

# Physiological characteristics of the anaerobic ammonium-oxidizing bacterium '*Candidatus Brocadia sinica*'

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The present study investigated the phylogenetic affiliation and physiological characteristics of bacteria responsible for anaerobic ammonium oxidization (anammox); these bacteria were enriched in an anammox reactor with a nitrogen removal rate of 26.0 kg N m<sup>-3</sup> day<sup>-1</sup>. The anammox bacteria were identified as representing '*Candidatus Brocadia sinica*' on the basis of phylogenetic analysis of rRNA operon sequences. Physiological characteristics examined were growth rate, kinetics of ammonium oxidation and nitrite reduction, temperature, pH and inhibition of anammox. The maximum specific growth rate ( $\mu_{\max}$ ) was 0.0041 h<sup>-1</sup>, corresponding to a doubling time of 7 days. The half-saturation constants ( $K_s$ ) for ammonium and nitrite of '*Ca. B. sinica*' were 28 ± 4 and 86 ± 4 μM, respectively, higher than those of '*Candidatus Brocadia anammoxidans*' and '*Candidatus Kuenenia stuttgartiensis*'. The temperature and pH ranges of anammox activity were 25–45 °C and pH 6.5–8.8, respectively. Anammox activity was inhibited in the presence of nitrite (50% inhibition at 16 mM), ethanol (91% at 1 mM) and methanol (86% at 1 mM). Anammox activities were 80 and 70% of baseline in the presence of 20 mM phosphorus and 3% salinity, respectively. The yield of biomass and dissolved organic carbon production in the culture supernatant were 0.062 and 0.005 mol C (mol NH<sub>4</sub><sup>+</sup>)<sup>-1</sup>, respectively. This study compared physiological differences between three anammox bacterial enrichment cultures to provide a better understanding of anammox niche specificity in natural and man-made ecosystems.

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## INTRODUCTION

Anaerobic ammonium oxidization (anammox), in which ammonium is directly oxidized to nitrogen gas under anoxic conditions with nitrite as the electron acceptor, was discovered by Mulder *et al.* (1995). Anammox is considered an efficient and cost-effective alternative to conventional processes of nitrogen removal from ammonia-rich wastewater because it demands less oxygen, requires no external carbon source and produces less excess sludge (Jetten *et al.*, 1997; Schmidt *et al.*, 2003).

**Abbreviations:** anammox, anaerobic ammonium oxidization; AOB, ammonia-oxidizing bacteria; DO, dissolved oxygen; DOC, dissolved organic carbon; ISR, 16S–23S intergenic spacer region;  $K_s$ , half-saturation constant;  $q_{\max}$ , maximum specific ammonium oxidation rate;  $\mu_{\max}$ , maximum specific growth rate; VSS, volatile suspended solids; Y, biomass yield.

The GenBank/EMBL/DBJ accession number for the AMX\_BG sequence is AB565477.

Four supplementary figures and two supplementary tables are available with the online version of this paper.

Anammox is mediated by a monophyletic group of obligately anaerobic chemoautotrophic bacteria that branch deeply in the order Planctomycetales (Strous *et al.*, 1999a). At the time of writing, five candidate genera have been identified in the bacterial order 'Brocadiales': '*Candidatus Brocadia*', '*Candidatus Kuenenia*', '*Candidatus Jettenia*', '*Candidatus Scalindua*' and '*Candidatus Anammoxoglobus*' (Kartal *et al.*, 2007; Krieg *et al.*, 2010; Quan *et al.*, 2008). Furthermore, Hu *et al.* (2010) recently proposed that anammox bacteria found in nitrogen removal reactors be classified as a novel taxonomic species, '*Candidatus Brocadia sinica*'.

Anammox bacteria or anammox activities have been detected in natural (Amano *et al.*, 2007; Kuypers *et al.*, 2003; Thamdrup & Dalsgaard, 2002) and man-made ecosystems such as wastewater treatment plants, and bacterial doubling times have been reported to be more than 8 days, even under laboratory conditions (Strous *et al.*, 1998; van der Star *et al.*, 2008a, b). Population shifts of anammox bacteria have been observed during the operation of anammox reactors (Kartal *et al.*, 2007; Park *et al.*,

2010; van der Star *et al.*, 2007, 2008a, b). van der Star *et al.* (2008a, b) reported the population shift of anammox bacteria in a membrane bioreactor under stable operating conditions. Initially, the dominant anammox bacteria in this reactor were members of 'Candidatus Brocadia', but the population shifted to 'Candidatus Kuenenia'. In contrast, a population shift from members of 'Candidatus Kuenenia' to those of 'Candidatus Brocadia' was observed during the long-term operation of a full-scale anammox reactor (van der Star *et al.*, 2007) and in biofilm-based, completely autotrophic nitrogen removal over nitrite bioreactors (Park *et al.*, 2010). One of the causes for the population shift is the difference in physiological characteristics between anammox bacteria (e.g. affinity constants and maximum growth rates). Physiological characteristics of 'Candidatus Brocadia anammoxidans' (Jetten *et al.*, 2005; Strous *et al.*, 1998, 1999b) and 'Candidatus Kuenenia stuttgartiensis' (Dapena-Mora *et al.*, 2007; Egli *et al.*, 2001; van der Star *et al.*, 2008a, b) have been examined, and the capacity for nitrate, iron and manganese respiration has been reported for members of 'Candidatus Scalindua' (van de Vossenberg *et al.*, 2008). Such information is, however, not available for other anammox bacteria, including 'Ca. B. sinica'.

