Discovery of a new genus of anaerobic ammonium oxidizing bacteria with a mechanism for oxygen tolerance

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Abstract

In the past 20 years, there has been a major stride in understanding the core mechanism of anaerobic ammonium-oxidizing (anammox) bacteria, but there are still several discussion points on their survival strategies. Here, we discovered a new genus of anammox bacteria in a full-scale wastewater-treating biofilm system, tentatively named "*Candidatus* Loosdrechtia aerotolerans". Next to genes of all core anammox metabolisms, it encoded and transcribed genes involved in the dissimilatory nitrate reduction to ammonium (DNRA), which coupled to oxidation of small organic acids, could be used to replenish ammonium and sustain their metabolism. Surprisingly, it uniquely harbored a new ferredoxin-dependent nitrate reductase, which has not yet been found in any other anammox genome and might confer a selective advantage to it in nitrate assimilation. Similar to many other microorganisms, superoxide dismutase and catalase related to oxidative stress resistance were encoded and transcribed by "*Ca*. Loosdrechtia aerotolerans". Interestingly, bilirubin oxidase (BOD), likely involved in oxygen resistance of anammox bacteria under fluctuating oxygen concentrations, was identified in "*Ca.* Loosdrechtia aerotolerans" and four *Ca*. Brocadia genomes, and its activity was demonstrated using purified heterologously expressed proteins. A following survey of oxygen-active proteins in anammox bacteria revealed the presence of other previously undetected oxygen defense systems. The novel *cbb3*-type cytochrome c oxidase and bifunctional catalase-peroxidase may confer a selective advantage to *Ca*. Kuenenia and *Ca*. Scalindua that face frequent changes in oxygen concentrations. The discovery of this new genus significantly broadens our understanding of the ecophysiology of anammox bacteria. Furthermore, the diverse oxygen tolerance strategies employed by distinct anammox bacteria advance our understanding of their niche adaptability and provide valuable insight for the operation of anammox-based wastewater treatment systems.

Keywords:

Anammox, New genus, Biofilm, Oxygen tolerance, Nitrate reduction

1. Introduction

The discovery of anaerobic ammonium oxidation (anammox) completely changed our understanding of the nitrogen cycle (Kuypers et al. 2018, Mulder et al. 1995). Anammox bacteria are affiliated with a monophyletic group in the phylum *Planctomycetes* (Strous et al. 1999), and have been detected in many natural and engineered ecosystems, where they facilitate the release of fixed nitrogen into the atmosphere. After the initial discovery of the anammox process, five candidate genera of anammox bacteria, including *Ca*. Scalindua (Kuypers et al. 2003), *Ca*. Kuenenia (Schmid et al. 2000, Schmid et al. 2003), *Ca*. Brocadia (Strous et al. 1999), *Ca*. Anammoxoglobus (Kartal et al. 2007b), and *Ca*. Jettenia (Quan et al. 2008), were consecutively identified.

Environmental metagenomics combined with continuous cultivation approaches and biochemical experiments resulted in the description of the core anammox metabolism, which can be described in three main catabolic reactions (Hu et al. 2019, Kartal et al. 2011): reduction of nitrite (NO_2^-) to nitric oxide (NO) by nitrite reductase (NIR or an unidentified enzyme, equation (1)), transformation of ammonium (NH_4^+) and NO to hydrazine (N_2H_4) by hydrazine synthase (HZS, equation (2)) (Kartal et al. 2011, Maalcke et al. 2014, Oshiki et al. 2016a), and oxidation of N_2H_4 into dinitrogen gas (N_2) by hydrazine dehydrogenase (HDH, equation (3)). The electrons released from hydrazine oxidation are used to establish a proton motive force for energy conservation and produce reducing equivalents needed for cellular anabolic activity (de Almeida et al. 2016, Hu et al. 2019). Whereas, hydroxylamine (NH_2OH) was found to be the intermediate of anammox process in *Ca*. Brocadia that an NH_2OH dependent metabolism was proposed for some anammox bacteria (Oshiki et al. 2016a). Anammox bacteria were assumed to oxidize a part of their substrate nitrite to nitrate by a nitrite oxidoreductase (NXR, equation (4)) to generate electrons for the reduction of nitrite to NO (equation [1]) (Hu et al. 2019, Kartal et al. 2013). Whereas HZS, HDH, and NXR are conserved throughout the known anammox genera, distinct anammox bacteria encode a cytochrome *cd*$_1$-type NIR (NirS), a copper-containing NIR (NirK), or some alternative protein for nitrite reduction such as a multiheme HAO-like protein complex (Ferousi et al. 2019, Kartal and Keltjens 2016). All of these catabolic reactions occur in the membrane-bound "prokaryotic organelle" called the anammoxosome, which comprises 50–70% of the cell volume (de Almeida et al. 2015).

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \ (E_0' = +0.38\ V) \quad [1]$$

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \ (E_0' = +0.06\ V) \quad [2]$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \ (E_0' = -0.75\ V) \quad [3]$$

