INTRODUCTION

In a previous study, we successfully reconstructed the whole-genome sequences of three currently non-isolatable major community members in an anaerobic ammonium oxidation (anammox) bioreactor, Candidatus (Ca.) Brocadia pituitae, Ca. Nitrosymbiomonas proteolyticus, and Ca. Desulfobacillus denitrificans (Okubo et al., 2021). Through comparative genomics of anammox bacteria, it was found that Ca. Brocadia pituitae lacked any genes encoding a canonical nitrite reductase (i.e., nirK and nirS), but possessed candidate genes (hao2 and hao3) encoding NO-forming or NH2OH-forming nitrite reductases. Also, analysis of the partial genome sequence of Ca. D. denitrificans revealed that this microbe is likely to be a nitric oxide (NO)-forming incomplete denitrifier because NO reductase genes (norBC) are missing, although a nirS gene was detected in the genome. Thus, it was hypothesized that Ca. B. pituitae uses not only self-produced NO and/or NH2OH, but also NO supplied by incomplete denitrifiers such as Ca. D. denitrificans. Ca. D. denitrificans has

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frequently been detected in other anammox bioreactors (Bae et al., 2010; Lawson et al., 2017) and its genome showed 99.0%-average nucleotide identity (ANI) to the draft genome of *Rhodocyclaceae* bacterium UTPRO2 from the metagenome of an anammox bioreactor (Lawson et al., 2017).

Recently, it has been reported that denitrification synergized with anammox could accelerate the anaerobic degradation of benzene, and *Rhodocyclaceae* bacteria might play a role in benzene degradation (Peng et al., 2017). Although one possible contribution of anammox bacteria could be to remove the nitrite that accumulates as a result of denitrification, benzene and metabolic intermediates such as toluene, phenol and benzoate were found to inhibit anammox activity (Peng et al., 2018). In addition to toxic aromatic compounds, anammox activity is also inhibited by non-toxic organic matter, salts, heavy metals, phosphate, and sulfide, which are commonly present in practical applications such as wastewater treatment (Jin et al., 2012). On the other hand, because 16S rRNA genes of *Denitrolinea* (Okubo et al., 2021) since this aerobic species was inevitably suggested to be an anammox bacterial community (ABC) member responsible for nitrite oxidation via consumption of O$_2$ in the anammox bioreactor (Okubo et al., 2021). Since *Ca. N*. proteolyticus possesses multiple secretory lytic enzymes and type II secretion systems, it was suggested that proteolysis of biomass from autolyzed cells and also the lysis of active cells sensitive to lytic enzymes may provide nutrients for itself as well as other heterotrophic members of the ABC (Okubo et al., 2021). Therefore, these predominant bacteria may be important cooperators that help to maintain a balanced population of ABC members and stable anammox activity in the bioreactor. Indeed, cooperative relationships were suggested in cross-feedings of nutrients such as amino acids, carbohydrates, and vitamins, and also in cell aggregation by supplying exopolysaccharides (Lawson et al., 2017; Zhao et al., 2019). However, the lifestyles of major cooperators in the ABC are still unclear, as complete genome sequences of non-isolatable cooperators have not yet been obtained although NO production by incomplete denitrification is not unusual (Schuster & Conrad, 1992).

In this study, we performed a detailed genomic analysis of *Ca. D*. denitrificans and examined the expression profile of its genes to determine why *Ca. D*. denitrificans is selected as a predominant species in the ABC and how it interacts with anammox bacteria, that is, *Ca. B*. pituitae.

## 2 MATERIALS AND METHODS

### 2.1 Genomic sequence

The ABC comprised members of four major species, anammox bacteria, *Ca. B*. pituitae (35%), nitrite-oxidizing bacteria, *Ca. N*. proteolyticus (10%) and imperfect denitrifiers, *Ca. D*. denitrificans (7%), and *Ca. Denitrolinea* symbiosum (3.4%) (Okubo et al., 2021). Among them, the genome sequence of *Ca. D*. denitrificans was used for detailed analysis in this study. To determine the phylogenetic position of *Ca. D*. denitrificans, a total of forty complete or draft genome sequences of bacteria, classified mainly into the orders *Rhodocyclales*, *Burkholderiales*, and *Nitrosomonadales*, were obtained from the DDBJ/EMBL/GenBank database.