The present study examined the phylogenetic affiliations and physiological characteristics of anammox bacteria enriched in our high-rate anammox reactor. Previously, we developed an up-flow, fixed-bed anammox biofilm reactor with non-woven fabric sheets as biomass carriers and attained a high nitrogen removal rate of 26.0 kg N m<sup>-3</sup> day<sup>-1</sup> (Tsushima *et al.*, 2007a, b). In the present study, a nucleotide sequence between the 16S and 23S rRNA genes was retrieved from the anammox biofilm and phylogenetic analysis was subsequently performed. To characterize physiological parameters, anaerobic batch experiments were performed to determine the following: (1) growth pH and temperature ranges, (2) tolerance to inhibitors, (3) utilization of organic matter, (4) accumulation and consumption of hydrazine after the addition of hydroxylamine, (5) half-saturation constants ( $K_s$ ) for ammonium and nitrite, (6) biomass yield and (7) dissolved organic carbon (DOC) yield in the culture supernatant. In addition, an up-flow column reactor was used to evaluate the maximum specific growth rate ( $\mu_{max}$ ). Finally, the ultrastructure of an anammox bacterium was examined by transmission electron microscopy to confirm the presence of the anammoxosome, which is a unique organelle of anammox bacteria.

## METHODS

**Biomass.** Biofilm samples were collected from the anammox reactor as described by Tsushima *et al.* (2007a). The previous study determined the microbial composition in the biofilm sample by fluorescence *in-situ* hybridization with probe AMX820 (Schmid *et al.*, 2001), and found that anammox bacteria accounted for approximately 90% of the total cells. In contrast, aerobic ammonia-oxidizing bacteria (AOB), detected with an NSO190 probe for betaproteobacterial AOB or an Nse1472

probe for AOB belonging to the *Nitrosomonas* group, comprised less than 0.1% of the total cells.

For the subsequent anaerobic batch experiments, the collected biofilm samples were dispersed by vigorous magnetic stirring for 2 h in an anaerobic chamber filled with 95% Ar/5% CO<sub>2</sub> gas. The aggregate diameter of the dispersed biomass was less than 100  $\mu$ m. Physiological characteristics, including the affinity constants for ammonium and nitrite, were determined using the dispersed biomass.

**Phylogenetic analysis.** DNA was extracted from the biomass with a Fast DNA SPIN kit (MP Biomedicals) according to the standard protocol provided with the instrument (Kindaichi *et al.*, 2007). rRNA gene fragments of anammox bacteria were PCR-amplified with the primer set Pla46f and 1037r, as described by Schmid *et al.* (2001). The nucleotide sequence of the amplicon was determined by direct sequencing with an ABI 3100 Avant sequencer (the sequencing primers used are shown in Supplementary Table S1). The nucleotide sequence identified was then subjected to a BLAST search (Altschul *et al.*, 1997), and sequence similarities with other anammox bacteria were evaluated. A phylogenetic tree based on the nucleotide sequence of the 16S rRNA genes was constructed using ARB software (Ludwig *et al.*, 2004). The nucleotide sequence was imported and aligned in ARB, and the phylogenetic tree was constructed by the parsimony method. Bootstrap resampling analysis for 100 replicates was performed to estimate the confidence of tree topologies.

**Anaerobic batch experiments.** Batch experiments were performed to determine the following physiological parameters: (1) temperature and pH ranges, (2) tolerance to inhibitors, (3) utilization of organic matter, (4) accumulation and consumption of hydrazine after the addition of hydroxylamine, (5)  $K_s$  values for ammonium and nitrite, (6) biomass yield and (7) DOC yield in the culture supernatant. The dispersed biomass (aggregate diameter <100  $\mu$ m) was washed twice and suspended in anammox minimal salts medium (van de Graaf *et al.*, 1996) containing ammonium (90 mg N l<sup>-1</sup>) and nitrite (80 g N l<sup>-1</sup>) at a volatile suspended solids (VSS) concentration of 0.2 mg VSS l<sup>-1</sup>. Eight millilitres of the suspension was dispensed into 10 ml serum vials, sealed with butyl rubber stoppers and purged with 95% Ar/5% CO<sub>2</sub> gas for 30 min. The pH value of the bulk solution after gas purging was 7.8. The vials were incubated in the dark for 1 day at 37 °C. The incubation period was prolonged to 40 days when DOC yield was determined.