$$NO_2^- \rightarrow NO_3^- + 2H^+ + 2e^- \ (E_0' = +0.42\ V) \quad [4]$$

While there have been advances made in understanding the core metabolism of anammox bacteria, there are still several discussion points on their physiological versatility and resistance to environmental factors including their alternative energy sources and oxygen defense systems. Anammox bacteria were assumed to have an exclusively chemolithotrophic lifestyle, acquiring energy for growth solely from anaerobic NH_4^+ oxidation, but they have the genomic potential of using formate, acetate, propionate, amino acid, hydrogen, and Fe^{2+} as electron donors to sustain their metabolism (Kartal et al. 2013, Lawson et al. 2020). Indeed, using enrichment cultures of anammox bacteria, their growth on anaerobic oxidation of short volatile fatty acids was demonstrated (Güven et al. 2005, Kartal et al. 2007b, Kartal et al. 2008, Tao et al. 2019). Distinct enrichments of anammox bacteria were reported to reduce NO_3^- to produce additional ammonium as a substrate for their main metabolism by dissimilatory nitrate reduction to ammonium (DNRA) coupled with short volatile fatty acids oxidation (Ali et al. 2020, Güven et al. 2005, Kartal et al. 2007a, Winkler et al. 2012). The known anammox bacteria appear to be oxygen sensitive and dissolved oxygen is an important parameter controlling anammox activity in manmade ecosystems such as wastewater treatment plants (WWTPs) (Cho et al. 2020, Kimura et al. 2011). Nevertheless, varying levels of oxygen tolerances were observed in different enrichment cultures of anammox bacteria (Oshiki et al. 2016b), but the underlying mechanisms are not yet clear, and oxygen defense systems of anammox bacteria have not been characterized. Altogether, these findings indicate greater ecophysiological flexibility of anammox bacteria and the presence of a specific niche for each distinct anammox genera or species.

In this study, we present the discovery of a new anammox genus in a full-scale wastewater treatment biofilm system treating landfill leachate. Using its metagenome-assembled genome (MAG) and metatranscriptomes, we constructed a metabolic blueprint of this new anammox bacterium. In addition, a detailed analysis of the MAG revealed a bilirubin oxidase, an enzyme that could be used in oxygen resistance, whose activity was validated using heterologously expressed proteins. We discuss the central metabolism of this new genus and its ecophysiological versatility related to its adaptation to unfavorable and fluctuating environmental conditions. Furthermore, the exploration of different oxygen defense mechanisms employed by distinct anammox bacteria advances our understanding of niche adaption and application of anammox bacteria in wastewater treatment systems.

2. Materials and Methods

2.1. Sample collection and sequencing

Two separate biofilm samples were collected from the activated carbon modules of a landfill leachate treatment plant called Zentraldeponie Emscherbruch (ZDE) WWTP (Table S1, Figure S1) located in Herten, Germany in November 2017 and April 2018, respectively. ZDE plant was primarily operated as an activated sludge system with a conventional nitrification-denitrification nitrogen removal process. The plant was upgraded in 2001 by equipping ultrafiltration and activated carbon modules after activated sludge systems (Figure S1). The activated carbon modules were kept at a high temperature (34 □) and nearly neutral pH (6.8) (Table S1) in which granular activated carbon acted as carrier materials for the establishment of anammox biofilm (Azari et al. 2017). On average 90.0% of the influent ammonium (758 mg/L) was removed and oxidized by the activated sludge systems; 24.8% of the inputted ammonium (76.6 mg/L), 98.5% of the inputted nitrite (45.8 mg/L), and 97.4% of the inputted nitrate (13.4 mg/L) were removed by the following biofilm system (Table S1). Previously, frequent sampling and independent analyses confirmed the enrichment of anammox bacteria in the activated carbon modules, and anammox biofilms were shaped as spherical granules with red color and different sizes (1 to 13 mm) (Azari et al. 2018, Azari et al. 2017) (Figure S1).

Biofilm samples collected in November 2017 were used for 16S rRNA gene high throughput sequencing, samples were stored at -20 °C. The specific primer pair of 515F/806R with a barcode was used to amplify the V4 regions of the total DNA. Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer2100 system. Libraries were sequenced on an Illumina HiSeq2500 platform with an insert size of 250 bp paired-ends. Sequences analysis was carried out using QIIME (Caporaso et al. 2010). Adapters, barcodes, and primers in the raw reads were trimmed off, the paired-end reads were merged using FLASH V1.2.7 and reads shorter than 170 bp were discarded. Chimera checking was performed by USEARCH2 (https://www.biorxiv.org/content/10.1101/074252v1) using the "Gold" database as a reference. All filtered sequences were clustered into one fasta file for OTU picking with ≥ 97% sequence identity cutoff and taxonomy assignment against SILVA 132 released reference database.

Two independent biofilm samples were collected from the same site in April 2018 for metagenomic and metatranscriptomic sequencing, samples for DNA isolation were stored at -

20 °C and samples for RNA isolation were preserved on site in LifeGuard Soil Preservation Solution (Qiagen, Germany). DNA and RNA extractions and the following metagenomic and metatranscriptomic sequencing methods were previously described (Yang et al. 2020c). In brief, total DNA and RNA were extracted, and genomic DNA was removed following RNA extraction. The ribosomal RNA was depleted from the total RNA using the Ribo-Zero rRNA removal kit (Illumina, Inc., San Diego, CA, USA), and the remaining mRNA was reverse-transcribed. DNA and cDNA were sequenced using an Illumina HiSeq sequencer (Illumina) with 150-bp paired-end reads at GENEWIZ (Suzhou, China).