### 2.2 Evaluation of the metabolic and physiological potential

The pattern of the metabolic and physiological potential of *Ca. D*. denitrificans was investigated using Genomaple™ (formerly MAPLE) ver. 3.2 (Arai et al., 2018; Takami et al., 2016). Genomaple™ is available through a web interface (https://maple.jamstec.go.jp/maple/maple-2.3.1/) and as a stand-alone package from Docker Hub (https://hub.docker.com/r/genomaple/genomaple). Genes were mapped to 795 functional modules defined by the KEGG (pathways, 305; complexes, 294; functional sets, 157; and signatures, 40), and the module completion ratio (MCR) was calculated according to a previously described Boolean algebra–like equation (Takami et al., 2012). To evaluate the MCR, Q-values suggesting the working probability of the modules were also calculated by Genomaple™. Q-values near zero indicate a high working probability of the module (Takami et al., 2016).

### 2.3 Analysis of RNA-seq data

To identify the actively working metabolic pathways in the anammox reactor, metatranscriptomic reads obtained in a previous study (Okubo et al., 2021) were mapped to the genome sequences with a cutoff identity of 95% using the Magic-BLAST program (Boratyn et al., 2019). The numbers of mapped reads were counted by SAMtools (Li et al., 2009) and HTSeq (Anders et al., 2015). The RPKM (reads per kilobase of exon per million mapped sequence reads) ratio, calculated by dividing the RPKM of each gene by the mean RPKM of all ribosomal proteins, was used to determine relative gene expression levels. Physiological and biochemical features of hydrogenases were estimated with the HydDB program (Søndergaard et al., 2016).

### 2.4 Phylogenetic analysis

Amino acid sequences of 45 ribosomal proteins, commonly detected by Genomaple™ ver. 2.3.0 (Arai et al., 2018; Takami et al., 2016) except for RpsN (K02954), RpmJ (K02919), RpsD (K2986), RpmH (K01914), RplI (K02939), RpsP (K02959), and RplS (K02884), were concatenated and aligned by the MUSCLE program (Edgar, 2004). A phylogenetic tree based on concatenated aligned sequences was constructed to determine the phylogenetic position of *Ca. D*. denitrificans by the maximum likelihood (ML) method with the LG+G+I+F model in the MEGA 10.1.8 package (Kuma et al., 2018).
Vulcanibacterium thermophilum KCTC 32020 (GCA_007923255.1) was used as an outgroup. A phylogenetic tree based on hydroxylamine oxidoreductase (Hao)-like proteins was constructed by the ML method with the WAG + G + I model with 500 bootstrap replicates. The oxidative and reductive types of dissimilatory sulfite reductase (dsrAB) were classified by the neighbor-joining method in the same package. The concatenated DsrAB amino acid sequence dataset (Müller et al., 2015) was used as a reference to infer the position of DsrAB from Ca. D. denitrificans.

3 | RESULTS AND DISCUSSION

3.1 | Denitrification pathway

Ca. D. denitrificans possesses all genes necessary for denitrification except for NO reductase (i.e., norBC) as shown in Figure 1. Also, narXL (DSYM_27930-27940), which are presumably involved in nitrate/nitrite dependent transcriptional activation of genes encoding nitrate oxidoreductase (i.e., narGHJI, DSYM_27970-8000) (Härtig et al., 1999) and the formate/nitrite transporter gene (DSYM_27950) were located upstream of narGHJI (Table S1: https://doi.org/10.5281/zenodo.5089211). Among these genes for nitrogen metabolism, expression of the genes encoding nitrite reductase (nirS) and nitrous oxide reductase (nosZ) was observed under the regular operating conditions of the anammox bioreactor (Figure 1 and Table S1: https://doi.org/10.5281/zenodo.5089211). The expression level of nosZ was much higher than that of nirS. Accordingly, it is thought that nitrous oxide (N2O) is used as a major electron acceptor in Ca. D. denitrificans; however, unexpectedly Ca. D. denitrificans lacks norBC, which encodes the enzyme responsible for the reduction of NO to N2O. Thus, we explored the possibility that N2O is produced by alternative enzymes. It is known that nitric oxide reductase is structurally similar to cytochrome oxidase (Zumft, 1997) and indeed, the cytochrome cbb3-type oxidase of Pseudomonas stutzeri is known to have nitric oxide reductase activity (Forte et al., 2001). Although Ca. D. denitrificans possesses two genes encoding a cytochrome cbb3-type oxidase (Table S2: https://doi.org/10.5281/zenodo.5089211), expression of these genes was not detected. On the other hand, NO-detoxifying enzymes can also reduce NO to N2O (Gardner et al., 2003), but Ca. D. denitrificans has no genes encoding this enzyme (norVW). These results suggest that Ca. D. denitrificans does not utilize self-produced N2O, but presumably it can use N2O supplied by other community members because norBC genes from minor community members were detected in the anammox bioreactor (Okubo et al., 2021) although alternative ways of producing N2O have been reported in other heterotrophic nitrifying bacteria (Zhang et al., 2015), aerobic ammonium oxidizing bacteria (AOB) (Caranto et al., 2016). On the other hand, since the nosZ gene is highly expressed, Ca. D. denitrificans is thought to reduce N2O emissions from the anammox bioreactor. It has been reported that only 0.0037% of the total nitrogen load in the anammox reactor was emitted as N2O even though N2O was detected within anammox granules (Rathnayake et al., 2018). N2O has a greenhouse effect more than 300 times that of carbon dioxide on a 100-y timescale and it also depletes the ozone layer (Solomon et al., 2007).