The influence of temperature on anammox activity was examined at 4–55 °C. Temperature control was performed with a water bath. Activation energy was calculated on the basis of the temperature dependency of the nitrogen removal rate, as described by Strous *et al.* (1999b). The influence of pH was examined at pH 5.0–10.0, and the initial pH of the medium was adjusted with 0.5 M H<sub>2</sub>SO<sub>4</sub> or 1 M NaOH. pH was not adjusted during incubation.

Anammox inhibition was evaluated under varying conditions of dissolved oxygen (DO), nitrite, phosphorus and salinity. Initial concentrations of DO, nitrite, phosphorus and salinity were <0.1–5.0 mg l<sup>-1</sup>, 0–350 mg N l<sup>-1</sup>, 0–20 mM and 0–3% (w/v), respectively. DO concentration was adjusted by bubbling N<sub>2</sub> gas.

Utilization of organic matter was examined after the addition of the following compounds at a final concentration of 1 mM: acetate, propionate, glucose, formate, ethanol and methanol. Biomass suspension was collected at appropriate time intervals and the nitrite-reducing activity was determined.

To confirm the accumulation and subsequent consumption of hydrazine, hydroxylamine was fed into the culture at a final concentration of 2.5 mM. Biomass suspension was collected at appropriate time intervals and hydrazine and hydroxylamine concentrations were determined.

Values of  $K_s$  for ammonium and nitrite were evaluated under nitrite- or ammonium-limiting conditions, respectively. When the  $K_s$  value for ammonium or nitrite was estimated, initial ammonium and nitrite concentrations were set at 5 and 30 mg N l<sup>-1</sup> or 15 and 5 mg N l<sup>-1</sup>, respectively.  $K_s$  values were calculated according to Michaelis–Menten kinetics.

DOC yield in the culture supernatant was determined during the 40 day incubation period. pH was adjusted by adding sulfuric acid at regular intervals. Whenever substrates were almost exhausted, variable amounts of ammonium and nitrite were added to the cultures with syringes.

**Maximum specific growth rate ( $\mu_{max}$ ).**  $\mu_{max}$  was calculated on the basis of the maximum specific ammonium oxidation rate [ $q_{max}$ , mol NH<sub>4</sub><sup>+</sup> (mol C)<sup>-1</sup> day<sup>-1</sup>] and biomass yield [ $Y$ , mol C (mol NH<sub>4</sub><sup>+</sup>)<sup>-1</sup>]. An up-flow column reactor was used to determine  $q_{max}$ . The volume of the column reactor was 67 ml, and the biofilm sample was inoculated as seed biomass. Anammox minimal salts medium containing ammonium (90 mg N l<sup>-1</sup>) and nitrite (80 mg N l<sup>-1</sup>) was continuously fed into the reactor, and the hydraulic retention time (HRT) was set at 0.12 h. The ammonium oxidation rate of the column reactor was calculated on the basis of the ammonium concentrations of influents and effluents and the HRT. To determine biomass concentration, the reactor was stopped after 22 days, all biomass retained in the column reactor was harvested and VSS were measured.  $q_{max}$  was calculated on the basis of the ammonium oxidation rate and biomass (VSS) concentration in the column reactor.

Biomass yield was calculated on the basis of the incorporation rate of [<sup>14</sup>C]bicarbonate and ammonium oxidation. The dispersed biomass was suspended in anammox minimal salts medium (NH<sub>4</sub><sup>+</sup>, 90 mg N l<sup>-1</sup>; NO<sub>2</sub><sup>-</sup>, 80 mg N l<sup>-1</sup>), and [<sup>14</sup>C]bicarbonate was supplemented at a final concentration of 20  $\mu$ Ci (mg VSS)<sup>-1</sup> [740 kBq (mg VSS)<sup>-1</sup>]. Four millilitres of mixture was dispensed into 5 ml serum vials and incubated at 37 °C for 3 days. Biomass suspension was collected each day, washed three times with PBS and mixed with scintillation cocktail (Ultima Gold XR). Radioactivity was determined with an LSC-1000 liquid scintillation counter (Miura & Okabe, 2008; Okabe *et al.*, 2005). Additional incubation in the absence of [<sup>14</sup>C]bicarbonate was performed in parallel to determine the ammonium oxidation rate. Biomass yield was calculated by dividing the incorporation rate of [<sup>14</sup>C]bicarbonate by the ammonium oxidation rate.

**Transmission electron microscopy.** Sample preparation for transmission electron microscopy was performed as described by van Niftrik *et al.* (2008). Briefly, the dispersed anammox biomass was cryofixed, and 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.