2.2. De novo metagenomic assembly, binning, and quality assessment

Raw shotgun sequencing reads were trimmed using BBduk (BBMap v. 36.32 - Bushnell B. - sourceforge.net/projects/bbmap/) with the following parameters: ktrim=r, k=21, mink=11, hdist=2, minlen=149, qtrim=r, trimq=15. Trimmed metagenomic reads were assembled using Spades v. 3.10.1 (Bankevich et al. 2012) with the k-mer sizes of 21, 33, 55, 77, 99, 127. All resulting scaffolds larger than 2,000 bp were binned using MetaBAT v. 2.12.1 with the setting '- minContig 2000' (Kang et al. 2015). The best resulting bins were chosen using DASTool (https://github.com/cmks/DAS_Tool). Finally, four putative anammox bacterial MAGs were identified from the metagenomic dataset by GTDB-Tk v1.1.0 (Chaumeil et al. 2019) using the GTDB database (https://doi.org/10.1101/771964), while a novel anammox bacterial MAG which was named YC1 ("*Ca*. Loosdrechtia aerotolerans"), could only be assigned to family *Brocadiaceae* and could not be classified as any known genera. The completeness and level of contamination of YC1 were estimated using CheckM v1.0.6 (Parks et al. 2015). YC1 was further iteratively assembled using a protocol as described in a previous study (Yang et al. 2020a). A high-quality MAG was acquired after 70 rounds of assembly (Table S2). Trimmed, paired-end metagenomic reads were mapped to the metagenomic assembly and predicted open reading frames (ORFs) by Prodigal v2.6.3 (Hyatt et al. 2010) using Bowtie2 v2.2.9 (Ben and Steven 2012) to calculate the genome and gene abundances as RPKM (Robinson and Oshlack 2010). The rRNA reads were identified and removed from metatranscriptomic datasets using SortMeRNA (version 2.1) (Kopylova et al. 2012) against both the SILVA 132 database and the default databases. Non-rRNA metatranscriptomic reads were mapped to the predicted ORFs by BWA v0.7.17 (Li and Durbin 2009) with the default settings to calculate the gene transcription as RPKM.

RPKM = (number of mapped reads)/[(gene length/1,000)*(total mapped reads/1,000,000)]

200 *2.3. Genome analyses*

201 The genome of "*Ca*. Loosdrechtia aerotolerans" was analyzed by the MicroScope
202 platform (http://www.genoscope.cns.fr/agc/microscope). The genome of "*Ca*. Loosdrechtia
203 aerotolerans" was further annotated by GhostKOALA, which is KEGG's internal annotation
204 tool for K number assignment of KEGG GENES using SSEARCH computation (Kanehisa et
205 al. 2016). In addition, predicted ORFs were also annotated by eggNOG-mapper (Huerta-Cepas
206 et al. 2017) and Rapid Annotation using Subsystem Technology (RAST) (Overbeek et al. 2014).
207 Predicted ORFs were also searched against the NCBI non-redundant (nr) database by BLASTP
208 using an E-value < 10^{-5} as the threshold with the setting '-max_target_seqs 3' to acquire more
209 annotation information. The above annotation results were summarized in Table S3. Potential
210 secreted proteins with the signal peptides were identified by SignalP 4.1, Signal-BLAST, and
211 PSORTb (Frank and Sippl 2008, Thomas Nordahl et al. 2011, Yu et al. 2010).

212 *2.4. Phylogenetic analyses*

213 All the putative anammox bacterial operational taxonomic units (OTUs) acquired from
214 the 16S rRNA gene high throughput sequencing were used for phylogenetic analysis with all
215 the 16S rRNA genes of anammox bacteria stored in the SILVA 138 SSU database. CheckM
216 v1.0.6 was used to produce an alignment of 43 concatenated markers (Parks et al. 2015) from
217 "*Ca*. Loosdrechtia aerotolerans", previously published anammox bacterial genomes available
218 by the end of December 2019 (Table S4), and 16 other Planctomycetes genomes. The full-
219 length 16S rRNA gene in the MAG was identified by BLASTN search against the SILVA 138
220 SSU database using an E value threshold of < 10^{-10}. 16S rRNA genes of anammox bacteria
221 stored in the SILVA 138 SSU database were used for phylogenetic analysis with the 16S rRNA
222 gene of "*Ca*. Loosdrechtia aerotolerans". HzsA sequences from previously published anammox
223 bacteria and "*Ca*. Loosdrechtia aerotolerans" were used for phylogenetic analysis. Sequences
224 of respiratory nitrate reductase (NarG), ferredoxin-dependent nitrate reductase (NarB),
225 periplasmic nitrate reductase (NapA), and nitrite oxidoreductase (NxrA) were downloaded
226 from NCBI and UniProtKB and clustered by usearch -cluster_fast (Edgar 2013) with an
227 identity of 85%, respectively, the cluster representatives were used for phylogenetic analysis
228 with the putative NarB from "*Ca*. Loosdrechtia aerotolerans". Laccase and bilirubin oxidase
229 amino acid sequences downloaded from NCBI and UniProt databases were clustered by
230 usearch -cluster_fast (Edgar 2013) with an identity of 85%, and the cluster representatives were

used for phylogenetic analysis with the putative bilirubin oxidases from anammox bacteria. All the above sequences used for phylogenetic analysis were aligned by MAFFT online service (Yamada et al. 2016), and gaps in the multiple sequence alignment were removed by trimAl V1.2 with the setting '-gt 0.1'. Maximum likelihood trees were built using IQ-TREE with the default settings (Nguyen et al. 2015) and visualized using iTOL (Letunic and Bork 2019). The models of sequence evolution HKY+F+R2, WAG+G4, LG+G4, Q.pfam+R10, VT+R10, and LG+F+R6 were chosen by ModelFinder (as implemented in IQ-TREE) to build 16S rRNA gene, HzsA, NxrA, nitrate reductases, BOD, and 43 concatenated markers phylogenetic trees, respectively.

2.5. Comparative genomic analyses

Average amino acid identity (AAI) was calculated between the genome of "*Ca*. Loosdrechtia aerotolerans" and 25 previously published anammox bacterial genomes (Table S4) using CompareM with the default setting (https://github.com/dparks1134/CompareM). Unique genes of "*Ca*. Loosdrechtia aerotolerans" compared to other anammox bacteria were identified by a reciprocal best BLAST (Altschul et al. 1990). BLAST hits with an E-value of 10^{-5}, amino acid identities < 40%, and a minimum alignment length < 50% were considered as unique genes.

