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Enrichment and physiological characterization of an anaerobic ammonium-oxidizing bacterium ‘*Candidatus Brocadia sapporoensis*.’

Short title: Physiological characterization of ‘*Candidatus Brocadia sapporoensis*.’

By

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Abstract

Anaerobic ammonium-oxidation (anammox) is recognized as an important microbial process in the global nitrogen cycle and wastewater treatment. In this study, we successfully enriched a novel anammox bacterium affiliated with the genus '*Candidatus Brocadia*' with high purity (>90%) in a membrane bioreactor (MBR). The enriched bacterium was distantly related to the hitherto characterized '*Ca. Brocadia fulgida*' and '*Ca. Brocadia sinica*' with 96% and 93% of 16S ribosomal RNA gene sequence identity, respectively. The bacterium exhibited the common structural features of anammox bacteria and the production of hydrazine in the presence of hydroxylamine under anoxic conditions. The temperature range of anammox activity was 20 - 45°C with a maximum activity at 37°C. The maximum specific growth rate (μ_{max}) was determined to be 0.0082 h⁻¹ at 37°C, corresponding to a doubling time of 3.5 days. The half-saturation constant (K_S) for nitrite was 5 ± 2.5 μM. The anammox activity was inhibited by nitrite with 11.6 mM representing the 50% inhibitory concentration (IC₅₀) but no significant inhibition was observed in the presence of formate and acetate. The major respiratory quinone was identified to be menaquinone-7 (MK-7). Comparative genome analysis revealed that the anammox bacterium enriched in present study shared nearly half of genes with '*Ca. Brocadia sinica*' and '*Ca. Brocadia fulgida*'. The bacterium enriched in this study showed all known physiological characteristics of anammox bacteria and can be distinguished from the close

relatives by its rRNA gene sequences. Therefore, we proposed the name ‘*Ca. Brocadia sapporoensis*’ sp. nov.

Keywords: Anammox enrichment culture, ‘*Ca. Brocadia sapporoensis*.’, physiological characteristics, comparative genome analysis

Introduction

Anaerobic ammonium oxidation (anammox) is a microbiological process in which ammonium is directly oxidized to nitrogen gas with nitrite as the electron acceptor under anoxic conditions [32]. Anammox bacteria or anammox activity has been ubiquitously detected in various anoxic environments and are considered to be the major stakeholders in the global nitrogen cycle. The anammox process is recognized as an environmental-friendly and cost-effective technology to remove nitrogen from wastewater because it requires no external organic compounds and less oxygen supply than the conventional nitrification-denitrification process.

Anammox bacteria are affiliated with the bacterial phylum *Planctomycetes* [55]. Although no pure culture has been established, five candidate genera (‘*Candidatus Brocadia*’ [55], ‘*Candidatus Kuenenia*’ [48], ‘*Candidatus Scalindua*’ [49], ‘*Candidatus Anammoxoglobus*’ [24] and ‘*Candidatus Jettenia*’ [45]) and about 20 candidatus species have been identified to date.

Anammox bacteria were usually enriched as mono-species [2, 6, 12, 14, 23, 38, 40, 45, 53] and population shifts in the reactors were often observed [24, 42, 43, 52, 53]. Coexistence of

two '*Ca. Scalindua*' species was also reported [26]. Difference in physiological characteristics is one of the important key factors to understand niche differentiation among anammox bacteria living in a wide variety of environments. For example, members of '*Ca. Kuenenia*' are considered as affinity strategists (i.e. high substrate affinity but low growth rate) and members of '*Ca. Brocadia*' are considered as growth-rate strategists (i.e. low substrate affinity but high growth rate) [53]. Members of '*Ca. Scalindua*' are exclusively detected from marine environment and lose their activity under low salinity. Thus, '*Ca. Scalindua*' is considered as marine anammox bacteria [47, 60]. However, only a few enrichment cultures are available despite their environmental and biological importance. Our current understanding of anammox physiology and biochemistry is mainly based on the enrichment culture of '*Ca. Kuenenia stuttgartiensis*'. We have established enrichment cultures of anammox bacteria affiliating with three out of five genera, namely, '*Ca. Brocadia*' [40], '*Ca. Scalindua*' [6] and '*Ca. Jettenia*' [2] with an enrichment of >90% and examined their physiological characteristics. However, since anammox bacteria are phylogenetically diverse, more enrichment cultures are needed to improve our understanding of their ecology, physiology and biochemistry.

In this study, we successfully developed an enrichment culture of a novel anammox bacterium affiliated with the genus '*Ca. Brocadia*'. The enriched bacterium named '*Ca. Brocadia sapporoensis*' was however, only distantly related to the hitherto characterized species in this genus, i.e., '*Ca. Brocadia sinica*' and '*Ca. Brocadia fulgida*'. In addition, we examined the following physiological features of the enriched '*Ca. B. sapporoensis*'; maximum specific growth rate (μ_{max}), half-saturation constant (K_S) for nitrite, phylogenetic affiliation, tolerance to nitrite, formate and acetate, temperature dependency of specific anammox activity, respiratory

quinone, and ultrastructure. Comparative genome analysis was also conducted among the genus of '*Ca. Brocadia*'.

