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 oxidation (anammox); these bacteria were enriched in an anammox reactor with a nitrogen removal rate of 26.0 kg N m⁻³ day⁻¹. 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 (µmax) was 0.0041 h⁻¹, corresponding to a doubling time of 7 days. The half-saturation constants (Ks) for ammonium and nitrite of ‘Ca. B. sinica’ were 28 ± 4 and 86 ± 4 μM, respectively, higher than those of ‘Candidatus Brocadia ammonoxidans’ 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 NH₄⁺)⁻¹, 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.

INTRODUCTION

Anaerobic ammonium oxidation (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).

Anammox is mediated by a monophyletic group of obligately anaerobic chemoeutrophic 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; Kriep 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; Kuyper 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⁻³ day⁻¹ (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) Kᵅ values for ammonium and nitrite, (6) biomass yield and (7) DOC yield in the culture supernatant. The dispersed biomass (aggregate diameter <100 μm) was washed twice and suspended in anammox minimal salin media (van de Graaf et al., 1996) containing ammonium (90 mg N l⁻¹) and nitrite (80 g N l⁻¹) at a volatile solids concentration (VSS) of 0.2 mg VSS l⁻¹. Eight millilitres of the suspension was dispensed into 10 ml serum vials, sealed with butyl rubber stoppers and purged with 95% Ar/5% CO₂ 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 H₂SO₄ 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⁻¹, 0–350 mg N l⁻¹, 0–20 mM and 0–3% (w/v), respectively. DO concentration was adjusted by bubbling N₂ 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.

**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 Nsr1472 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% CO₂ gas. The aggregate diameter of the dispersed biomass was less than 100 μm. 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 16S rRNA of anammox bacteria was determined by direct sequencing with an ABI 3100 Avant sequencer (the sequencing primers 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) Kᵅ values for ammonium and nitrite, (6) biomass yield and (7) DOC yield in the culture supernatant. The dispersed biomass (aggregate diameter <100 μm) was washed twice and suspended in anammox minimal salin medium (van de Graaf et al., 1996) containing ammonium (90 mg N l⁻¹) and nitrite (80 g N l⁻¹) at a volatile suspended solids (VSS) concentration of 0.2 mg VSS l⁻¹. Eight millilitres of the suspension was dispensed into 10 ml serum vials, sealed with butyl rubber stoppers and purged with 95% Ar/5% CO₂ 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.

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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$^{-1}$ or 15 and 5 mg N L$^{-1}$, 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 ($\mu_{\text{max}}$).** $\mu_{\text{max}}$ was calculated on the basis of the maximum specific ammonium oxidation rate ($q_{\text{max}}$, mol NH$_4^+$ (mol C)$^{-1}$ day$^{-1}$) and biomass yield ($Y$, mol C (mol NH$_4^+$)$^{-1}$).

An up-flow column reactor was used to determine $q_{\text{max}}$. 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$^{-1}$) and nitrite (80 mg N L$^{-1}$) 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. $q_{\text{max}}$ 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 $[^{14}C]$bicarbonate and ammonium oxidation. The dispersed biomass was suspended in anammox minimal salts medium (NH$_4^+$, 90 mg N L$^{-1}$; NO$_3^-$, 80 mg N L$^{-1}$), and $[^{14}C]$bicarbonate was supplemented at a final concentration of 20 µCi (mg VSS)$^{-1}$ [740 kBq (mg VSS)$^{-1}$]. Four milliliters 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 $[^{14}C]$bicarbonate was performed in parallel to determine the ammonium oxidation rate. Biomass yield was calculated by dividing the incorporation rate of $[^{14}C]$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$_4^+$, NO$_3^-$ and NO$_2^-$ were determined using ion chromatographs (DX-100, Dionex) with an IonPac CS3 cation column and IonPac AS9 anion column after filtration using 0.2 µm 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-3000 organic carbon analyser after filtration using 0.45 µm 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)$^{-1}$], which was derived from an assumption of the elemental composition of anammox bacteria as CH$_2$O$_{0.5}$N$_{0.15}$ (Strous et al., 1998). Protein concentration was determined with an RC DC Protein Assay kit (Bio-Rad). Biomass was pelletled 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$^{-3}$ day$^{-1}$ (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 µm, which is comparable with that in previous studies (Jetten et al., 2005; Strous et al., 1998, 1999b)
reporting the affinity constants and inhibitory concentrations of ‘Ca. B. anammoxidans’ (Table 1). Therefore, the affinity constants determined in this study and those of 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 ± 3 kJ mol⁻¹ (± 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’