The haoA (DSYM_27790) gene product (i.e., hydroxylamine oxidoreductase) from Ca. D. denitrificans clustered with those of aerobic AOB in the genera Nitrosospira and Nitrosomonas (Figure A1). The haoB (DSYM_27800) gene, encoding another whose functional role remains to be elucidated, is adjacent to haoA and the genes encoding cytochrome c554 (DSYM_27810) and cytochrome c (DSYM_27820), proteins that would relay the electrons to the quinone pool. Among these neighboring genes, only haoA and haoB were expressed (Figure 1). Although the Hao protein catalyzes the oxidation of hydroxylamine to NO (Caranto & Lancaster, 2017) in aerobic AOB, it has also been reported to catalyze the reduction of NO to hydroxylamine and ammonium, which are substrates for anammox bacteria (Kostera et al., 2010). Accordingly, Ca. D. denitrificans is thought to provide not only NO, but also hydroxylamine and ammonium to anammox bacteria. However, because the reaction pathway catalyzed by the Hao protein is still unclear, further study on the role of this protein in Ca. D. denitrificans is required to fully understand the nitrogen flow in the anammox community.

FIGURE 1  Nitrogen metabolism pathway. Values in parentheses represent RPKM ratios. The dotted arrow shows the missing process. By-products though imperfect denitrification such as NO, NH2OH, and NH4+ are thought to be supplied to anammox bacteria.
3.2 Aromatic compound metabolism

A wide range of organic and inorganic compounds can act as electron donors for denitrifying bacteria (Capua et al., 2019), although aromatic compounds are known to inhibit anammox activity and decrease the population of anammox bacteria (Peng et al., 2018; Pereira et al., 2014). Here, we predicted the prefered electron donors for the incomplete denitrifier, Ca. D. denitrificans under the regular operating conditions of the anammox bioreactor (Okubo et al., 2021) based on genomic and transcriptomic data. Ca. D. denitrificans contains a complete module for the ATP-dependent benzoyl-CoA degradation pathway (M00541), which converts benzoyl-CoA to 3-hydroxypimeloyl-CoA (Figure 2 and Table 1). Benzoyl-CoA is a key in the anaerobic degradation of many aromatic compounds (Fuchs et al., 2011). Expression of the genes encoding the conversion of 3-hydroxypimeloyl-CoA to acetyl-CoA (step 5–10 in Figure 2) was also observed except for step 6. The acetyl-CoA generated through this pathway would be used in the TCA cycle and glyoxylate cycle for not only ATP and NADH production but also carbon assimilation (Table 1). Transcripts for the gene encoding step 6 may have been undetected because the number of transcript reads that mapped to genes associated with this module was small overall. Ca. D. denitrificans also possessed all of the genes necessary for the metabolism of benzene, benzoate, phenylphosphate, 4-hydroxybenzoate, and protocatechuato into benzoyl-CoA (Figure 2). Ca. D. denitrificans is thought to be a member of a new family within the order Rhodocyclales. The genes encoding formate dehydrogenase-N (see Phylogenetic position section), but other bacteria within this order have also been detected in benzene-degrading nitrate-reducing microbial consortia (Atashgah et al., 2018; Zaan et al., 2012). Ca. D. denitrificans also possessed genes encoding benzoate-CoA ligase (badA) and benzoyl-CoA 2,3-epoxidase (boxABC), which catalyze the degradation of benzene to 3,4-dehydrodipicolyl-CoA semialdehyde and formate under aerobic conditions (Figure 3). However, the genes encoding NADP+-specific aldehyde dehydrogenase and β-ketoacyl-CoA thiolase (boxDE), which are required to convert 3,4-dehydrodipicolyl-CoA semialdehyde to succinyl-CoA and acetyl-CoA (Gescher et al., 2006; Fuchs et al., 2011) were not detected in the Ca. D. denitrificans genome. Given these results, Ca. D. denitrificans presumably degrades benzoate partially under aerobic conditions to produce formate, which could be utilized for further metabolism such as formate oxidation. Accordingly, Ca. D. denitrificans seems to use aromatic compounds derived from secondary metabolites and cell lysates of other ABC members. Indeed, aromatic compounds such as naringenin and flavanone, which are synthesized from L-phenylalanine, were detected in the centrifugal supernatant of wet biomass scraped from the non-woven fabric in the anammox bioreactor by metabolome analysis using LC-TOFMS (Figure A2). However, since the genes responsible for each metabolic step have not been identified although the synthetic pathways of both compounds are known, the producers of such aromatic compounds could not be specified. In general, because the metabolites are usually utilized immediately in the active cell growth phase, these detected aromatic compounds may be the final metabolites that are no longer metabolized or slowly metabolized. Unlike these aromatic compounds, benzoyl-CoA which is a key intermediate in the anaerobic degradation of many aromatic compounds was not detected and in fact, expression of the genes responsible for benzoyl-CoA metabolism was observed (Figures 2 and 3). Although it is difficult to specify the origin of aromatic compounds, it is thought that such compounds are presumably supplied to Ca. D. denitrificans through the lysis of active ABC members sensitive to the lytic enzymes in addition to autolyzed old cells because Ca. N. proteolyticus possesses various lytic enzymes (Okubo et al., 2021).