Materials and methods

Reactor configuration

Biomass was collected from the non-woven fabric sheets installed in an up-flow biofilm reactor [14] and used as inoculum for a 3 L jar fermenter (MBF-500ME, EYELA; Tokyo, Japan) installed with a hollow-fiber membrane unit. The membrane unit was composed of 300 polyethylene tubes (pore size 0.1 μm , tube diameter 1 mm, length 70 mm) [38]. Cultivating condition was similar to those previously described for the cultivation of '*Ca. Brocadia sinica*' [38] and '*Ca. Jettenia caeni*' [2]. The MBR was operated at room temperature (20 - 25 °C) and pH 6.9 - 7.5. The culture liquid in the MBR was continuously mixed with a metal propeller at a stirring speed of 150 rpm and purged with 95% Ar - 5% CO₂ at a flow rate of 10 mL min⁻¹ to maintain anoxic condition. Inorganic synthetic medium was fed continuously at a rate of 3.3 L d⁻¹, resulting in a hydraulic retention time (HRT) of 1 d. The medium was prepared by adding following constituents in our university tap water (treated groundwater); (NH₄)₂SO₄, 14-210 mg-N L⁻¹; NaNO₂ 14-210 mg-N L⁻¹; FeSO₄•7H₂O, 9.0 mg L⁻¹; EDTA•4Na, 5.0 mg L⁻¹; NaCl, 1.0 mg L⁻¹; KCl, 1.4 mg L⁻¹, CaCl₂•2H₂O, 1.4 mg L⁻¹, MgSO₄•7H₂O, 1.0 mg L⁻¹; NaHCO₃, 84 mg L⁻¹; KH₂PO₄, 54 mg L⁻¹. During the first 3 days, 35 mg-N L⁻¹ of ammonium and nitrite were fed at a flow rate of 1.75 L d⁻¹. Then the flow rate was increased to 3.3 L d⁻¹ and concentrations of ammonium and nitrite were gradually increased to 210 mg-N L⁻¹. Ammonium and nitrite were

fed at a 1:1 ratio; therefore, nitrite was limiting nutrient in the MBR. When the anammox activity was suddenly dropped, the concentration of each substrate in the medium was decreased to 14 mg-N L⁻¹ and gradually increased again after the activity recovered. Sludge retention time (SRT) was maintained at 60 d by withdrawing 50 mL of mixed liquor from the MBR with a syringe every day. Anammox activity was monitored by measuring the concentrations of NH₄⁺, NO₂⁻, and NO₃⁻ in the feed solution and permeate. The membrane unit was not replaced unless it was accidentally broken. The biomass attached on the membrane surface was removed by shaking the membrane in the mixed liquor.

Abundance of anammox bacteria

The preliminary Miseq analysis of microbial community in the inoculated biomass revealed that the original biomass contained two anammox species affiliating with the genus ‘*Ca. Brocadia*’ and ‘*Ca. Jettenia*’, respectively (data not shown). Therefore, the 16S rRNA gene copy numbers of those two anammox species were monitored by quantitative PCR (qPCR) with specific primer sets during the MBR operation. The qPCR primer set for ‘*Ca. Brocadia* sp.’ was newly designed in this study (**Table 1**). The specific primer for ‘*Ca. Jettenia* sp’ and TaqMan^{TM} probe were previously designed [61]. PCR mixture was prepared using Premix Ex Taq (Perfect Real Time) (TaKaRa Bio; Kusatsu, Japan), following the company’s protocol. The PCR mixture contained 5 µl of Premix Ex Taq (2×), 0.2 pmol µl⁻¹ of each primer, 4 pmol µl⁻¹ of TaqMan probe, 0.2 µL of ROX Reference Dye II (50×) and 1 µl of template DNA (< 50 ng µl⁻¹) at a final volume of 10 µl. The thermal profile was 95 °C for 30 sec, 40 cycles of 95 °C for 5 sec and 60 °C for 34 sec. Temperature control and data analysis was performed using Applied

Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific K.K.; Yokohama, Japan). The enrichment level was indicatively confirmed by fluorescence *in situ* hybridization (FISH) with probe AMX156 [48] for anammox bacteria and EUB338 mix (composed of EUB338 [5], EUB338II and EUB338III [10]) for total bacteria.

DNA extraction and phylogenetic analysis

The phylogenetic affiliation of the anammox bacterium enriched in the MBR was determined by 16S-23S rRNA gene sequence analysis. A 1.5 mL of liquid culture sample was collected from the MBR and centrifuged at 15,000 rpm for 8 min. Then total DNA was extracted from the pellet with a Fast DNA SPIN Kit for soil (MP biomedical; Santa Ana, CA, US) following the manufacturer's instructions. The 16S-23S rRNA genes were PCR-amplified with the primer set Pla46f and 1037r, as described previously [46]. The PCR products were submitted to Eurofins Genomics for sequence analysis after purification with LaboPass^{TM} PCR (COSMO Genetech; Seoul, Korea). The PCR primers used in the sequencing were shown in **Table S1**.