**Analytical procedures.** The concentrations of NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> were determined using ion chromatographs (DX-100, Dionex) with an IonPac CS3 cation column and IonPac AS9 anion column after filtration using 0.2  $\mu$ m pore-size membranes (Cho *et al.*, 2010). Hydroxylamine and hydrazine concentrations were determined by the colorimetric method (Frear & Burrell, 1955; Watt & Chrisp, 1952). DOC concentration was determined with a Shimadzu TOC-5000 organic carbon analyser after filtration using 0.45  $\mu$ m pore-size membranes. Salinity was determined with an IS Mill-E refractometer. Biomass dry weight was determined as VSS according to standard methods (Eaton *et al.*, 2005). Unit conversion of biomass dry weight from g VSS to mol C was performed by using a conversion factor of 0.0357 [mol C (g VSS)<sup>-1</sup>], which was derived from an assumption of

the elemental composition of anammox bacteria as CH<sub>2</sub>O<sub>0.5</sub>N<sub>0.15</sub> (Strous *et al.*, 1998). Protein concentration was determined with an RC DC Protein Assay kit (Bio-Rad). Biomass was pelleted and suspended in 10% (w/v) SDS. After centrifugation, the protein in the supernatant was determined according to the standard protocol provided by the manufacturer. BSA was used as the protein standard. Biomass protein content was calculated by dividing the protein concentration by the VSS concentration.

## RESULTS AND DISCUSSION

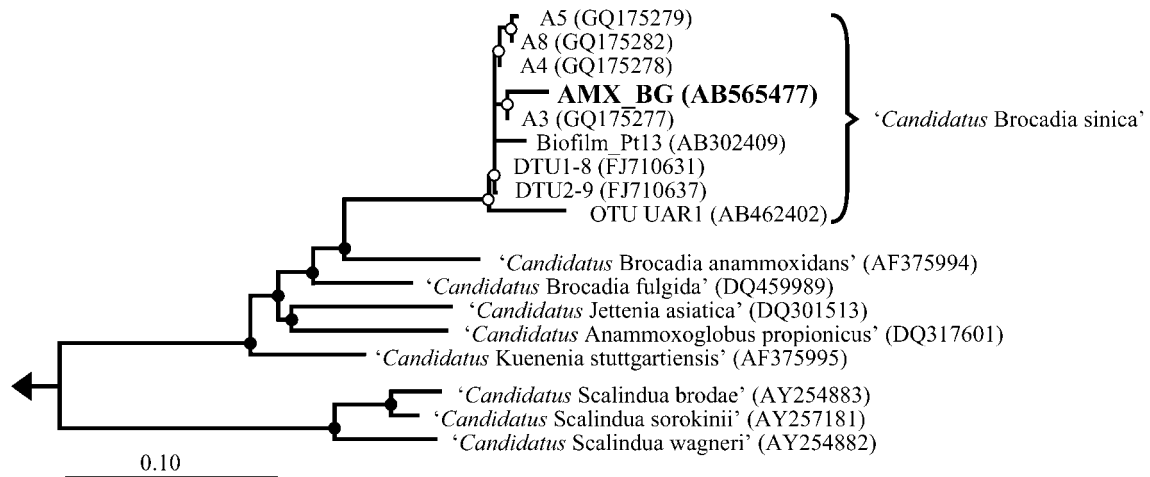
### Phylogenetic affiliation of the anammox bacterium enriched in the high-rate anammox reactor

The nucleotide sequence of the 16S–23S rRNA genes was retrieved from the anammox reactor achieving a high nitrogen removal rate of 26.0 kg N m<sup>-3</sup> day<sup>-1</sup> (Tsushima *et al.*, 2007a, b) and phylogenetic analysis was performed. A phylogenetic tree based on the nearly full-length 16S rRNA gene sequence is shown in Fig. 1, and levels of 16S rRNA gene, 16S–23S intergenic spacer region (ISR) and 23S rRNA gene sequence similarity between AMX\_BG retrieved in the present study and all other known anammox bacteria are given in Supplementary Table S2. AMX\_BG and ‘*Ca. B. sinica*’ shared 99% 16S rRNA gene sequence similarity, which indicates that the anammox bacterium represented by the nucleotide sequence AMX\_BG is closely related to the group ‘*Ca. B. sinica*’. Single nucleotide polymorphism was not found in the nucleotide sequence of AMX\_BG retrieved by the direct sequencing method, which indicates that a single species of anammox bacterium predominated in the biomass.

Taxonomic classification of ‘*Ca. B. sinica*’ has been recently proposed by Hu *et al.* (2010) on the basis of the 16S rRNA gene sequence. We further examined the taxonomic classification of ‘*Ca. B. sinica*’ on the basis of the 23S rRNA gene and ISR in addition to the 16S rRNA gene. The findings shown in Supplementary Table S2 support the taxonomic classification of ‘*Ca. B. sinica*’ as an anammox bacterium distinct from ‘*Ca. B. anammoxidans*’. The presence of ‘*Ca. B. sinica*’ in anammox reactors has been previously reported in China (A3, A4, A5 and A8 in Fig. 1), Japan (OTU UAR1 and Biofilm\_Pt13) and Germany (DTU1-8 and DTU2-9), which implies the wide distribution of this bacterium in anammox reactors.