The distribution of specific genes in anammox bacterial genomes was investigated. Specific gene sequences identified in known anammox bacterial genomes were downloaded from NCBI depending on the annotation. Furthermore, the manually annotated Swiss-Prot sequences were downloaded from UniProt and merged into the above corresponding sequence pools and the BLAST databases (Table S5) were built using these downloaded sequences by the makeblastdb tool of NCBI. The predicted ORFs from "*Ca*. Loosdrechtia aerotolerans" and 25 known anammox bacterial genomes (Table S4) were searched by BLASTP against these functional gene databases with E-value < 10^{-5}, amino acid identity > 40% and minimum alignment length (length of aligned query sequence/length of database sequence) > 50% (Yang et al. 2020a, Yang et al. 2020b). To further examine the accuracy of gene annotation based on the above custom databases, the extracted gene sequences from anammox genomes were BLASTP against the NCBI RefSeq protein database with E-value < 10^{-10}, and the results were manually checked.

2.6. Global distribution

The 16S rRNA gene sequence of "*Ca*. Loosdrechtia aerotolerans" was submitted to IMNGS (Ilias et al. 2016) with the setting "min threshold 94.5%, min size 250 bp" to investigate the occurrence of the new genus in different ecosystems. All acquired sequences were searched back to the 16S rRNA gene sequence of "*Ca*. Loosdrechtia aerotolerans" with E-value < 10^{-10} by BLASTN and only the identity larger than 94.5% (Yarza et al. 2014) was considered. In the rRNA amplicon datasets, the relative abundance of the target sequences larger than 0.1% was considered.

2.7. Heterologous expression and purification of bilirubin oxidases

The BOD sequences from "*Ca*. Loosdrechtia aerotolerans" (E3K32_04160), "*Ca*. Brocadia caroliniensis" (OOP56653.1), and "*Ca*. Brocadia sp. AMX2" (RIK02322.1) were codon optimized by GeneDesign (http://54.235.254.95/gd/), synthesized (BGI Genomics Co Ltd., China), and cloned into the NdeI/HindIII site of pCold-TF vector with an N-terminal His_6 tag and a soluble trigger factor chaperone tag (Takara Bio Co Ltd., Japan). *E. coli* BL21 (Takara Bio Co Ltd., Japan) was used as the host strain for the recombinant vectors. The recombinant *E. coli* were inoculated into a LB medium containing 100 μg/mL ampicillin and incubated at 37 °C until the OD_{600} reached 0.6-0.8. Isopropyl-d-1-thiogalactopyranodside and $CuSO_4$ were added to the culture at final concentrations of 0.1 mM and 0.25 mM, respectively, and then the culture was incubated at 15 °C for 18 h. The cell pellets were collected and resuspended in 20 mL binding buffer (20 mM phosphate buffer (pH 7.4), 500 mM NaCl, 50 mM imidazole, 1 mM dithiothreitol, 1 mM lysozyme, and 1 mM phenylmethylsulfonyl fluoride, and 0.25 mM $CuSO_4$), followed by ultrasonic decomposition. The target proteins were further purified by Mag-Beads His-Tag Protein Purification Kit (BBI Co Ltd., China) with washing buffer (20 mM phosphate buffer (pH 7.4), 500 mM NaCl, 100 mM imidazole, 0.1% NP-40, 0.25 mM $CuSO_4$) and elution buffer (20 mM phosphate buffer (pH 7.4), 500 mM NaCl, and 500 mM imidazole). The purified proteins were concentrated by 30K Amicon Ultra-15 (Millipore Co Ltd., USA). The concentration of the purified proteins was measured by Bradford Protein Assay Kit (Beytotime Bio Co Ltd., China) and analyzed by a 12% SDS-PAGE gel (Biomed Co Ltd., China). The three-dimensional structures of BODs were built by the I-TASSER server (Zhang 2008).

2.8. Bilirubin oxidation activity measurement

The BOD activity was measured as previously described (Sakasegawa et al. 2006). Briefly,

10 μL of 40 μM purified proteins were inoculated into a 300 μL reaction system containing 240 μL citrate-phosphate buffer (0.1 M, pH 7.5) and 50 μL bilirubin (2 mM) (Sigma, USA). The oxidation of bilirubin at 40 °C by the purified proteins was monitored by a spectrophotometer (Infinite® M Plex, Tecan Trading AG) at 450 nm for 3 min. One unit of BOD activity was defined as the amount of BOD (mmol) that oxidized 1 mmol of bilirubin per minute.

2.9. Data availability

The 16S rRNA gene raw reads were deposited into the NCBI short-reads archive database under the accession number SRR11309782. Raw metagenomic and metatranscriptomic sequences were submitted to NCBI under BioProject PRJNA526440. The MAG of *Ca*. Loosdrechtia aerotolerans is available in NCBI GenBank under the accession number SOER00000000.

3. Results and Discussion

3.1. Identification of a new genus of anammox bacteria

Zentraldeponie Emscherbruch (ZDE) WWTP was designed to treat landfill leachate. The plant was designed to remove ammonium through a two-stage process, in which part of ammonium was oxidized to nitrite by the activated sludge system and the remaining ammonium and oxidized nitrite were then transferred to nitrogen gas by anammox in the activated carbon modules under anaerobic condition (Table S1, Figure S1). Biofilm samples were collected from the activated carbon modules of the plant in November 2017 and April 2018 for 16S rRNA gene amplicon sequencing and metagenomic and metatranscriptomic sequencing, respectively. In total 27 OTUs of *Brocadiales* anammox bacteria were obtained from 16S RNA gene amplicon sequencing, and most of them were assigned to *Ca*. Brocadia, but only OTU27 could not be assigned to any of the known anammox genera (Figure S2).