16S rRNA gene, inter spacer region (ISR) and 23S rRNA gene sequences were compared with those on the public database by using the Basic Local Alignment Search Tool (BLAST; NCBI). Phylogenetic trees were constructed by four methods, namely, maximum likelihood (ML), neighbor joining (NJ), maximum parsimony (MP) and unweighted pair group method with arithmetic mean (UPGMA) using a software MEGA6.06.

Physiological characterization

Anaerobic batch experiments were performed to examine temperature dependency of anammox activity and tolerance to inhibitors and to estimate half-saturation constant (K_S). For the determination of temperature dependency and tolerance, firstly the biomass collected from the MBR was washed twice and suspended in the inorganic medium without ammonium and nitrite. Then the cell suspension was dispensed into 12.5 mL glass serum vials sealed with butyl rubber septum and aluminum caps. The headspace was exchanged with helium gas four times, and then the vials were incubated overnight. The experiment was started by adding $^{15}\text{NH}_4\text{Cl}$ and $\text{Na}^{14}\text{NO}_2$ at a final concentration of 3 mM. Production of $^{15}\text{N}^{14}\text{N}$ was measured every hour using gas chromatography mass spectrometry (GC/MS) system GCMS-QP2010SE (SHIMADZU; Kyoto, Japan). Specific anammox activity (SAA) was determined as production rate of $^{15}\text{N}^{14}\text{N}$ per protein. All the above batch experiments were performed in triplicate.

Temperature dependency was examined at 4 - 45 °C. The temperature was controlled by using incubators. Activation energy was calculated based on the Arrhenius relation. Inhibitory effect was evaluated under various concentrations of nitrite (2.5 - 20 mM), formate (0 - 10 mM) and acetate (0 - 10 mM), respectively.

The value of K_S for nitrite was estimated under nitrite-limiting condition. Anammox biomass was washed and suspended as described above. The suspension was diluted to OD_{600} value of 0.1 with the same medium and dispensed into 20 mL glass serum vials. After overnight incubation, ammonium and nitrite were added at final concentrations of 1,000 μM and 350 μM , respectively. Nitrite concentration was measured every 10 min by naphthylethylendiamine method at an absorbance wavelength of 540 nm [21]. The K_S value was calculated according to the Monod kinetics.

Hydroxylamine consumption and subsequent hydrazine production was examined by incubating the enriched anammox bacterial cells with hydroxylamine (the initial concentration of 2.5 mM) at 37°C under anoxic conditions. The liquid sub-samples were collected to determine hydroxylamine and hydrazine concentrations every hour using disposable syringes and filtrated with 0.2 mm-pore-size cellulose acetate membranes immediately.

Maximum specific growth rate (μ_{max})

μ_{max} was estimated by gel-immobilization method as described previously [3, 61]. Briefly, a 100 mL of free-living planktonic cells of enriched '*Ca. B. sapporoensis*' was mixed with the same volume of polyvinyl alcohol (6%, w/v) and sodium alginate (2%, w/v) solution. Then the mixture was dropped into calcium chloride solution (4%, w/v), forming spherical gel beads with diameters of 3.5 - 4.5 mm. The gel beads were cured in the same solution overnight and then washed with our university tap water. The gel beads were inoculated in a 255 mL glass column reactor and cultivated at 37°C. The inorganic nutrient medium described above was fed continuously. Nitrogen loading rate (NLR) was gradually increased as cells grow to avoid nitrite limitation. Ten gel beads were collected every week and the 16S rRNA gene copy number of '*Ca. B. sapporoensis*' in the gel beads was determined in triplicate by qPCR as described above.

Analysis of respiratory quinone

Quinone was extracted with a chloroform-methanol mixture and separated by reversed-phase high performance liquid chromatography (HPLC) as previously described [18]. Briefly,

the anammox cell suspension was centrifuged at 10,000 rpm for 10 min. Then the pellet was resuspended in phosphate buffer (50 mM, pH6.8) containing 1 mM ferricyanide. Quinone was extracted with an equal volume of chloroform-methanol mixture (2:1, v/v), concentrated with a rotary evaporator and reextracted with n-hexane-water (1:1, v/v). Thereafter, quinone components were separated and identified with a reverse-phase HPLC with a UV-VIS detector. The standards of each quinone were prepared from known bacterial species as described previously [9].

Transmission electron microscopy

A biomass sample for transmission electron microscopy (TEM) was prepared according to a previous study [33]. The anammox biomass was dispersed and cryofixed, and then freeze-substitution was performed with osmium tetroxide and acetone. After dehydration with acetone, the cells were embedded in Quetol 651 resin. Ultrathin sections (80 - 90 nm) were obtained with an ultramicrotome. These sections were stained with uranyl acetate and lead citrate and observed with an electron microscope (JEM-1200EX) at 80 kV.

