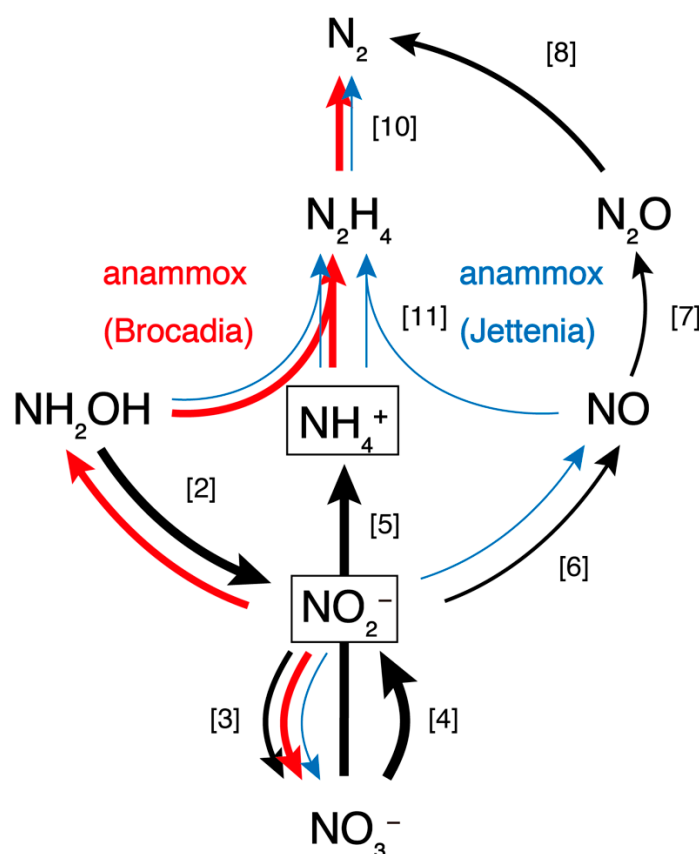
3.4. Nitrogen Cycle

To understand the contribution of bacteria to the nitrogen cycle in the anammox reactor, we focused on the following reconstructed nitrogen maker genes: ammonia oxidation (*amoA and amoBC*), hydroxylamine oxidation (*hao*), nitrite oxidation (*nxrAB*), dissimilatory nitrate reduction (*narGH* and/or *napAB*), dissimilatory nitrate reduction to ammonium (DNRA; *nirBD* and/or *nrfAH*), nitrite reduction (*nirK* or *nirS*), nitric oxide reduction (*norBC*), nitrous oxide reduction (*nosZ*), nitrogen fixation (*nifKDH*), hydrazine dehydrogenase (*hdh*), and hydrazine synthase (*hzs*; Figure 3). The dominant anammox bacterial bin BroJett002 (*Candidatus* Brocadia sinica) and the minor anammox bacterial bin BroJett041 (*Candidatus* Jettenia caeni) contained key genes for nitrite reduction, hydrazine synthesis, and hydrazine dehydrogenation. However, bin BroJett002 lacked nitrite reduction genes (*nirS* or *nirK*), as previously reported [26,27]. Bins BroJett020 and BroJett029 contained genes for the complete denitrification of nitrate to dinitrogen gas. Lau et al. [28] reported that *Owenweeksia hongkongensis*, which is closely related to bin BroJett020 based on the 16S rRNA gene, cannot reduce nitrate. In contrast, Ali et al. [26] reported that the metagenome assembly BCD5 has key enzymes for denitrification, such as *nar*, *nir*, and *noz*. In addition, *Dongia mobilis* and *Oceanibaculum indicum*, closely related to bin BroJett029, can reduce nitrate [29,30]. Most of the bins, except for Patescibacterial bins, contained genes related to nitrate reduction and DNRA (Figure 3), and their contribution was relatively high (Figure 4). The production of NH_4^+ and NO_2^- in the anammox reactor plays an important role in supporting the anammox process. Anammox bacteria are well known to lack the N_2O production pathway [31]. Although bins BroJett010 and BroJett038 can produce N_2O and lack N_2O reduction genes, indicating the release of N_2O outside the up-flow column reactor, most of the bins can reduce N_2O to N_2 gas, except for *Patescibacteria*. The emission of N_2O from the up-flow column reactor must be investigated. Interestingly, no genes related to the nitrogen cycle were detected in bins classified as *Patescibacteria*.