Shotgun metagenomic and metatranscriptomic sequencing of the biofilm produced 251.6 million DNA and 66.4 million cDNA reads. In total 198 MAGs were obtained after *de novo* assembly and binning of the metagenomic DNA reads, in which 106 MAGs had high completeness (> 80%) and low contamination (< 10%) (Table S6). Four high-quality anammox bacterial MAGs (Table S7) were identified based on the taxonomic assignments of GTDB-Tk (Chaumeil et al. 2019), including two *Ca*. Kuenenia MAGs (YC6 and YC7), one *Ca*. Brocadia

MAG (YC2), and the MAG YC1. *Ca*. Kuenenia YC6 dominated the anammox community (5.82%), then followed by YC2 (1.85%), YC7 (0.78%), and YC1 (0.6%) (Table S7 and Figure S3). YC1 was assigned to the family *Brocadiaceae* but could not be assigned to any known genera. In line with this observation, the 16S rRNA gene of YC1 had a 100% identity with OTU27 (Figure S4). In the V4 region of the 16S rRNA gene from YC1 and OTU27, a 7-bps gap was found compared to the 16S rRNA genes from other known anammox bacteria (Figure S4).

In line with the finding in "*Ca*. Jettenia asiatica" (Quan et al. 2008), the 16S rRNA, intergenic spacer region, and 23S rRNA coding genes were arranged in a large rRNA gene fragment in YC1. Phylogenetic analyses of the full-length 16S rRNA genes (Figure 1a) and 23S rRNA genes (Figure S5c), 43 concatenated markers (Figure S5a), and HzsA sequences of anammox bacteria (Figure S6) confirmed the placement of YC1 in the phylum *Planctomycetes*. Based on the phylogenetic analyses of the 16S rRNA gene (Figure 1a) and HzsA (Figure S6), YC1 had the closest phylogenetic relationship with *Ca*. Anammoxoglobus. The highest 16S rRNA gene sequence identity between YC1 and known anammox bacteria was 92.79% with *Ca*. Anammoxoglobus propionicus (KC862502.1) (Figure 1b), which is much lower than the minimum similarity cutoff demarcating the genus level (94.5%) (Yarza et al. 2014). AAI between YC1 and non-marine genera *Ca*. Jettenia, *Ca*. Brocadia, and *Ca*. Kuenenia was 66.4-75.2% which is larger than the defined minimum genus demarcation using AAI (60-80%) (Konstantinidis et al. 2017, Luo et al. 2014) (Figure S5b). YC1 had the lowest AAI (< 60%) with marine genus *Ca*. Scalindua (Figure S5b). However, AAI among the other three non-marine genera *Ca*. Jettenia, *Ca*. Brocadia, and *Ca*. Kuenenia were also larger than 60% (Figure S5b), illustrating the minimum genus demarcation of AAI for non-marine anammox bacteria should be larger than 60%. Therefore, we propose YC1 represents a new genus and have tentatively named it "*Candidatus* Loosdrechtia aerotolerans".

Description of *Candidatus* Loosdrechtia aerotolerans **gen. nov., sp. nov.** The genus name *Loosdrechtia* (Latin substantive in nominative singular, gender: feminine), is Latinized from van Loosdrecht, in honor of Mark van Loosdrecht for his contribution to the field of anammox research. The specific epithet *aerotolerans* (compounded from L. *aer-*, air, and L. *tolerans*, tolerating, treated as Latin adjective in feminine nominative singular), referring to the oxygen (air) tolerance of the niche of the species.

The analogous 16S rRNA gene sequence of "*Ca.* Loosdrechtia aerotolerans" could be detected in 831 rRNA amplicon datasets indexed by IMNGS (Ilias et al. 2016) with a 94.5%

identity cutoff (Yarza et al. 2014) and had considerable abundance (> 0.1%) in 379 amplicon datasets. It appeared to be widely distributed in freshwater, sediment, and soil ecosystems as well as in wastewater, activated sludge, and biofilm niches of wastewater treatment systems (Figure S7).

3.2. Central anammox catabolism of "Ca. Loosdrechtia aerotolerans"

The production and oxidation of hydrazine are central for energy conservation in anammox bacteria (Kartal and Keltjens 2016). The biochemically unique multiheme cytochrome *c* protein hydrazine synthase (HZS) produces hydrazine from nitric oxide and ammonium in a slow, two-step reaction mechanism with hydroxylamine as an enzyme-bound intermediate (Dietl et al. 2015, Kartal et al. 2011). HZS and its associated electron transfer module (ETM) are conserved throughout the known anammox genera (Kartal et al. 2011). In line with this observation, HZS and its ETM were encoded by "*Ca*. Loosdrechtia aerotolerans" and were highly conserved (Figure S8a-c, Figure S9). It has been postulated that HZS receives electrons from a tetraheme *c*-type cytochrome, encoded by kuste2854 in *K. stuttgartiensis* as a part of the ETM (Ferousi et al. 2019). The homolog of this protein in "*Ca*. Loosdrechtia aerotolerans" (E3K32_13910) shared a 73% sequence identity with kuste2854, and the four heme-binding motifs, including the naturally occurring contracted CKCH heme-binding motif, were all conserved in E3K32_13910 (Figure S10). The amino acid sequences of the three catalytic subunits of HZS (HzsABC) from "*Ca*. Loosdrechtia aerotolerans" were also fully conserved, and superimposable with those in *K. stuttgartiensis* and shared highly similar catalytic sites in the α and γ subunits, including the heme and Zn^{2+} binding sites as well as the specific Hzsβ loop in the β subunit (Dietl et al. 2015) (Figure S8a-c, Figure S9). In line with previous findings, the approximately two-fold higher coverage of *hzs* gene than the whole genome (Figure S9) suggests that "*Ca*. Loosdrechtia aerotolerans" also harbors two identical copies of *hzs* gene cluster (Frank et al. 2018, Speth et al. 2015, Yang et al. 2018). As isolated, hydrazine synthase is a relatively slow enzyme, if turnover rate of this enzyme is also slow in the cellular environment, encoding multiple copies might facilitate the increased levels of HZS protein in the cells in order to reach appreciable hydrazine production rates (Kartal et al. 2013). All the three subunits of HZS coding genes were highly transcribed and among the most abundant transcripts (Figure 2, Figure S13), illustrating "*Ca*. Loosdrechtia aerotolerans" participated in nitrogen removal with high activity in the full-scale biofilm system.