### Physiological characteristics

Table 1 shows the physiological characteristics of ‘*Ca. B. sinica*’ and other anammox bacteria, namely ‘*Ca. B. anammoxidans*’ and ‘*Ca. K. stuttgartiensis*’. Of note, the degree of biomass aggregation significantly affects the determination of affinity constants and inhibitory concentrations because of the occurrence of diffusion limitation in the biomass. The mean aggregate diameter in the present study was <100  $\mu$ m, which is comparable with that in previous studies (Jetten *et al.*, 2005; Strous *et al.*, 1998, 1999b)



**Fig. 1.** Phylogenetic maximum-parsimony tree showing the relationships among anammox bacteria based on 16S rRNA genes retrieved from the high-rate anammox reactor. Filled circles, bootstrap values >90% based on 100 resamplings; open circles, bootstrap values <90% based on 100 resamplings. Bar, 0.1% sequence divergence.

reporting the affinity constants and inhibitory concentrations of 'Ca. B. anammoxidans' (Table 1). Therefore, the affinity constants determined in this study and those of 'Ca. B. anammoxidans' were regarded as apparent values. For 'Ca. K. stuttgartiensis', the affinity constants for nitrite and inhibitory concentrations were determined using free-living cells (van der Star *et al.*, 2008a, b) and aggregating biomass (no details of aggregate diameter were given) (Dapena-Mora *et al.*, 2007; Egli *et al.*, 2001), respectively.

The effect of temperature on the anammox activity of 'Ca. B. sinica' is shown in Fig. 2(a). The temperature range of anammox activity was 25–45 °C, and the highest nitrogen removal rate was obtained at 35–40 °C. Mean activation energy was  $56 \pm 3 \text{ kJ M}^{-1}$  ( $\pm \text{SD}$ ) on the basis of the Arrhenius plot of the rate constant for nitrogen removal (Fig. 2b). The effect of pH is shown in Supplementary Fig. S1. The pH range of anammox activity was 6.5–8.8, and the highest nitrogen removal rate was observed between pH 8.0 and pH 8.2.

**Table 1.** Physiological characteristics of 'Ca. B. sinica' and two anammox bacteria identified elsewhere, 'Ca. B. anammoxidans' and 'Ca. K. stuttgartiensis'

ND, Not determined because comparable data were unavailable in other studies. A comparable dataset was not available for 'Ca. B. fulgida', 'Candidatus Jettenia asiatica', 'Candidatus Anammoxoglobus propionicus' or 'Candidatus Scalindua brodae'.

Physiological characteristic	'Ca. B. sinica'	'Ca. B. anammoxidans'*	'Ca. K. stuttgartiensis'†
Growth temperature (°C)	25–45	20–43	25–37
Growth pH	7.0–8.8	6.7–8.3	6.5–9.0
Growth rate ( $\text{h}^{-1}$ )	0.0041	0.0027	0.0026–0.0035
Biomass yield [ $\text{mmol C (mmol N)}^{-1}$ ]	0.063	0.07	ND
Affinity for ammonium (mmol)	$28 \pm 4$	<5	ND
Affinity for nitrite (mmol)	$86 \pm 4$	<5	0.2–3
Activation energy ( $\text{kJ mol}^{-1}$ )	$56 \pm 3$	70	ND
Protein content of biomass [ $\text{g protein (g VSS)}^{-1}$ ]	0.61	0.6	ND
Yield of DOC production [ $\text{mmol C (mmol N)}^{-1}$ ]	0.005	ND	ND
Tolerance:			
Nitrite (mmol)	<16	7	13, 25
DO (mmol)	<63	<1	0–200
Phosphate (mmol)	<20	ND	20, 21
Salinity	<3%	ND	200 $\text{mmol}\ddagger$

\*Data from Jetten *et al.* (2005) and Strous *et al.* (1998, 1999b).

†Data from Dapena-Mora *et al.* (2007), Egli *et al.* (2001) and van der Star *et al.* (2008a, b).

‡Concentration of chloride ions.

Regarding tolerance to inhibitors, 50 % inhibitory concentrations for nitrite and DO were 16 mM and 63  $\mu\text{M}$ , respectively. Phosphorus and salinity did not significantly interfere with anammox activity, and 80 and 70 % anammox activity was maintained even in the presence of 20 mM phosphorus and 3 % (w/v) salinity, respectively. '*Ca. B. sinica*' exhibited significantly higher tolerance to the presence of DO ( $<63 \mu\text{M}$ ) than '*Ca. B. anammoxidans*' ( $<1 \mu\text{M}$ ). Such high DO tolerance might reflect the occurrence of oxygen diffusion limitation in the aggregating biomass. However, the effect of oxygen diffusion limitation may be minor given that the aggregate biomass was dominated by anammox bacteria (i.e. the abundance of AOB and nitrite-oxidizing bacteria, scavengers of DO, was less than 0.1 %) and the mean aggregate diameter was less than 100  $\mu\text{m}$ . The physiological characteristics of high DO tolerance facilitate the use of '*Ca. B. sinica*' for