		[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]
BroJett025	*Patescibacteria*, *Candidatus* Pacebacteria											
BroJett032	*Patescibacteria*, *Candidatus* Pacebacteria											
BroJett037	*Patescibacteria*, Candidate division WS6											
BroJett019	*Patescibacteria*, Candidate division WS6											
BroJett008	*Patescibacteria*											
BroJett034	*Patescibacteria*, Berkelbacteria											
BroJett039	*Chloroflexi*			X	X	X			X			
BroJett021	*Chloroflexi*			X	X	X			X			
BroJett001	*Chloroflexi*			X	X	X	X					
BroJett015	*Chloroflexi*				X	X						
BroJett018	*Chloroflexi*						X		X			
BroJett007	*Chloroflexi*						X	X	X			
BroJett038	*Chloroflexi*						X	X				
BroJett033	*Chloroflexi*								X			
BroJett011	*Chloroflexi*								X			
BroJett009	*Armatimonadetes*			X	X	X						
BroJett022	*Actinobacteria*			X	X		X					
BroJett024	*Alphaproteobacteria*			X	X		X					
BroJett030	*Alphaproteobacteria*			X	X		X		X			
BroJett013	*Alphaproteobacteria*				X		X					
BroJett029	*Alphaproteobacteria*				X			X	X			
BroJett010	*Gammaproteobacteria*			X			X	X				
BroJett026	*Betaproteobacteria*						X					
BroJett012	*Betaproteobacteria*				X	X		X				
BroJett006	*Betaproteobacteria*		X		X	X	X	X				
BroJett031	*Betaproteobacteria*			X	X	X		X				
BroJett040	*Oligoflexia*						X					
BroJett028	*Deltaproteobacteria*			X	X			X	X			
BroJett017	*Ignavibacteriae*			X	X	X						
BroJett005	*Ignavibacteriae*			X	X							
BroJett020	*Bacteroidetes*			X	X			X	X			
BroJett042	*Bacteroidetes*							X	X			
BroJett014	*Planctomycetes*			X	X	X						
BroJett041	*Planctomycetes* (Jettenia)	X		X		X					X	X
BroJett002	*Planctomycetes* (Brocadia)	X		X	X						X	X
BroJett003	*Planctomycetes*			X	X							
BroJett004	*Planctomycetes*			X	X				X			

Figure 3. Presence/absence of nitrogen marker genes annotated using KEGG (Kyoto Encyclopedia of Genes and Genomes) and Blastp. The numbers in square brackets on the horizontal axis represent the following genes: [1] ammonia oxidation (amoA and amoBC, pmmo), [2] hydroxylamine oxidation (hao), [3] nitrite oxidation (nxrAB), [4] dissimilatory nitrate reduction (narGH and/or napAB), [5] DNRA (nirBD and/or nrfAH), [6] nitrite reduction (nirK or nirS), [7] nitric oxide reduction (norBC), [8] nitrous oxide reduction (nosZ), [9] nitrogen fixation (nifKDH), [10] hydrazine dehydrogenase (hdh), and hydrazine synthase (hzs). The colored lines next to the bins correspond to the phyla shown in Figure 2. The text colors of BroJett041 and BroJett002 correspond to Figure 4 and represent *Candidatus* Jettenia caeni and *Candidatus* Brocadia sinica, respectively.

Figure 4. Nitrogen cycle in the reactor. Red and blue arrows represent the anammox processes of *Candidatus* Brocadia sinica and *Candidatus* Jettenia caeni, respectively. Black arrows represent reactions involved in the nitrogen cycle other than the anammox process. The number of each reaction corresponds to those shown in Figure 3, and represents the following genes: [2] hydroxylamine oxidation (hao), [3] nitrite oxidation (nxrAB), [4] dissimilatory nitrate reduction (narGH and/or napAB), [5] DNRA (nirBD and/or nrfAH), [6] nitrite reduction (nirK or nirS), [7] nitric oxide reduction (norBC), [8] nitrous oxide reduction (nosZ), [10] hydrazine dehydrogenase (hdh), and hydrazine synthase (hzs). The width of each arrow reflects the total abundance of the bins, except for *Candidatus* Brocadia sinica because its abundance was too high.