The oxidation of hydrazine to N_2 results in highly energized, low-potential electrons, which

388 are used to establish a proton motive force for energy conservation and produce reducing
389 equivalents needed for cellular anabolic activity in anammox bacteria. Hydrazine oxidation is
390 carried out by a dedicated octaheme hydroxylamine oxidoreductase-like (HAO-like) protein,
391 hydrazine dehydrogenase (HDH) (Akram et al. 2019, Kartal et al. 2011, Maalcke et al. 2016).
392 The HDH homolog in "*Ca*. Loosdrechtia aerotolerans" (E3K32_09170) was highly conserved,
393 including the catalytic heme and the unusual heme-binding motif (CXXXXCH) (Figure S8d,
394 Figure S11a). HDH is severely inhibited by hydroxylamine which is suggested to leak out of
395 HZS during hydrazine production (Akram et al. 2021, Dietl et al. 2015, Kartal and Keltjens
396 2016, Maalcke et al. 2014). To prevent this inhibition, anammox bacteria encode a dedicated
397 hydroxylamine oxidizing protein (HOX) that recycles hydroxylamine back to NO (Figure 2),
398 which can then be used by HZS (Kartal et al. 2011, Maalcke et al. 2014). In "*Ca*. Loosdrechtia
399 aerotolerans", HOX was also conserved (E3K32_09155) with highly similar heme placements
400 to HOX (kustc1061) of *K. stuttgartiensis* (Figure S8e, Figure S11b). Like HZS, the HDH and
401 HOX coding genes were all highly transcribed and among the most abundant transcripts,
402 indicating the central roles of these enzymes in anammox metabolism (Figure 2, Figure S13).
403 Based on growth experiments with *K. stuttgartiensis* using NO and ammonium, it has been
404 suggested that the core anammox catabolic unit initially consisted of HZS and HDH, and nitrite
405 reduction (NIR) and oxidation (NXR) pathways were acquired later in its evolutionary history
406 (Hu et al. 2019). Indeed, HZS and HDH are conserved throughout the known anammox
407 bacteria, whereas distinct anammox genera utilize different NO-generating nitrite reductases
408 (NIR) (Kartal and Keltjens 2016). *Ca*. Kuenenia and *Ca*. Scalindua species encode cytochrome
409 cd_1 NIR (NirS), whereas *Ca*. Jettenia species encode a copper-containing NIR (NirK) (Hira et
410 al. 2012, Hu et al. 2012) (Figure 3). "*Ca*. Loosdrechtia aerotolerans" contained a *nirK*, but it
411 was very lowly transcribed (Figure S13). The low transcript of *nir* gene in anammox bacteria
412 has widely been reported (Bagchi et al. 2016, Kartal et al. 2011, Smeulders et al. 2020, Yang
413 et al. 2020c). In striking contrast, NirS is one of the most abundant proteins in "*Ca*. Scalindua
414 profunda" (van de Vossenberg et al. 2013). Although not biochemically characterized, based
415 on physiological experiments as well as transcriptomic and proteomic analyses, the HAO-like
416 protein cluster kustc0458/kustc0457 was implicated in nitrite reduction to NO in *K.*
417 *stuttgartiensis* (Hu et al. 2019). Interestingly, "*Ca*. Loosdrechtia aerotolerans" also encoded
418 and highly transcribed a kustc0458/kustc0457 homolog (Figure S8f, Figure S14), which could
419 fulfill the function of nitrite reduction to NO in this microorganism.
420 The electrons required for nitrite reduction to NO are most likely derived from nitrite

oxidation to nitrate catalyzed by NXR (Hu et al. 2019). The highly complex NXR gene cluster identified in *K. stuttgartiensis* (Kartal et al. 2013) was completely conserved in "*Ca*. Loosdrechtia aerotolerans" in a single gene cluster (E3K32_04900 to E3K32_04970), including the E3K32_04960, E3K32_04945, and E3K32_04940 encoding NxrA, NxrB, and NxrC, respectively. The transcriptions of these genes were detected, albeit at lower amounts than HZS, HDH, and HOX coding genes (Figure 2, Table S8).

3.3. Dissimilatory and assimilatory nitrate reduction

DNRA has been observed in the enrichment cultures of anammox bacteria that could supply anammox bacteria with both nitrite and ammonium (Kartal et al. 2007a). In anammox bacteria, the first step of DNRA, nitrate reduction to nitrite, was proposed to be catalyzed by NXR operating in the reverse as nitrate reductase has not been observed in these microorganisms (Chicano et al. 2021, Kartal et al. 2007a). The close affiliation of the "*Ca*. Loosdrechtia aerotolerans" NxrA with homologs from the other known terrestrial anammox bacteria was confirmed by phylogenetic analysis (Figure S15), indicating it could be used for nitrate reduction. Pentaheme cytochrome *c* nitrite reductase (NrfA), which catalyzes the six-electron reduction of nitrite into ammonium has been observed in *Ca*. Brocadia, *Ca*. Jettenia, and one *Ca*. Scalindua species (Figure 3). The presence and transcription of NrfA coding genes (Figure 2) are consistent with the possible availability of DNRA can be coupled to the anaerobic oxidation of small organic acids in wastewater treatment systems (Kartal et al. 2007b, Kartal et al. 2008, Lawson et al. 2020, Tao et al. 2019).