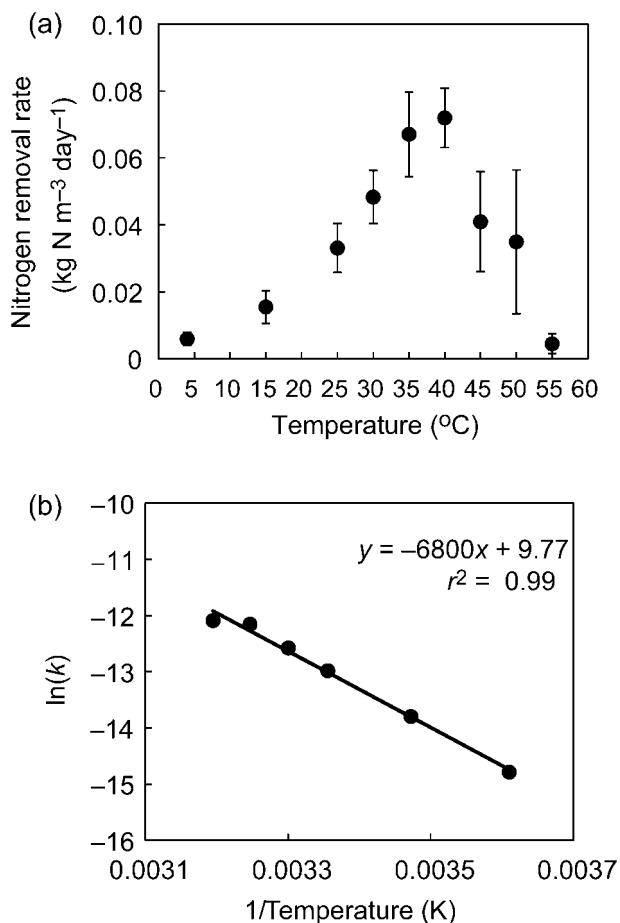
wastewater treatment because it can withstand temporary exposure to or contamination with oxygen.

The presence of acetate, propionate and glucose did not affect nitrite-reducing activity significantly, while formate, ethanol and methanol decreased this activity (Table 2). In particular, ethanol and methanol strongly inhibited nitrite-reducing activity, which agrees with the previously reported inhibitory effect of organic matter in these bacteria (Güven *et al.*, 2005; Tsushima *et al.*, 2007c).

Hydrazine accumulated rapidly immediately after the addition of hydroxylamine. Thereafter, hydrazine was consumed once hydroxylamine had been depleted. Hydrazine and hydroxylamine metabolism has been regarded as a unique process of anammox bacteria (van de Graaf *et al.*, 1997; van der Star *et al.*, 2008a, b). Thus, our findings support the identity of '*Ca. B. sinica*' as an anammox bacterium.

The mean ( $\pm$ SD)  $K_s$  values for nitrite and ammonium were  $86 \pm 4 \mu\text{M}$  (Supplementary Fig. S2) and  $28 \pm 4 \mu\text{M}$ , respectively. These  $K_s$  values were higher than those of '*Ca. B. anammoxidans*' and '*Ca. K. stuttgartiensis*'. However, the aggregate diameter of the dispersed biomass affects the  $K_s$  value because of the occurrence of oxygen diffusion limitation. The aggregate diameter of the dispersed biomass used in the present study was  $<100 \mu\text{m}$ , which is comparable with that reported by Strous *et al.* (1999b), who gave  $K_s$  values for ammonium and nitrite ( $<5$  and  $<5 \mu\text{M}$ , respectively) of '*Ca. B. anammoxidans*'. On the other hand, the  $K_s$  value for nitrite of free-living cells of '*Ca. K. stuttgartiensis*' has been given as 0.2–3  $\mu\text{M}$  (van der Star *et al.*, 2008a, b). Therefore, the  $K_s$  values obtained in the present study and those of '*Ca. B. anammoxidans*' should be regarded as apparent values. Our  $K_s$  values are generally higher than those of free-living '*Ca. K. stuttgartiensis*'.

The incorporation of [ $^{14}\text{C}$ ]bicarbonate with ammonium consumption is shown in Supplementary Fig. S3. The biomass yield of '*Ca. B. sinica*' was 0.062 mol C (mol  $\text{NH}_4^+$ ) $^{-1}$ , similar to the value reported for '*Ca. B.*



**Fig. 2.** Influence of temperature on anammox. (a) Nitrogen removal rate at 4–55 °C; values are means  $\pm$  SD of triplicate experiments. The temperature range of anammox activity was 25–45 °C, and the highest nitrogen removal rate was at 35–40 °C. (b) Arrhenius plot of nitrogen removal at different temperatures. Activation energy was obtained as the slope of the regression line,  $56 \pm 3 \text{ kJ M}^{-1}$ .