Comparative genome analysis

The bacterial genome sequences previously constructed were obtained from the public database; '*Ca. Brocadia* sp. 40', '*Ca. Brocadia fulgida*', '*Ca. Brocadia sinica*', '*Ca. Jettenia caeni*', '*Ca. Kuenenia stuttgartiensis*', '*Ca. Scalindua brodae*'. Brief descriptions of the genomes

are shown in **Table S2**. Homology search was performed using programs blast-2.2.31 and in silico Molecular Cloning (in silico biology; Yokohama, Japan).

Analytical procedure

Concentrations of NH_4^+ , NO_2^- and NO_3^- were measured using ion chromatography system IC-2010 (TOSOH; Tokyo, Japan) equipped with TSKgel IC-Anion HS or TSKgel IC-Cation HS (TOSOH; Tokyo, Japan). The feed solution and permeate of the MBR were filtered with 0.2 mm-pore-size cellulose acetate membranes (Advantec Toyo, Tokyo, Japan).

Protein concentration in the biomass suspension was determined by using DC^{TM} Protein Assay kit (BIO-RAD; Hercules, CA, US). The 1 mL of biomass was pelleted and suspended in 1 mL of 10% sodium dodecyl sulfate (SDS) solution. After heating at 99 °C for 15 min, the suspension was centrifuged and protein concentration in the supernatant was measured. Quick Start BSA Standard (BIO-RAD; Hercules, CA, US) was used as the protein standard.

The concentrations of hydrazine and hydroxylamine were determined colorimetrically as previously described [13, 59]. Briefly, hydrazine was derivatized using *p*-dimethylaminobenzaldehyde, and absorbance at a wave length of 490 nm was measured [59]. For hydroxylamine, liquid samples were mixed with 8-quinolinol solutions, heated at 99°C. After cooling down for 15 min, absorbance at 705 nm was measured [13].

Nucleic acid sequence accession number

The GenBank/EMBL/DDBJ accession number for 16S - 23S rRNA gene sequence of '*Ca. Brocadia sapporoensis*' is KY659581.

Results and discussion

Reactor operation

The membrane bioreactor (MBR) was inoculated with biomass originating from an up-flow biofilm reactor and operated at SRT of 60 d and at room temperature (20-25°C). According to microbial community analysis by Miseq analysis, the original biomass contained at least two anammox species, '*Ca. Brocadia* sp' (21.2 % of total reads) and '*Ca. Jettenia* sp' (2.9 %), respectively (data not shown). Each genus contained only one species and other candidate genera ('*Ca. Kuenenia*', '*Ca. Anammoxoglobus*' and '*Ca. Scalindua*') were not detected. Thus, only the populations of detected '*Ca. Jettenia* sp.' and '*Ca. Brocadia* sp.' were monitored by qPCR. Nitrogen loading rate was increased stepwise from 0.035 to 0.420 kg-N m⁻³ d⁻¹ by increasing NH₄⁺ and NO₂⁻ concentrations in the influent within one month (**Figure S1(a)**). The nitrogen removal rate (NRR) reached 0.354 kg-N m⁻³ d⁻¹ and the biomass color had turned to bright red after 37 days (**Figure S2**). Although the NO₂⁻ concentration in the MBR was monitored and kept below 10 mg L⁻¹, the NO₂⁻ concentration suddenly increased sometimes due to unknown reasons. When this happened, the influent was stopped and then NLR was gradually increased again to prevent nitrite inhibition. During the entire period, the average molar ratios between nitrite consumed and ammonium consumed (NO₂⁻ / NH₄⁺) and between nitrate produced and

ammonium consumed ($\text{NO}_3^- / \text{NH}_4^+$) were 1.30 ± 0.24 and 0.31 ± 0.16 , respectively, which were close to the previously reported molar ratios of anammox reaction [31].

The qPCR-based 16S rRNA gene analysis revealed that '*Ca. B. sapporoensis*' was a dominant anammox species (5×10^{10} - 3×10^{11} copies mL^{-1}) in the MBR during the entire culturing period (**Figure S1 (c)**), whereas '*Ca. Jettenia* sp' was present but less than 0.4 % of '*Ca. Brocadia sapporoensis*'. FISH analysis revealed that the enrichment level of probe AMX 156-hybridized '*Ca. B. sapporoensis*' accounted for more than 95% of total bacterial cells, which was predominantly present as free-living planktonic cells (**Figure 1**). This indicated that '*Ca. B. sapporoensis*.' was successfully enriched. The enriched '*Ca. B. sapporoensis*.' was subjected to the following experiments.

Phylogenetic affiliation

The 16S-23S rRNA gene sequence of the enriched anammox bacterium was determined by direct sequencing method. The enriched anammox bacterium showed 99% 16S rRNA gene similarity with '*Ca. Brocadia* sp. 40' (AM285341) [25] and Asahi BRW1 (AB456583) [34], 96% with '*Ca. Brocadia fulgida*' [23] and 93% with '*Ca. Brocadia sinica*' [40], respectively (**Table 2**).