Interestingly, in contrast to other anammox microorganisms, a soluble, ferredoxin-dependent nitrate reductase (NarB) and its associated ferredoxin related to electron donation (Jepson et al. 2004) were encoded by "*Ca*. Loosdrechtia aerotolerans" (Figure 4a, Figure S12) and had the similar sequencing depth with other genes in the same scaffold (Figure S16a). The nitrate reductase is classified within assimilatory nitrate reductases, had the highest amino acid identity (62.37%) to NarB from the Planctomycete *Aquisphaera giovannonii* (WP_148594665.1) (Figure 4a). NarB is described as a soluble and monomeric enzyme, which contains a [4Fe-4S] cluster and a Mo bismolybdopterin guanine dinucleotide cofactor (Moco) as its prosthetic groups (Rubio et al. 2002). The conserved catalytic sites of the well-studied NarB (CAA52675.1) from *Synechococcus elongatus* (Srivastava et al. 2015, Srivastava et al. 2013), including Lys58, Arg70, Cys148, Asp163, and Arg351, were conserved in the "*Ca*. Loosdrechtia aerotolerans" NarB (Figure 4b, Figure S16b). The finding of NarB in "*Ca*.

Loosdrechtia aerotolerans" was unexpected because it has not yet been found in any other anammox bacteria (Figure 3). According to the close lineage between NarB from "*Ca*. Loosdrechtia aerotolerans" and other Planctomycete strains (Figure 4a), we propose that the ancestor of anammox bacteria should encode NarB, but it was lost by most of the known anammox bacteria in the long-term evolution might be because of the dramatic changes in the habitat. Nevertheless, the possibility that "*Ca*. Loosdrechtia aerotolerans" acquired the *narB* through horizontal gene transfer from another Planctomycete organism cannot be excluded.

Ferredoxin-dependent NarB has been characterized as a crucial enzyme for nitrate assimilatory purposes in diverse microorganisms by catalyzing the two-electron reduction of nitrate to nitrite (Feng et al. 2014, Rubio et al. 1996, Wang et al. 2011). Although the *in situ* transcription of the genes encoding NarB and its associated ferredoxin was not detected (Figure 2), "*Ca*. Loosdrechtia aerotolerans" could potentially use this as an additional pathway for nitrate reduction under nitrate replete conditions. As using ferredoxin to produce auxiliary nitrite would be a huge energy waste that nitrate reduction catalyzed by NarB may probably be restricted to ammonium limited conditions for assimilation purposes. But gene coding for known assimilatory nitrite reductase was not found in the recovered part of "*Ca*. Loosdrechtia aerotolerans". Possessing multiple routes for small organic acid utilization coupled with nitrate reduction to ammonium and the ability to use different nitrogen sources could be associated with the niche adaptability of "*Ca*. Loosdrechtia aerotolerans".

3.4. Utilization of organic substrates

In anammox bacteria, nitrate reduction to ammonium via nitrite can be coupled to the anaerobic oxidation of small organic acids (Kartal et al. 2007b, Kartal et al. 2008, Lawson et al. 2020, Tao et al. 2019). In agreement with previous findings that at least three different acetyl-CoA synthetases (ACS) were found in anammox bacterial genomes (Kartal et al. 2007b, Kartal et al. 2008), four ACSs were encoded by "*Ca*. Loosdrechtia aerotolerans" and transcription of three of them were detected (Figure 2, Table S8). ACS, which catalyzes the ligation of acetate with CoA for acetyl-CoA production has been implicated in acetate oxidation by anammox bacteria (Russ et al. 2012), but the exact propionate oxidation pathway is still unclear. Nevertheless, substrate specificity studies in different organisms demonstrated that propionate could also be used by ACS with propionate-CoA production (Hele 1954, Li et al. 2011, Sealy-Lewis 1994). Additionally, the propionate activation capability of an ACS-like protein (kustc1128) found in *K. stuttgartiensis* was demonstrated in a heterologous host (Russ

et al. 2012). The produced propionate-CoA could be further transformed to succinyl-CoA through an α-carboxylation reaction and then to acetyl-CoA through the TCA cycle (Figure 2), which is the pathway for propionate conversion in a syntrophic propionate-oxidizing bacterium (Plugge et al. 1993). All three genes encoding the α-carboxylation pathway were encoded and two of them were transcribed by "*Ca*. Loosdrechtia aerotolerans" (Figure 2, Table S8). The transcribed genes related to acetate and propionate oxidation, suggesting that small organic acid oxidation was active in "*Ca*. Loosdrechtia aerotolerans". Therefore, we propose that "*Ca*. Loosdrechtia aerotolerans" could use the available small organics in wastewater as electron donors for certain redox reactions, such as nitrate/nitrite assimilatory or oxygen elimination (see below). This capability would give "*Ca.* Loosdrechtia aerolerans" a competitive advantage over other microorganisms in the dynamic wastewater treatment environments, where substrate concentrations might fluctuate. All known anammox bacteria have the genetic potential for acetate conversion, but the homologs of the complete α-carboxylation pathway for propionate transformation were identified only in "*Ca*. Loosdrechtia aerotolerans" and three *Ca*. Brocadia genomes (Figure 3). The capabilities and efficiencies of co-oxidation of ammonium and acetate/propionate likely determine the ecological niches of distinct anammox bacteria.

"*Ca*. Loosdrechtia aerotolerans" also encoded the genetic potential to replenish the intracellular carbon cycle using other extracellular organic compounds, such as proteins, peptides, amino acids, long-chain fatty acids, maltodextrin, and C4-dicarboxylate, and to store carbon and energy in the form of glycogen (see the supplemental material: Utilization of other organic substrates and Storage compound).

3.5. Diverse oxygen resistance strategies of anammox bacteria

Next to the utilization of alternative energy conservation pathways, resistance and adaptability of anammox bacteria to changing environmental conditions plays an important role in the niche differentiation of these microorganisms (Kartal et al. 2013). The reports from distinct anammox enrichment cultures suggest that anammox bacteria are most likely oxygen tolerant rather than strictly anaerobic (Oshiki et al. 2016b). Nevertheless, the underlying mechanism of oxygen tolerance and the activity of a protein used against oxygen toxicity has not been shown in anammox bacteria before.