**Table 2.** Effect of organic matter (all at 1 mM) on the nitrite-reducing activity of '*Ca. B. sinica*'

Compound	Relative nitrite-reducing activity (%)*
Acetate	98
Propionate	99
Glucose	105
Formate	64
Methanol	14
Ethanol	9

\*Activity in the vials without carbon compounds was set at 100 %. Values below 100 % indicate the inhibitory effect of specific carbon compounds. The SD obtained from five replicates was below 10 %.

anammoxidans' [ $0.07 \text{ mol C (mol N)}^{-1}$ ] (Strous *et al.*, 1999b). No incorporation of [ $^{14}\text{C}$ ]bicarbonate or ammonium consumption occurred in the control experiments using pasteurized biomass.

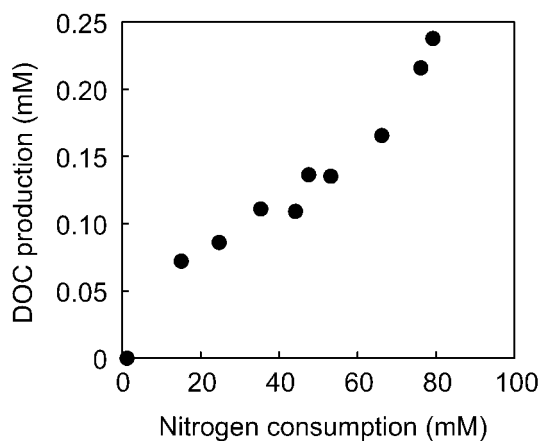
DOC production occurred concurrently with nitrogen removal (Fig. 3). DOC yield was  $0.005 \text{ mol C (mol NH}_4^+)^{-1}$  on the basis of the slope of the regression line. Carbon flow in the biomass estimated from DOC and biomass yields [ $0.062 \text{ mol C (mol NH}_4^+)^{-1}$ ] indicated that approximately 7% of fixed carbon was secreted as DOC into the culture supernatant. This provides a first insight into carbon flow in anammox bacterial cells, which should be useful for the future design of mathematical models simulating the growth of anammox bacteria.

The value of  $q_{\text{max}}$  was determined in a column anammox reactor. The performance of the column reactor is shown in Supplementary Fig. S4. The maximum volumetric ammonium oxidation rate of  $22.2 \text{ kg N m}^{-3} \text{ day}^{-1}$  was obtained after 21 days of operation, and the biomass concentration in the column reactor was  $11 \text{ g VSS l}^{-1}$ . Thus, the  $q_{\text{max}}$  of our anammox enrichment culture was  $1.58 \text{ mol NH}_4^+ (\text{mol C})^{-1} \text{ day}^{-1}$ , corresponding to  $75.0 \text{ } \mu\text{M NH}_4^+ (\text{g protein})^{-1} \text{ min}^{-1}$ . This  $q_{\text{max}}$  was higher than that reported in earlier studies:  $25 \text{ } \mu\text{M NH}_4^+ (\text{g protein})^{-1} \text{ min}^{-1}$  (Strous *et al.*, 1999a),  $30 \text{ } \mu\text{M NH}_4^+ (\text{g protein})^{-1} \text{ min}^{-1}$  (Egli *et al.*, 2001) and  $45 \text{ } \mu\text{M NH}_4^+ (\text{g protein})^{-1} \text{ min}^{-1}$  (Strous *et al.*, 1998). One possible explanation for such a high ammonium oxidation rate by 'Ca. B. sinica' is the activation energy of  $56 \pm 3 \text{ kJ M}^{-1}$ , which is lower than that of 'Ca. B. anammoxidans' ( $70 \text{ kJ M}^{-1}$ ) (Strous *et al.*, 1999b). The relationship

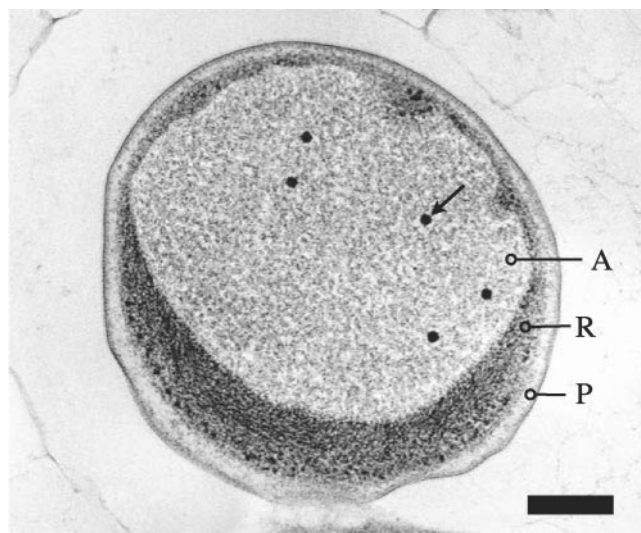
between activation energy and the specific reaction rate can be expressed by the following equation:  $V = \mu[S] \times 10^{-(\Delta G/2.3RT)}$  (Lodish *et al.*, 2000), where  $V$  is nitrogen removal rate,  $S$  is a reaction intermediate with the highest free energy,  $\mu$  is the rate constant for conversion of  $S$  into nitrogen gas,  $\Delta G$  is activation energy,  $R$  is the gas constant and  $T$  is temperature. The nitrogen removal rate increases 10-fold for every  $5.7 \text{ kJ M}^{-1}$  decrease in the activation energy. This equation suggests that 'Ca. B. sinica' can oxidize ammonium at a higher rate than 'Ca. B. anammoxidans'. However, activation energy is unmeasurable and errors in the data can cause a large under- or overestimation. Further evidence is required to determine the difference in maximum ammonium oxidation rates between 'Ca. B. sinica' and 'Ca. B. anammoxidans'.