Phylogenetic trees based on 16S rRNA gene sequences were constructed by four different methods, namely, maximum likelihood method (ML), neighbor joining method (NJ), maximum parsimony method (MP) and unweighted pair group method with arithmetic mean (UPGMA) and integrated in **Figure 2**. According to the phylogenetic tree, the members of '*Ca. Brocadia*' are divided into two groups; one including '*Ca. B. fulgida*', '*Ca. B. caroliniensis*' and the

enriched '*Ca. B. sapporoensis*' and the other including '*Ca. B. anammoxidans*' and '*Ca. B. sinica*', and the two groups shared up to 94% of 16S rRNA gene sequence. Particularly, 93% of 16S rRNA gene sequence similarity between '*Ca. B. sapporoensis*' and '*Ca. B. sinica*' was as low as that between '*Ca. B. fulgida*' and '*Ca. J. caeni*'. Based on this, we proposed the name '*Ca. Brocadia sapporoensis*' sp. nov. for the bacterial clade of the '*Ca. Brocadia* sp.40'.

Ultrastructure

TEM was performed to confirm the cellular structure of the enriched '*Ca. B. sapporoensis*' (**Figure 3**). The cells consisted of typical three separated compartments, anammoxosome, riboplasm and paryphoplasm, which was unique to anammox bacterial cells. An electron-dense particle was also found in the anammoxosome. It was reported that '*Ca. B. fulgida*' exhibited autofluorescence with two excitation (352 and 442 nm) and two emission (464 and 521 nm) maxima [23]. However, '*Ca. B. sapporoensis*', which shared 96% of 16S rRNA gene sequence with '*Ca. B. fulgida*', did not show autofluorescence (data not shown).

Physiological characteristics

When almost all hydroxylamine was consumed, transient accumulation of hydrazine was observed by incubating the enriched '*Ca. B. sapporoensis*' cells in a glass serum vials with hydroxylamine under anoxic conditions (**Figure 4**). This is the common feature of anammox bacteria; therefore, '*Ca. B. sapporoensis*' shares the common physiological feature with known anammox bacteria.

Since the enrichment temperature of MBR was changed from room temperature to 37°C on day 170 (**Figure S1**), temperature dependency of SAA was determined using the biomass enriched at 20 - 25°C and 37°C. Specific anammox activities (SAA) were determined at 20 - 45 °C and maximum anammox activity was detected at 37°C in both experiments (**Figure 5**). That means optimum temperature for '*Ca. B. sapporoensis*' was independent of the temperature of continuous cultivation. Interestingly, the biomass enriched at room temperature (20-25°C) showed higher activity than that enriched at 37 °C under low temperature range (< 30°C). In contrast, biomass enriched at 37°C lost 16% of its activity at 45 °C while biomass enriched at room temperature lost 32% of the activity. Activation energies were calculated from an Arrhenius plot of SAA at 4 to 37 °C for both enrichment temperatures. The activation energy of biomass enriched at 20-25°C (27.6 kJ mol⁻¹) was half of that of biomass enriched at 37°C (56.0 kJ mol⁻¹). Deterioration of nitrogen removal rate under low temperature range has been regarded as one of the main obstacles in application of anammox-based process to main stream wastewater treatment.

Inhibitory effect by nitrite was expressed as 50% inhibitory concentration (IC₅₀). IC₅₀ for nitrite was determined to be 11.6 mM, which was comparable to that of other anammox bacteria [2, 8, 11, 12, 40].

Effects of formate and acetate were examined at a concentration range of 0 - 10 mM, but no significant inhibition was observed. Since the activity of '*Ca. B. sinica*' and '*Ca. J. caeni*' were inhibited in the presence of 1 mM formate [2, 40], '*Ca. B. sapporoensis*' could be more tolerable to formate than other species. Further experiments should be conducted to study the

effect of other organic matter such as methanol which inhibits the activity of most of the anammox bacteria.

Anammox bacteria are known to utilize formate, acetate, propionate, methylamine, ferrous iron and hydrogen as electron donors to reduce nitrate to ammonium (respiratory ammonification) [16, 23, 24, 39, 58]. Particularly, '*Ca. B. fulgida*' showed high oxidation rate of acetate and became dominant in the presence of acetate [23]. As '*Ca. B. sapporoensis*' was related to '*Ca. B. fulgida*', '*Ca. B. sapporoensis*' may have an ability to oxidize some organic compounds. The key enzymes responsible for respiratory ammonification such as *nrfA* have been detected in the genome of '*Ca. B. sapporoensis*' [1]. Expression of those genes and oxidation capacity of organic matter should be confirmed in future.

The K_S value for NO_2^- was determined to be $5.0 \pm 2.5 \mu\text{M}$ from batch experiment based on the Monod equation. This value was comparable to the previously reported value obtained by using MBR culture of '*Ca. Brocadia* sp. 40' [31] and thus can be considered as the intrinsic value without substrate diffusion limitation.