Like in many other microorganisms, superoxide dismutase and catalase related to oxidative stress resistance were encoded and expressed by "*Ca*. Loosdrechtia aerotolerans" (Figure 2) and thus are well prepared for defense against reactive oxygen species (ROS).

Interestingly, the catalase coding gene was absent in some *Ca.* Brocadia and *Ca*. Jettenia strains suggesting that these microorganisms employ different oxygence defense systems (Figure 3). An additional bifunctional catalase-peroxidase was uniquely encoded by *Ca*. Scalindua strains (Figure 3), with both catalase (catalyzes high concentration of H_2O_2) and peroxidase (catalyzes low concentration of H_2O_2) activities (Hillar et al. 2000), may confer a selective advantage to *Ca*. Scalindua when facing frequent oxygen intrusions in the oxygen minimum zones of the ocean (Ulloa et al. 2012). The absence of oxygen tolerance systems in "*Ca*. Scalindua rubra" BSI-1 obtained from the anoxic deep sea (Speth et al. 2017) would make sense (Figure 3), which are not necessary for the niche adaptability of anammox bacteria to the anoxic environment and would be additional energy consumption burdens in cell replication and growth under the oligotrophic environment.

Unexpectedly, "*Ca*. Loosdrechtia aerotolerans" encoded a CotA-like multicopper oxidase (MCO) with three specific copper-binding sites (Figure 5a, b, Figure S17). A signal peptide identified at the N-terminal of its sequence indicates that the protein will not reside in the cytoplasm. Whether it is transported to the periplasm or the anammoxosome compartment remains to be determined. The CotA-like protein had a high amino acid sequence identity with the oxygen-utilizing laccases and bilirubin oxidases (BOD), and it clustered into the BOD group (Figure 5c, d). A detailed analysis revealed that putative BOD-like proteins were also found in four *Ca*. Brocadia strains, albeit with low sequence identities (< 40%) with the "*Ca*. Loosdrechtia aerotolerans" BOD (Figure 5a, d, Figure S17). Consequently, BODs from "*Ca*. Loosdrechtia aerotolerans" (E3K32_04160), "*Ca*. Brocadia caroliniensis" (OOP56653.1), and "*Ca*. Brocadia sp. AMX2" (RIK02322.1) were selected for heterologous expression. The BODs expressed in *E. coli* were further purified and displayed one major band in a denaturing SDS polyacrylamide gel (Figure 5e). The activities of the purified BODs were determined towards bilirubin oxidation (Figure 5f). In line with the suggested role of BODs in oxygen resistance, higher oxygen tolerances were previously observed in the two BOD-positive "*Ca*. Brocadia caroliniensis" and "*Ca*. Brocadia fulgida" compared to the two BOD-negative "*Ca*. Brocadia sinica" and "*Ca*. Brocadia anammoxidans" (Oshiki et al. 2016b). Although the *in situ* transcription of gene encoding BOD was not detected in "*Ca*. Loosdrechtia aerotolerans" (Figure 2), the oxygen-converting BOD could be used as a protective protein involved in low potential electron carriers used by the anammox bacteria for example in the anammoxosome.

A survey of oxygen-active proteins revealed the presence of a varying number of genes that could be involved in oxygen defense in anammox bacteria. *Ca*. Kuenenia and *Ca*. Scalindua

contain a terminal *cbb3*-type cytochrome *c* oxidase (Figure 3), which is normally involved in aerobic respiration. In line with this finding, the transcriptions of terminal oxidases were detected previously in an anaerobic anammox bioreactor likely associated with protection against oxygen (Lawson et al. 2017, Richardson 2000). The *cbb3*-type cytochrome *c* oxidase has a high affinity for oxygen (Hamada et al. 2014, Pitcher and Watmough 2004), which might function as an oxygen detoxifying enzyme to protect anammox bacteria against oxygen intrusion in dynamic natural (e.g. oxygen minimum zones in the ocean) and engineered environments such as wastewater treatment plants.

4. Conclusions and Implications

In the present study, we described the discovery of a new anammox bacterium in the full-scale biofilm landfill leachate treating bioreactor. The nearly complete genome of the novel anammox bacterium, tentatively named "*Ca*. Loosdrechtia aerotolerans", was constructed by metagenomic analysis. All the genes related to anammox core metabolism are conserved in "*Ca*. Loosdrechtia aerotolerans". As expected, core genes essential for anaerobic ammonia oxidization were among the most highly transcribed genes, reflecting a high nitrogen removal potential of "*Ca*. Loosdrechtia aerotolerans" in the full-scale biofilm system. The low transcribed *nir* gene indicates "*Ca*. Loosdrechtia aerotolerans" might heavily rely on the HAO-like protein to fulfill the function of nitrite reduction to NO as previously obsevred in *K. stuttgartiensis* (Ferousi et al. 2019). Furthermore, its metabolic versatility related to its survival in unfavorable and fluctuating environmental parameters was investigated. Earlier observations and our findings show that anammox bacteria have a versatile lifestyle, are able to utilize different substrates to survive in distinct dynamic environments. Furthermore, these microorganisms are equipped with diverse strategies to protect themselves against changing environmental conditions, such as oxygen fluctuations, in both manmade and natural ecosystems where they remove ammonium from wastewater or contribute to the input of dinitrogen gas into our atmosphere. Overall, the examined full-scale anammox biofilm system provides an interesting case study of anammox-based nitrogen removal. The discovery of this new genus with high transcriptional activity and metabolic versatility advances our understanding of the phylogenetic diversity, ecophysiology, and niche adaptability of anammox bacteria in full-scale wastewater treatment systems. Future research efforts might aim to exploit the enrichment strategy and unique physiological versatility of novel anammox organisms to

optimize anammox-based nitrogen removal from sewage using currently existing and new bioreactor designs.

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Competing interests

All other authors have no competing interests.

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