The value of  $\mu_{\text{max}}$  was calculated to be  $0.0041 \text{ h}^{-1}$  (doubling time=7 days), which corresponds to around half the maximum growth rate of aerobic ammonia oxidizers (Prosser, 1989). This high growth rate is one of the key physiological characteristics of 'Ca. B. sinica'. This rate was higher than that reported for other anammox bacteria. Strous *et al.* (1998) first determined the doubling time of 'Ca. B. anammoxidans' to be 11 days on the basis of the maximum ammonium oxidation rate and biomass yield; this method used for determining doubling time is similar to that used in the present study. Based on biomass retention time in a membrane bioreactor, van der Star *et al.* (2008a, b) estimated the doubling time of 'Ca. K. stuttgartiensis' to be 8.3–11 days. Given the low  $\mu_{\text{max}}$  of anammox bacteria, the preparation of seeding biomass for the start-up of a new anammox reactor requires a long incubation period. As 'Ca. B. sinica' has a lower  $\mu_{\text{max}}$  (7 days) than 'Ca. B. anammoxidans' (11 days) and 'Ca. K. stuttgartiensis' (8.3–11 days), the preparation of seeding biomass can be achieved with shorter incubation periods, which is clearly of value.

To better understand the niche differentiation of anammox genera in natural and man-made ecosystems, the physiological characteristics listed in Tables 1 and 2 are useful. 'Ca. B. sinica' was able to out-compete 'Ca. K. stuttgartiensis' and 'Ca. B. anammoxidans' only under high ammonium and nitrite conditions because they exhibit relatively high  $\mu_{\text{max}}$  ( $0.0041 \text{ h}^{-1}$ ) and  $K_s$  values ( $28 \text{ } \mu\text{M}$  for ammonium and  $86 \text{ } \mu\text{M}$  for nitrite). These physiological characteristics can explain the dominance of 'Ca. B. sinica' in the anammox reactor operated at high ammonium and nitrite load in the present study. However, 'Ca. B. sinica' will be out-competed in natural ecosystems, where the availability of ammonium and nitrite is limited. The physiological characteristics of anammox bacteria other than 'Ca. Brocadia' and 'Ca. Kuenenia' have not yet been studied. Such further studies are essential for a comprehensive understanding of niche differentiation of anammox genera in natural and man-made ecosystems.



**Fig. 3.** DOC production by anammox bacteria in the anaerobic batch experiment. Biomass was incubated for 40 days and DOC concentration in the culture supernatant increased concurrently with nitrogen consumption. The correlation coefficient of the regression line between DOC concentration and nitrogen consumption was 0.94. The slope of the regression line indicates DOC yield,  $0.005 \text{ mol C (mol NH}_4^+)^{-1}$ .



**Fig. 4.** Transmission electron micrograph of a ‘*Ca. B. sinica*’ cell. A, anammoxosome; R, riboplasm; P, parryphoplasm. The arrow indicates condensed and electron-dense particles. Bar, 200 nm.

### Ultrastructure

As shown in Fig. 4, ‘*Ca. B. sinica*’ contains a membrane-bound intracytoplasmic compartment, the anammoxosome. Condensed and electron-dense particles were present in the anammoxosome. Similarly, van Niftrik *et al.* (2008) also found electron-dense particles inside cells of ‘*Ca. K. stuttgartiensis*’ and ‘*Candidatus Brocadia fulgida*’, and identified them as stored iron particles based on transmission electron microscopy and energy-dispersive X-ray analysis. The presence of the anammoxosome inside cells of ‘*Ca. B. sinica*’ provides further strong evidence for the classification of anammox bacteria. This study has provided a comprehensive survey of the physiological characteristics of ‘*Ca. B. sinica*’, which has been detected in anammox reactors operated in China, Japan and Germany. It exhibited higher  $\mu_{\max}$  and  $K_s$  values than ‘*Ca. B. anammoxidans*’ and ‘*Ca. K. stuttgartiensis*’, which explains why it dominates in the anammox reactor operating under high ammonium and nitrite loading rates.

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