The μ_{max} was directly estimated from an exponential increase in 16S rRNA gene copy number in the gel beads. The 16S rRNA gene copy number started to increase exponentially after 23 days of lag phase (**Figure S3**). Assuming day 32 - 46 as exponential growth phase, the μ_{max} was calculated to be 0.0082 h^{-1} (corresponding doubling time (t_d) = 3.5 d). This μ_{max} value is lower than the previously reported value ($\mu_{max} = 0.0138 \text{ h}^{-1}$, $t_d = 2.1 \text{ d}$) for '*Ca. Brocadia* sp. 40', which was estimated by decreasing SRT stepwise in an MBR at 30°C [29]. Recent study also reported the higher μ_{max} value for '*Ca. B. sinica*' ($\mu_{max} = 0.0138 \text{ h}^{-1}$, $t_d = 2.1 \text{ d}$) using the PVA-SA immobilization technique at 37°C [61]. These results indicated that the growth rate of anammox

bacteria is in line with other lithoautotrophs like ammonium oxidisers. In this study, μ_{max} was determined by directly measuring temporal increases in 16S rRNA gene copy numbers of immobilized anammox bacterial cells. The qPCR-based quantification assay of 16S rRNA gene copy number has been successfully used to estimate μ_{max} of anammox bacteria [57, 61]. The PVA-SA immobilization technique was also used, because new-born daughter cells could be completely retained in the gel matrix without energy loss for synthesis of extracellular polymeric substances (EPS), which resulted in high growth rates, unless the substrates are sufficiently supplied [3, 61].

Quinones were extracted from the '*Ca. B. sapporoensis*' predominated biomass and analyzed by reversed-phase HPLC. Menaquinon-7 (MK-7) was detected as the predominant respiratory quinone (**Figure S4**). Previous studies revealed MK-7 was dominant in enrichment cultures of '*Ca. J. caeni*', '*Ca. B. sinica*' and '*Ca. K. stuttgartiensis*' [2, 4, 36]. Therefore, most of the anammox bacteria utilize mainly MK-7 in their electron transfer systems.

Kinetic and physiological characteristics of '*Ca. B. sapporoensis*' described above are summarized and compared with other anammox species (**Table 3**). It should be noted that since these kinetic and physiological characteristics are highly dependent on the experimental conditions (e.g., enrichment culture conditions and biomass forms) and measurement methods, detailed experimental conditions and procedures must be carefully evaluated for meaningful comparisons.

Comparative genome analysis

A draft genome sequence of the enriched '*Ca. B. sapporoensis*' in this study was recently determined by shotgun sequencing method [1]. Briefly, a 2.93-Mb genome sequence composed of 122 contigs (G + C contents; 42.7%) was annotated, and 2,521 gene-coding regions, 41 tRNAs, and a single *rrn* operon were annotated. The completeness was estimated to be 93% by CheckM [1]. Most of the essential genes for anammox metabolism, for example, ammonium transport protein (*amtB/Rh*) and hydroxylamine oxidoreductase/hydrazine dehydrogenase (*hao/hdh*) like protein were encoded in the genome. Intriguingly, the genes that encode canonical NO-forming nitrite reductases (*nirK* or *nirS*) were not identified in the '*Ca. B. sapporoensis*' genome like '*Ca. B. sinica*' [37], which was further confirmed by PCR with primers targeting *nirK* and *nirS* [27, 28, 35, 56] (data not shown). It is known that '*Ca. Kuenenia*' and '*Ca. Scalindua*' encode *nirS* [51, 54], whereas '*Ca. Jettenia*' encodes *nirK* [17]. Nine copies of *hao* like gene were detected with threshold E value of 10^{-15} (BIY37_00135, BIY37_01405, BIY37_01555, BIY37_04505, BIY37_05450, BIY37_07425, BIY37_07875, BIY37_10925 and BIY37_11225). Genes encoding key enzymes for carbon fixation through Acetyl-CoA pathway and reductive TCA cycle were identified. However, citrate synthase was missing like other freshwater anammox bacterial species. Therefore, '*Ca. B. sapporoensis*' may use acetyl-CoA pathway for CO₂ fixation as previously suggested [50].

'*Ca. B. sapporoensis*' showed the AAI value of 80% with '*Ca. B. fulgida*', 77% with '*Ca. B. sinica*', and 71% with '*Ca. J. caeni*'. (**Table S2**). The AAI values suggested that '*Ca. B. sapporoensis*' is a new species of the genus '*Ca. Brocadia*' but not classified as a member of any other genera although it shared only 93% of 16S rRNA gene sequence. A Venn diagram was generated to compare the genes located in the genomes of '*Ca. B. sapporoensis*', '*Ca. B. sinica*' and '*Ca. B. fulgida*' (**Figure 6**). Half of genes were commonly shared among three *Ca.*

Brocadia' species, 'while only one third of genes shared with different genera 'Ca. J. caeni', 'Ca. K. stuttgartiensis' and 'Ca. S. profunda' [36]. Interestingly, 'Ca. B. fulgida' and 'Ca. B. caroliniensis' possessed a *nirK*-like gene [15, 41], which was however not found in the genome of 'Ca. B. sinica' and 'Ca. B. sapporoensis'. Hydroxylamine-dependent anammox process by 'Ca. B. sinica' cells was recently proposed instead of the conventionally proposed NO-dependent anammox process [35]. Further studies are required to elucidate the anammox pathway employed by 'Ca. B. sapporoensis'. It seems that 'Ca. S. japonica' and 'Ca. K. stuttgartiensis', which possessed canonical *nirS*-type nitrite reductase, showed higher nitrite affinity than 'Ca. B. fulgida' and 'Ca. B. caroliniensis', which possessed *nirK*- type nitrite reductase (**Table 5**). Since nitrite affinity is absolutely one of key factors determining niche differentiation of anammox bacteria, nitrite reductase of 'Ca. B. sapporoensis' is of great interest and thus must be identified in the future.