As mentioned above, because aromatic compounds are known to inhibit anammox activity and decrease the population of anammox bacteria (Peng et al., 2018; Pereira et al., 2014), the degradation of aromatic compounds by Ca. D. denitrificans is expected to help maintain the activity and population of anammox bacteria in the reactor. Interestingly anammox-enhanced benzene degradation by denitrifiers has been reported, although the enhancement mechanism is not fully understood (Peng et al., 2017). This interrelationship through aromatic compounds seems to be one of the reasons why Ca. D. denitrificans and Ca. B. pituitae are linked in the anammox bioreactor and may be one reason that makes isolating ABC members difficult. Based on genomic analyses, it is expected that Ca. D. denitrificans can degrade aromatic compounds under both aerobic and anaerobic conditions. Since dissolved oxygen in the synthetic medium is consumed by nitrite oxidizer, Ca. N. proteolyticus, grown on the surface of biomass, it is thought that anoxic-anoxic interface occurs between the surface and inside of the biomass with a thickness of several millimeters formed on the non-woven fabric often used as a carrier of up-flow anammox bioreactor. This phenomenon was also observed in another type of anammox reactor (Nielsen et al., 2005). Therefore, the presence of both anaerobic and aerobic pathways for aromatic compound degradation would facilitate the colonization of Ca. D. denitrificans in the ABC.

Ca. D. denitrificans has two formate dehydrogenases, classified as formate dehydrogenase-N and NAD-dependent formate dehydrogenase. The genes encoding formate dehydrogenase-N were organized to encode the α- (DSYM_14690), β- (DSYM_14700), and γ- subunits were expressed in Ca. D. denitrificans. On the other hand, the NAD-dependent formate dehydrogenase-N couples formate oxidation to nitrate reduction (Maia et al., 2015), but the expression of the narGHJI genes was not detected in our analyses (Figure 1). Thus, formate oxidation by formate dehydrogenase-N is not necessarily coupled to the reduction of nitrate in Ca. D. denitrificans. On the other hand, the NAD-dependent formate dehydrogenase is considered to couple formate oxidation to...
FIGURE 2  Anaerobic pathway for the degradation of aromatic compounds. The gene encoding the phenylphosphate synthase alpha subunit (PpsA) was missing. M00541: module for Benzoyl-CoA degradation (benzoyl-CoA → 3-hydroxypimeloyl-CoA). The dotted line shows the reaction by the incomplete phenylphosphate synthase (pps) lacking subunit A.
the reduction of NAD+, providing reducing equivalents in the form of NADH (Maia et al., 2015). The genes encoding this enzyme were organized in the order γ-, β-, α- and δ-subunits (DSYM_25570-600), but the gene encoding the accessory protein (fdsC) was missing. In addition, only the α-subunit gene was expressed with an RPKM ratio of 0.1.