3.5. Genomic Features of Patescibacteria Bins

We successfully recovered six draft genome bins of *Patescibacteria* from a long-term-operated freshwater anammox column reactor (BroJett008, 019, 025, 032, 034, and 037). The taxonomic assignments of these metagenomic bins were classified as *Pacebacteria* (BroJett025 and 032), *Dojkabacteria* (BroJett019 and 037), *Patescibacteria* (BroJett008), and *Berkelbacteria* (BroJett034), based on 400 conserved protein sequences (Figure 2). The genome size and the GC-content ranged from 0.57 to 1.18 Mb and from 34.3% to 48.8%, respectively, with high completeness values of 93.0% and 100%, respectively, estimated using the CheckM software package based on the 43 CPR marker genes set [10] (Table 1). Although the incomplete Patescibacterial genomes could not conclude their whole metabolic capacities, most of the gene sets for major biosynthesis pathways, such as the tricarboxylic acid cycle, gluconeogenesis, and prerequisite electron carriers, were lacking (Figure S2). In addition, there was a lack of genomes of de novo amino acid biosynthesis pathways, except for partial biosynthesis genes for serine/glycine (BroJett008, 025, 032, 034, and 037), threonine/asparagine (BroJett037), glutamine (BroJett025), and aspartate/glutamate (BroJett032 and BroJett019; Figure S2). Similarly, there were no genes relevant to the nitrogen and sulfur cycles, suggesting that these *Patescibacteria* acquire essential nutrients from other microorganisms with symbiotic lifestyles for their growth in the reactor, which is similar to the results obtained

in previous studies [32]. In contrast, a recent cell–cell association analysis based on a single amplified genome of 4829 individual cells of prokaryotes collected from subsurface field samples revealed that most of the *Patescibacteria* populations in the studied subsurface environments may not form specific physical associations with other microorganisms [33]. Instead, it was speculated that the *Patescibacteria* may rely solely on fermentation for energy conservation. In our anammox reactor, fermentative pathways for lactate (L-lactate dehydrogenase: BroJett025, 032, and 037) and formate (formate C-acetyltransferase: BroJett025) were found in Patescibacteria metagenomic bins. This suggests that *Patescibacteria* provide these fermentative by-products to bins 4 and 15, which possess lactate dehydrogenase [34] and formate dehydrogenase [35], respectively (Figure 5 and Figure S2). Although the cometabolism of these bacteria must be further studied, *Patescibacteria* may support their growth in the reactor. With respect to other possible features of the carbon cycle in the anammox reactor, we identified chitinase (BroJett037), diacetylchitobiose deacetylase (BroJett019 and BroJett032), and beta-*N*-acetylhexosaminidase (BroJett025 and BroJett032). Based on the function of the abovementioned chitin degradation-related genes, we speculate that chitin is converted to *N*-acetylglucosamine via chitobiose, *N*-acetylglucosamine hydrolyzes to acetate via diacetylchitobiose deacetylase [36], and acetate could be a useful carbon source for other microorganisms in the anammox reactor. Generally, chitin is a component of eukaryotic cells, such as protozoa, fungi, insects, crustaceans, and arthropods [37]. In prokaryotes, several bacteria can produce poly-*N*-acetylglucosamine (PGA), which is known as chitin-like polysaccharide, for biofilm formation [38]. Interestingly, metagenomic bins associated with *Candidatus* Brocadia (BroJett002), *Candidatus* Jettenia (BroJett041), and Ignavibacteria (BroJett005) encode poly-beta-1,6 *N*-acetyl-D-glucosamine synthase (PgaC), which is key for the biosynthesis of PGA (Table S2). This enzyme catalyzes the polymerization of uridine