As mentioned above, MK-7 was dominant in the enrichment cultures of 'Ca. B. sapporoensis', 'Ca. B. sinica', 'Ca. J. caeni' and 'Ca. K. stuttgartiensis'. Most of the genes, except for *add/mqnB* and *mqnX*, responsible for menaquinone biosynthesis in futasine pathway [19] were detected on their genomes, even though few genes encoding for conventional pathway in which menaquinone is produced from chorismate through *o*-succinylbenzoate were also found. It should be confirmed whether anammox bacteria synthesize MK-7 through the futasine pathway in future.

Description of 'Candidatus Brocadia sapporoensis'

‘*Ca. Brocadia sapporoensis*’ (sap.po.ro.en’sis. N.L. neut. adj. sapporoensis pertaining to Sapporo city, Hokkaido, Japan, where this organism was first enriched).

Cells are cocci (0.7 – 1.0 μm) and consisted of typical three separated compartments, anammoxosome, riboplasm and paryphoplasm. Cells grow autotrophically and anoxically with NH_4^+ and NO_2^- as an electron donor and acceptor, respectively. Nitrogen gas and nitrate are the products of anaerobic NH_4^+ oxidation. Cells produced hydrazine in the presence of hydroxylamine under anoxic conditions. Cells grow at a maximum specific growth rate (μ_{max}) of 0.0082 h^{-1} at 37°C . Anammox activity occurs at mesophilic conditions ($20 - 45^\circ\text{C}$). Cells have the half-saturation constant (K_S) for nitrite of $5 \pm 2.5 \mu\text{M}$ and 50% nitrite inhibitory concentration (IC_{50}) of 11.6 mM. Cells contain MK-7 as a predominant respiratory quinone. 16S rRNA gene sequence similarity to the closest relative (‘*Ca. Brocadia fulgida*’) is 96%. Unlike ‘*Ca. B. fulgida*’, cells do not exhibit autofluorescence.

The ‘*Ca. B. sapporoensis*’ was enriched from biomass collected from an up-flow biofilm reactor treating industrial wastewater in Kumamoto city, Japan. The enrichment was performed using MBR in Sapporo city, Japan. Here, the taxonomic classification of ‘*Ca. Brocadia sapporoensis*’ is proposed for the bacterial clade of ‘*Ca. Brocadia* sp.40’. The accession number of the 16S rRNA gene is KY659581. The GenBank accession number of the ‘*Ca. Brocadia sapporoensis*’ genome is MJUW000000000.2. The taxonumber is CA00020.

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Figure Legends

Figure 1. FISH image of enriched free-living planktonic cells of ‘*Ca. Brocadia*’ after hybridization with Alexa555 (red)-labeled AMX156 probe (specific for ‘*Ca. Brocadia*’) and Alexa488 (green)-labeled EUB338, EUB338II and EUB338III probes (bacterial universal probes). The cells hybridized both probes labeled with red and green fluorochromes are shown as yellow cells. Scale represents 20 μm .

Figure 2. Phylogenetic tree based on 16S rRNA gene sequences of known anammox bacteria. The scale represents the number of nucleotide changes per sequence position. Pie charts at the nodes represent the confidence of the branch topology results, and bootstrap (1000 replication) greater than 75% are filled in black. ML; maximum likelihood method, NJ; neighbor joining method, UPGMA; unweighted pair group method with arithmetic mean, MP; maximum parsimony method.

Figure 3. Transmission electron micrograph of ‘*Ca. B. sapporoensis*’ cells. (a) densely populated cells; scale bar represents 2 μm . (b) Single cell; scale bar represents 200 nm. P, paryphoplasm; R, riboplasm; A, anammoxosome. An arrow indicates an electron-dense particle in the anammoxosome.

Figure 4. Time course of the concentration of hydroxylamine and hydrazine in the batch experiment.

Figure 5. Temperature dependency of anammox activity of ‘*Ca. B. sapporoensis*’ cultivated at 37 °C (a) and at room temperature (25°C) (b). The activation energy was 56.0 kJ mol⁻¹ in (a) and 27.6 kJ mol⁻¹ in (b).

Figure 6. Venn diagram showing shared genes among the genomes of three anammox bacteria affiliated with '*Ca. Brocadia*'.