3.3 | Glucose metabolism

Ca. D. denitrificans has 9 of the 10 enzymes of glycolysis (Embden-Meyerhof pathway), glucose ⇄ pyruvate (M00001; MCR: 90.0%; Table S3: https://doi.org/10.5281/zenodo.5089211), but lacks ATP-dependent phosphofructokinase (ATP-PFK) (Figure 4). ATP-PFK is a regulatory enzyme in glycolysis that catalyzes the irreversible phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate using ATP. Ca. D. denitrificans possessed a diphosphate-dependent phosphofructokinase, which catalyzes the reversible phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate (PPI-PFK, DSYM_10110) (Alves et al., 2001; Mertens, 1991). Considering these results, Ca. D. denitrificans would have the metabolic potential to route glucose through the Embden-Meyerhof pathway utilizing PPI-PFK instead of ATP-PFK. The Entner-Doudoroff pathway (M00008) is not functional because Ca. D. denitrificans lacks the enzymes phosphogluconate dehydratase and 2-dehydro-3-deoxyphosphogluconate aldolase, which catalyze the last two steps of this 4-step pathway (i.e., MCR: 50%; Table 1 and Figure 4). On the other hand, since Ca. D. denitrificans possessed all enzymes of the pentose phosphate pathway, it can presumably metabolize glucose through this pathway as well as the PPI-PFK dependent Embden-Meyerhof pathway. However, since MCRs of these glycolytic pathway modules based on the transcriptome data were low (30.0% and 42.9%, respectively; Table 1), Ca. D. denitrificans is not expected to use glucose as a major carbon and energy source in the anammox bioreactor under regular operating conditions.

3.4 | Inorganic compound metabolism

Because inorganic compounds can act as electron donors for denitrification (Capua et al., 2017), we search for genes that would suggest potential inorganic electron donors that could drive denitrification. Ca. D. denitrificans possesses two sox gene clusters, soxXAYZ (DSYM_07930-60) and soxYZAXB (DSYM_23290-330) (Table 2), but soxCD was not found. The reaction catalyzed by the Sox system in the absence of SoxCD proteins is considered to convert thiosulfate to elemental sulfur (S0) or polysulfide, which produces 2 mol of electrons per mol of thiosulfate (Friedrich et al., 2005). Ca. D. denitrificans also possesses cysDN (DSYM_2680-90) and sat (DSYM_23250), fccAB (DSYM_09470-80), dsrAB (DSYM_11230-40) and aprAB (DSYM_23230-40), which are also involved in sulfur metabolism (Table 2). The dissimilatory sulfate reductase (DsrAB) catalyzes the reduction of sulfate to sulfide but DsrAB from sulfur-oxidizing bacteria catalyzes an oxidative reaction. Since the oxidative and reductive type

| TABLE 1 | Module completion ratios of carbon metabolism pathways |
|---|---|---|
| Carbon metabolism | Module ID | MCR (%) |
| Carbohydrate metabolism | | |
| Glycolysis (Embden-Meyerhof pathway), glucose ⇄ pyruvate | M00001 | 90.0 |
| Entner-Doudoroff pathway, glucose–6P ⇄ glyceraldehyde-3P + pyruvate | M00008 | 50.0 |
| Pentose phosphate pathway | M00004 | 100.0 |
| Citrate cycle (TCA cycle) | M00009 | 87.5 |
| Glyoxylate cycle | M00012 | 100.0 |
| PRPP biosynthesis | M00005 | 100.0 |
| Aromatics degradation | | |
| Benzoyl-CoA degradation, benzoyl-CoA ⇄ 3-hydroxyphenyl-CoA | M00541 | 100.0 |
| CO₂ fixation | | |
| Reductive pentose phosphate cycle (Calvin cycle) | M00165 | 90.9 |
| Reductive citrate cycle | M00173 | 90.0 |
| Reductive acetyl-CoA pathway | M00377 | 28.6 |
| 3-Hydroxypropionate bi-cycle | M00376 | 30.8 |
| Dicarboxylate-hydroxybutyrate cycle | M00374 | 46.2 |
| Hydroxypropionate-hydroxybutyrate cycle | M00375 | 14.3 |
| Abbreviations: MCR, module completion ratio; TRP, transcriptome. |
enzymes are phylogenetically distinct (Müller et al., 2015), a phylogenetic analysis was performed to determine its type. Because the dsrAB genes from Ca. D. denitrificans clustered with oxidative type enzymes (Figure A3), Ca. D. denitrificans is predicted to oxidize sulfide into sulfate via elemental sulfur (SO), sulfite, and adenosine 5'-phosphosulfate (Kappler & Dahl, 2001; Russ et al., 2014). The oxidation of sulfur compounds can be coupled with denitrification (Chung et al., 2014; Russ et al., 2014) and these reactions can also be catalyzed by enzymes encoded by cysDN and sat, fccAB, and aprAB, respectively (Table 2). Thus, Ca. D. denitrificans may also contribute to the removal of sulfide, which is known to inhibit anammox activity (Russ et al., 2014).