diphosphate-*N*-acetylglucosamine, which is synthesized from beta-D-fructose 6-phosphate generated during glycolysis, to produce PGA. In addition, an anammox bacterial bin of *Candidatus* Brocadia (BroJett002) possesses putative poly-beta-1,6 *N*-acetyl-D-glucosamine export porin (PgaA) and poly-beta-1,6-*N*-acetyl-D-glucosamine N-deacetylase (PgaB). Similar PgaABC proteins were also found in the *Candidatus* Brocadia sinica JPN1 genome. These observations imply that major microbial constituents of the reactor, including anammox bacteria, may produce PGA and that some Patescibacteria populations may utilize parts of the PGA (e.g., *N*-acetylglucosamine) for their growth. On the other hand, there were no genes of the biofilm PGA synthesis protein (PgaD) in the investigated anammox bacterial bins, which is necessary for the formation of PGA that functions as a helper protein of PgaC [38,39]. Therefore, further gene expression studies and identification of PGA materials are required for the confirmation of actual PGA production from anammox bacteria in the reactor. The utilization of organic compounds by coexisting heterotrophic bacteria has also been reported for autotrophic nitrifying biofilms, which are fed with ammonia as the sole energy source [40]. Moreover, we newly discovered that *Pacebacteria* and unclassified *Patescibacteria*, other than *Dojkabacteria* and *Microgenomates* [12], may support fermentative lifestyles in the anammox granule. Overall, *Patescibacteria* populations in the anammox reactor may play ecological roles, such as in short-chain fatty acid production and the degradation of chitin-related compounds, and they may survive depending on the PGA production by major anammox bacteria based on metagenomic information.
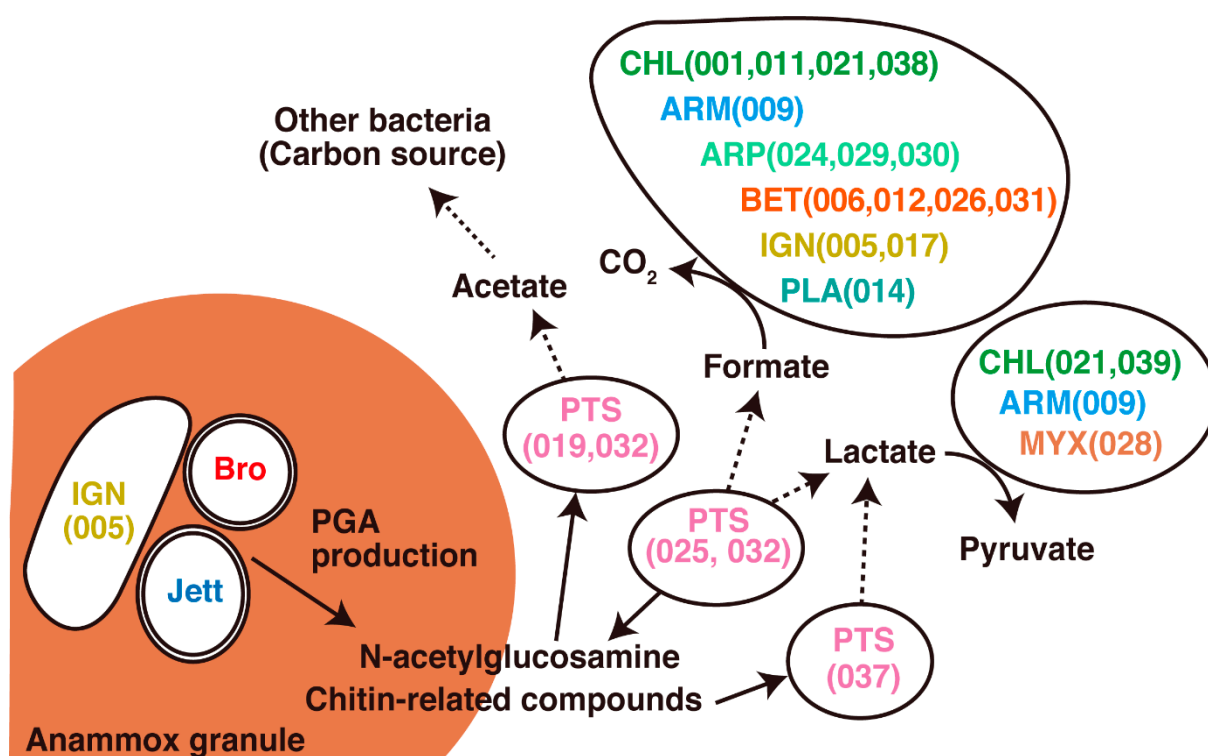
Figure 5. Putative carbon metabolic interactions among *Patescibacteria* and predominant bacteria. The numbers on the bacterial cells indicate the bin IDs shown in Table 1. PTS: *Patescibacteria*, IGN: *Ignavibacteriae*, Bro: *Candidatus* Brocadia, Jett: *Candidatus* Jettenia, CHL: *Chloroflexi*, ARM: *Armatimonadetes*, ARP: *Alphaproteobacteria*, BET: *Betaproteobacteria*, PLA: *Planctomycetes*, MYX: *Myxococcales*. Solid arrows indicate the degradation and metabolic flows of poly-*N*-acetylglucosamine/chitin-related compounds. Dashed arrows indicate the by-products from fermentation and poly-*N*-acetylglucosamine degradation. Curved solid arrows indicate metabolic reactions via formate or lactate dehydrogenases.

4. Conclusions

Six draft genome bins of *Patescibacteria* were recovered from freshwater anammox column reactors operated for more than 15 years and fed with an inorganic and synthetic nutrient medium by metagenomic deep-sequencing analysis. The metabolic capacities predicted for the six Patescibacterial bins with high completeness suggest that *Patescibacteria* can utilize chitin-related compounds and produce fermentation by-products of lactate and formate in the anammox reactor. The phylogenetically and metabolically diverse *Patescibacteria* as well as other coexisting heterotrophic bacteria ensure the effective utilization of chitin-related compounds produced by anammox bacteria, which may create a stable anammox ecosystem without other by-products. Further studies involving metatranscriptomics and metabolomics may help to elucidate the in situ ecological functions of Patescibacteria and the biological interactions with anammox bacteria in the reactor.

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Data Availability Statement: The sequence data were deposited in the DDBJ database under the DDBJ/EMBL/GenBank accession number DRA011208.

Conflicts of Interest: The authors declare no conflict of interest.

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