Figure 1 Narita *et al.*, (2017)

Figure 2 Narita *et al.*, (2017)

Figure 3 Narita *et al.*, (2017)

Figure 4 Narita *et al.*, (2017)

Figure 5 Narita *et al.*, (2017)

Figure 6 Narita *et al.*, (2017)

Table 1. 16S rRNA gene-targeted primers and probes for qPCR assay.

Primer/probe name	Specificity	Sequence (5'-3')	Reference
BRSP454F	Ca. B. sapporoensis	GCAAGGATGTTAATAGCGTTC	This study
BRSP660R	Ca. B. sapporoensis	TCAAGCCATGCAGTATCGGAT	
JEC447F	Ca. J. caeni	GTAAGGGGGTGAATAGCCCTC	[66]
JEC629R	Ca. J. caeni	TCCAGCCCTATAGTATCAACT	
JEC512P	Ca. B. sapporoensis Ca. J. caeni	5'-FAM-CCGAAAGGGTTGCTAATTCTCA-MGB-3'	

16S rRNA position was counted according to 16S rRNA gene sequence of *Escherichia coli*.

Table 2. 16S-23S rRNA gene sequence similarity among the hitherto characterized anammox bacteria.

	Ca. B. sp.40			Ca. B. fulgida			Ca. anammoxidans			B. Ca. B. sinica			Ca. J. caeni			Ca. asiatica			J. Ca. stuttgartiensis			K. Ca. propionicus			A. Ca. brodae			S.		
	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S
Ca. sapporoensis	B. 99	n.d	n.d	96	86	97	94	87	91	93	86	92	92	84	90	92	84	90	93	83	88	90	78	n.d	87	n.d	n.d			
Ca. B. sp.40				96	n.d	n.d	94	n.d	n.d	93	n.d	n.d	92	n.d	n.d	92	n.d	n.d	92	n.d	n.d	90	n.d	n.d	86	n.d	n.d			
Ca. B. fulgida							94	84	86	93	85	86	93	80	89	93	86	86	92	89	94	92	88	87	87	n.d	n.d			
Ca. anammoxidans										95	92	97	92	81	90	92	85	90	90	89	89	91	90	83	86	n.d	n.d			
Ca. B. sinica													92	75	91	92	81	91	91	89	89	91	91	85	90	n.d	n.d			
Ca. J. caeni																98	88	98	90	88	88	93	80	86	90	n.d	n.d			
Ca. J. asiatica																			91	87	88	94	80	86	90	n.d	n.d			
Ca. stuttgartiensis																						90	89	81	87	n.d	n.d			
Ca. propionicus																									85	n.d	n.d			

n.d. = no data

Unit: %

Table 3. Summary of physiological characteristics of ‘*Ca. B. sapporoensis*’ and other known anammox bacteria.

	<i>Ca.</i> <i>sapporoensis</i>	^{B.} <i>Ca. B. sp. 40</i>	<i>Ca. B. fulgida</i>	<i>Ca. B. sinica</i>	<i>Ca.</i> <i>stuttgartiensis</i>	^{K.} <i>Ca. J. caeni</i>	<i>Ca. S. japonica</i>
K_S for NO_2^- (μM)	5.0 ± 2.5^a	2.5^a	350 ± 90^b	34 ± 21^a	0.2-3	35.6 ± 0.92^a	0.45^a
μ_{max} (h^{-1})	$0.0082^{d, e}$	$0.0139^{a, f}$	<i>n.d.</i>	$0.0142^{d, e}$	$0.0026-0.0035^{a, f}$	$0.0075^{d, e}$	$0.0071^{a, e}$
Growth temperature ($^{\circ}\text{C}$)	20-45	30^f	$30-33^f$	25-45	25-37	20-42.5	10-30
Activation energy (kJ mol^{-1})	56^a	<i>n.d.</i>	65.7^a	56 ± 3^c	<i>n.d.</i>	55.4 ± 6.8^a	81.4 ± 3^a
Respiratory quinone	MK-7	<i>n.d.</i>	<i>n.d.</i>	MK-7	MK-7	MK-7	<i>n.d.</i>
IC_{50} for NO_2^- (mM)	11.6^a	<i>n.d.</i>	13.2^b	$< 16^c$	$13^b, 25^b$	11^a	7.5^a
Relative nitrite-reducing activity under presence of 1 mM organic matter (%)							
Formate	101^a	<i>n.d.</i>	<i>n.d.</i>	64^c	89^b	19.8^a	<i>n.d.</i>
Acetate	104^a	<i>n.d.</i>	<i>n.d.</i>	98^c	102^b	83.7^a	<i>n.d.</i>
Reference	This study	[31, 33]	[8, 24, 32, 48]	[39, 41, 43, 66]	[4, 11, 12, 17, 58]	[2, 66]	[6, 66]

n.d. = no data.

^a Values determined using free-living cells.

^b Values determined using aggregated cells.

^c Values determined using aggregated cells after mechanical dispersion.

^d Values determined using cells immobilized in gel beads.

^e μ_{max} based on qPCR.

^f μ_{max} based on substrate conversion rate.

^g Temperature at which the bacterium was enriched.