<table>
<thead>
<tr>
<th>Physiological characteristic</th>
<th>‘Ca. B. sinica’</th>
<th>‘Ca. B. anammoxidans’ *</th>
<th>‘Ca. K. stuttgartiensis’ †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth temperature (°C)</td>
<td>25–45</td>
<td>20–43</td>
<td>25–37</td>
</tr>
<tr>
<td>Growth pH</td>
<td>7.0–8.8</td>
<td>6.7–8.3</td>
<td>6.5–9.0</td>
</tr>
<tr>
<td>Growth rate (h⁻¹)</td>
<td>0.0041</td>
<td>0.0027</td>
<td>0.0026–0.0035</td>
</tr>
<tr>
<td>Biomass yield [mmol C (mmol N)⁻¹]</td>
<td>0.063</td>
<td>0.07</td>
<td>ND</td>
</tr>
<tr>
<td>Affinity for ammonium (mmol)</td>
<td>28 ± 4</td>
<td>&lt;5</td>
<td>ND</td>
</tr>
<tr>
<td>Affinity for nitrite (mmol)</td>
<td>86 ± 4</td>
<td>&lt;5</td>
<td>0.2–3</td>
</tr>
<tr>
<td>Activation energy (kJ mol⁻¹)</td>
<td>56 ± 3</td>
<td>70</td>
<td>ND</td>
</tr>
<tr>
<td>Protein content of biomass [g protein (g VSS)⁻¹]</td>
<td>0.61</td>
<td>0.6</td>
<td>ND</td>
</tr>
<tr>
<td>Yield of DOC production [mmol C (mmol N)⁻¹]</td>
<td>0.005</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Tolerance:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite (mmol)</td>
<td>&lt;16</td>
<td>7</td>
<td>13, 25</td>
</tr>
<tr>
<td>DO (mmol)</td>
<td>&lt;63</td>
<td>&lt;1</td>
<td>0–200</td>
</tr>
<tr>
<td>Phosphate (mmol)</td>
<td>&lt;20</td>
<td>ND</td>
<td>20, 21</td>
</tr>
<tr>
<td>Salinity (mmol)</td>
<td>&lt;3 %</td>
<td>ND</td>
<td>200 mmol‡</td>
</tr>
</tbody>
</table>

*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 μM, 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 μM) than ‘Ca. B. anammoxidans’ (≤ 1 mM). 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 μm. 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 (Guven 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.

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative nitrite-reducing activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>98</td>
</tr>
<tr>
<td>Propionate</td>
<td>99</td>
</tr>
<tr>
<td>Glucose</td>
<td>105</td>
</tr>
<tr>
<td>Formate</td>
<td>64</td>
</tr>
<tr>
<td>Methanol</td>
<td>14</td>
</tr>
<tr>
<td>Ethanol</td>
<td>9</td>
</tr>
</tbody>
</table>

*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 mol C (mol N)⁻¹] (Strous et al., 1999b). No incorporation of [¹⁴C]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 mol C (mol NH₄⁺)⁻¹ on the basis of the slope of the regression line. Carbon flow in the biomass estimated from DOC and biomass yields [0.062 mol C (mol NH₄⁺)⁻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 qmax 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 kg N m⁻³ day⁻¹ was obtained after 21 days of operation, and the biomass concentration in the column reactor was 11 g VSS l⁻¹. Thus, the qmax of our anammox enrichment culture was 1.58 mol NH₄⁺ (mol C)⁻¹ day⁻¹, corresponding to 75.0 μM NH₄⁺ (g protein)⁻¹ min⁻¹. This qmax was higher than that reported in earlier studies: 25 μM NH₄⁺ (g protein)⁻¹ min⁻¹ (Strous et al., 1999a), 30 μM NH₄⁺ (g protein)⁻¹ min⁻¹ (Egl et al., 2001) and 45 μM NH₄⁺ (g protein)⁻¹ 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 ± 3 kJ M⁻¹, which is lower than that of ‘Ca. B. anammoxidans’ (70 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 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 h⁻¹ (doubling time=7 days), which corresponds to about 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 h⁻¹) and Kₛ values (28 μM for ammonium and 86 μM 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](http://mic.sgmjournals.org)

**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 mol C (mol NH₄⁺)⁻¹.
It exhibited higher characteristics of 'Ca. K. stuttgartiensis' and 'Ca. B. sinica', which has been detected in

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 $\mu_{\text{max}}$ and $K_i$ values than ‘Ca. B. anamnoodians’ 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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Physiological characteristics of 'Ca. B. sinica'


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