![Figure 3](Image)

**Figure 3** Aerobic pathway for benzoate degradation. Values in parentheses represent RPKM ratios.

Ca. D. denitrificans possessed four hydrogenase genes (DSYM_11670, DSYM_11790, DSYM_19030 and DSYM_28330). DSYM_11670 and DSYM_28330 were classified into [NiFe]-hydrogenase Groups 1c and 1e, respectively (Søndergaard et al., 2016). Enzymes of these groups are considered to be oxygen-sensitive and support anaerobic hydrogenotrophic respiration linked to the reduction of various electron acceptors. However, only DSYM_11670 was expressed with an RPKM ratio of 0.2. The remaining two genes, DSYM_11790 and DSYM_19030, were classified [NiFe]-hydrogenase Groups 3d and 2b, both of which are O2-tolerant; expression of these genes, however, was not observed.

### 3.5 | CO2 fixation

Genome analyses revealed that Ca. D. denitrificans has the potential to utilize various inorganic electron donors such as reduced sulfur compounds and hydrogen in addition to organics to carry electrons to the electron acceptor nitrate. Autotrophic denitrifiers fix inorganic carbon (CO2 and HCO3−), but Ca. D. denitrificans does not have complete modules for any of the six known carbon fixation pathways (Table 1). Ca. D. denitrificans lacks ATP citrate lyase and citryl-CoA synthetase, which are key enzymes for the reductive citrate cycle, although the MCR of this cycle is high (90%; Table S2: https://doi.org/10.5281/zenodo.5089211). Also, because Ca. D. denitrificans lacks the gene encoding sedoheptulose-bisphosphatase, which catalyzes the 9th reaction step in the 11-step reductive pentose phosphate cycle (Calvin cycle), the completion ratio of this module was 90.9% (Table 1). This missing enzyme is considered to be unique to the Calvin cycle (Atomi, 2002). On the other hand, it was recently shown that transaldolase (EC 2.2.1.2) can substitute for sedoheptulose-bisphosphatase and sedoheptulose-1,7-bisphosphate aldolase. Ca. D. denitrificans possesses a transaldolase (DSYM_09660) gene, although expression of the gene was not observed (Frolov et al., 2019). Therefore, Ca. D. denitrificans presumably has the potential to carry out CO2 fixation via this irregular Calvin cycle using transaldolase instead of sedoheptulose-bisphosphatase. The fixed carbon seems to be used for the biosynthesis of amino acids (Figure A4), nucleotides, and sugars (Table 1). Expression of the genes for nine of the ten steps of the irregular Calvin cycle was observed, including genes encoding ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (DSYM_24900) and phosphoribulokinase (DSYM_24930) (Figure 5). Considering the limited number of metatranscriptomic reads that mapped to the coding regions of Ca. D. denitrificans (2937 reads in total), gene expression may be underestimated. Therefore, the Calvin cycle is presumed to work in the anammox bioreactor, even if only at low levels. Nevertheless, since experimental evidence for CO2 fixation through the irregular Calvin cycle has been confirmed only in *Thermodesulfobium acidiphilum*, a member of the Firmicutes (Frolov et al., 2019), further biochemical experiments are required to conclude whether Ca. D. denitrificans can fix CO2 in the ABC. This type of metabolic versatility, which suggests a mixotrophic lifestyle,
**Figure 4** Carbohydrate metabolism pathway. The dotted arrow represents the missing enzyme. Blue, brown, and green lines show the flow of Embden-Meyerhof (M00001), pentose phosphate (M00004), and Entner-Doudoroff (M00008) pathways, respectively. Dashed lines show missing reaction steps. Blue and green lines show reversible and irreversible reactions respectively. The brown line shows the reactions focused on in this study.

**Table 2** Gene repertoire and the expression profile for sulfur metabolism

| Sulfur metabolism                                    | Enzyme                                             | Gene       | Locus ID   | RPKM ratio |
|-------------------------------------------------------|----------------------------------------------------|------------|------------|------------|
| Thiosulfate oxidation (S₂O₃²⁻ → S⁰ or polysulfide)     | L-cysteine S-thiosulfotransferase                  | soxX       | DSYM_07930 | 0.0        |
|                                                       | L-cysteine S-thiosulfotransferase                  | soxA       | DSYM_07940 | 0.0        |
|                                                       | Sulfur-oxidizing protein                           | soxY       | DSYM_07950 | 0.0        |
|                                                       | Sulfur-oxidizing protein                           | soxZ       | DSYM_07960 | 0.0        |
|                                                       | Sulfur-oxidizing protein                           | soxY       | DSYM_23290 | 0.0        |
|                                                       | Sulfur-oxidizing protein                           | soxZ       | DSYM_23300 | 0.0        |
|                                                       | L-cysteine S-thiosulfotransferase                  | soxA       | DSYM_23310 | 0.2        |
|                                                       | L-cysteine S-thiosulfotransferase                  | soxA       | DSYM_23320 | 0.0        |
|                                                       | S-sulfosulfanyl-L-cysteine sulfohydrolase          | soxB       | DSYM_23330 | 0.2        |
| Sulfide dehydrogenase (H₂S/HS⁻ → S⁰)                  | NADPH-dependent 2,4-dienoyl-CoA reductase, sulfur reductase | fccB       | DSYM_09470 | 1.1        |
|                                                       | Cytochrome c-type                                   | fccA       | DSYM_09480 | 0.0        |
| Reverse dissimilatory sulfite reductase (S⁰ → SO₃²⁻)   | Dissimilatory sulfite reductase α subunit          | dsrB       | DSYM_11230 | 0.0        |
|                                                       | Dissimilatory sulfite reductase β subunit          | dsrA       | DSYM_11240 | 0.0        |
| Adenylylsulfate reductase (SO₃²⁻ → adenosine 5′-phosphosulfate) | Adenylylsulfate reductase, subunit A | aprA       | DSYM_23230 | 0.4        |
|                                                       | Adenylylsulfate reductase, subunit B               | aprB       | DSYM_23240 | 0.3        |
| Sulfate adenylytransferase (adenosine 5′-phosphosulfate → SO₄²⁻) | Sulfate adenylytransferase subunit 2 | cysD       | DSYM_26280 | 0.0        |
|                                                       | Sulfate adenylytransferase subunit 1               | cysN       | DSYM_26290 | 0.0        |
|                                                       | Sulfate adenylytransferase                         | sat        | DSYM_23250 | 0.0        |
would be helpful for Ca. D. denitrificans to colonize an anammox bioreactor and also natural environments.

3.6 | Phylogenetic position of Ca. D. denitrificans

A phylogenetic tree based on a concatenated alignment of 45 ribosomal proteins was constructed to infer the phylogenetic position of Ca. D. denitrificans. Ca. D. denitrificans mapped to a large cluster mainly comprised of various species within the order Rhodocyclales. However, Ca. D. denitrificans formed a cluster with a sub-cluster comprised of 4 species within the order Nitrosomonadales and Rugosibacter aromaticivorans within the Rhodocyclales, whereas other species within the Nitrosomonadales independently formed their large own cluster (Figure A5). Also, Methylversatilis discipultum within the Nitronomadales formed a cluster with a sub-cluster of Rhodocyclales. In short, these five Sterolibacteriaceae species within the Nitronomadales were nested in the large cluster of Rhodocyclales. Thus, it appears that these species assigned as Sterolibacteriaceae species in the Rhodocyclales cluster are misidentified and should be reclassified as members of the order Rhodocyclales. Although Ca. D. denitrificans is phylogenetically distant from Sterolibacteriaceae species due to the low bootstrap value of 50% (Figure A5). Therefore, Ca. D. denitrificans is presumed to be a member of a new family within the order Rhodocyclales.

4 | CONCLUSION

Through a series of analyses, it was found that Ca. D. denitrificans has versatile potential to exploit various compounds such as aromatic compounds, glucose, sulfur compounds, and hydrogen as electron donors for denitrification, but the most favorable compounds were aromatics, which inhibit anammox. In addition, Ca. D. denitrificans also possessed hydroxylamine oxidoreductase, which catalyzes the oxidation of hydroxylamine to NO and potentially the reverse reaction, and the potential for CO₂ fixation via an irregular Calvin cycle, implying mixotrophic potential. Thus, we revealed the metabolic versatility that may facilitate the colonization of Ca. D. denitrificans in the anammox bioreactor. Our findings will not only boost our understanding of the functional relationships between incomplete denitrifiers and anammox bacteria, but also a potential isolation strategy for non-isolatable anammox community members.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Takashi Okubo: Data curation (lead); Formal analysis (equal); Methodology (equal); Visualization (lead); Writing-original draft (lead). Hideto Takami: Conceptualization (lead); Formal analysis (equal); Funding acquisition (lead); Methodology (equal); Project administration (lead); Writing-review & editing (lead).

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

RNA sequence (RNA-seq) data for the anammox bacterial community and the genome sequence of Ca. D. denitrificans are available in the NCBI databases with the accession numbers DRA009157 and AP021857, respectively: https://www.ncbi.nlm.nih.gov/sra/DEA009157, https://www.ncbi.nlm.nih.gov/nuccore/AP021857. Supporting Tables (Tables S1–S3) are available at https://doi.org/10.5281/zenodo.5089211
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Figure A1  Phylogenetic tree based on hydroxylamine oxidoreductase (Hao)-like proteins. Hao proteins from aerobic and anaerobic ammonia-oxidizing bacteria were used in this figure as the reference proteins. Noc, Nitrosoccus oceani ATCC19707; NE, Nitrosomonas europaea ATCC 19718; Nmul, Nitrosospira multiformis ATCC 25196; BPIT, Ca. Brocadia pituitae; BROSI, Ca. B. sinica JPN1; EX330, Ca. Brocadia sp. BROELECO1; B6D34, Ca. Brocadia sp. UTAMX1; B6D35, Ca. Brocadia sp. UTAMX2; KSU1, Ca. Jettenia caeni KSU1; KSMBR1, Ca. Kuenenia stuttgartiensis MBR1; SCALIN, Ca. Scalindua japonica husup-a2. The red character shows Hao protein from Ca. Desulfobacillus denitrificans.
Figure A2  Aromatic compound detected in the centrifugal supernatant of wet biomass. (a) Detection of aromatic compounds by LC-TOFMS. P (positive mode), N (negative mode), Measurement conditions are as follows. LC system: Agilent 1200 series RRLC system SL, Column: ODS column, 2 × 50 mm, 2 μm, MS system: Agilent LC/MSD TOF, Column temp.: 40°C, Mobile phase (a: H2O/0.1% HCOOH, b: Isopropanol: Acetonitrile: H2O (65:30:5)/0.1% HCOOH, 2 mM HCOONH4), Flow rate: 0.3 mL/min, Run time: 20 min, Post time: 7.5 min, Gradient condition: 0–0.5 min: B 1%, 0.5–13.5 min: B 1–100%, 13.5–20 min: B 100%, MS ionization mode: ESI negative or positive, MS Nebulizer pressure: 40 psi, MS dry gas flow: 10 L/min, MS dry gas temp: 350°C, MS capillary voltage: 3500 V, MS scan range: m/z 100–1,700, Sample injection: 1 μL, m/z, molecular mass/charge; MT, migration time; RT, retention time; KEGG, Kyoto Encyclopedia of Genes and Genomes; HMDB, Human Metabolome Database. (b) Synthetic pathway for naringenin. (c) Synthetic pathway for flavanone
Figure A3  Phylogenetic tree based on concatenated amino acid sequences of DsrAB. The dot represents the phylogenetic placement of the Ca. D. denitrificans enzyme.
Figure A4  Amino acid biosynthesis pathways. Dotted lines represent missing or incomplete steps. Biosynthetic pathways for shaded amino acids are missing. PEP, phosphoenolpyruvate; PRPP, 5'-Phospho-alpha-D-ribose 1-diphosphate. K and M numbers represent KEGG Orthology and module IDs.

Figure A5  Phylogenetic tree based on concatenated amino acid sequences of ribosomal proteins. Numbers at the nodes are the percentages of 1000 bootstrap replications. Ca. D. denitrificans appears in blue character.