RESEARCH ARTICLE

**Candidatus 'Brocadia fulgida': an autofluorescent anaerobic ammonium oxidizing bacterium**

Boran Kartal1, Laura van Niftrik1, Jayne Rattray2, Jack L.C.M. van de Vossenberg1, Markus C. Schmid1, Jaap Sinninghe Damsté2, Mike S.M. Jetten1,3 & Marc Strous1

1Department of Microbiology, Institute for Water and Wetland Research, Radboud University Nijmegen, Nijmegen, The Netherlands; 2Department of Marine Biogeochemistry and Toxicology, Netherlands Institute for Sea Research, Den Burg, The Netherlands; and 3Department of Biotechnology, Delft University of Technology, Delft, The Netherlands

Correspondence: Boran Kartal, Department of Microbiology, Institute for Water and Wetland Research, University of Nijmegen, Toernooiveld 1, Nijmegen 6525 ED, The Netherlands. Tel.: +0031 24 3652569; fax: +0031 24 3652830; e-mail: kartal@science.ru.nl

Received 25 July 2007; revised 27 September 2007; accepted 7 October 2007.
DOI:10.1111/j.1574-6941.2007.00408.x

Editor: Alfons Stams

Keywords
anaerobic ammonium oxidation; acetate; anammox; wastewater; ladderane lipids.

Abstract
Anaerobic ammonium oxidizing (anammox) bacteria are detected in many natural ecosystems and wastewater treatment plants worldwide. This study describes the enrichment of anammox bacteria in the presence of acetate. The results obtained extend the concept that the anammox bacteria can be enriched to high densities in the presence of substrates for heterotrophic growth. Batch experiments showed that among the tested biomass, the biomass from the **Candidatus 'Brocadia fulgida'** enrichment culture oxidizes acetate at the highest rate. Continuous cultivation experiments showed that in the presence of acetate, ammonium, nitrite and nitrate, **Candidatus 'Brocadia fulgida'** out-competitive other anammox bacteria. The results indicated that **Candidatus 'Brocadia fulgida'** did not incorporate acetate directly into their biomass. **Candidatus 'Brocadia fulgida'** exhibited the common characteristics of anammox bacteria: the presence of an anammoxosome and ladderane lipids and the production of hydrazine in the presence of hydroxylamine. Interestingly, the biofilm aggregates of this species showed strong autofluorescence. It is the only known anammox species exhibiting this feature. The autofluorescent extracellular polymeric substance had two excitation (352 and 442 nm) and two emission (464 and 521 nm) maxima.

Introduction
Anaerobic ammonium oxidizing (anammox) bacteria conserve energy via oxidation of ammonium with nitrite in the absence of oxygen. The discovery of the anammox process in the second half of the 1990s was the start of a series of fundamental changes in our understanding of the biological nitrogen cycle (Strous & Jetten, 2004). Many studies in the last decade showed that the anammox bacteria are ubiquitous in natural ecosystems and contribute significantly to the global loss of fixed nitrogen (Kuypers et al., 2003, 2005; Hamersley et al., 2007; Jaeschke et al., 2007; Schmid et al., 2007). Furthermore, the anammox process has been applied successfully for ammonium removal from wastewater on both full (van der Star et al., 2007) and lab scale (Pynaert et al., 2003; Kartal et al., 2004; Jetten et al., 2005).

In nature, anammox bacteria were first detected in the Black Sea, the world’s largest anoxic basin (Kuypers et al., 2003). Since then, they have been shown to contribute significantly to nitrogen gas production in two important sites for primary production: the Benguela and Peru upwelling systems (Kuypers et al., 2005; Hamersley et al., 2007). Besides, anammox bacteria and their activity have also been detected in the Arabian Sea (Jaeschke et al., 2007), which contains the largest oxygen minimum zone in the world, and in many natural systems (Risgaard-Petersen et al., 2004; Rysgaard & Glud, 2004; Meyer et al., 2005; Trimmer et al., 2005; Penton et al., 2006; Schubert et al., 2006). Still, it is a challenge to cultivate anammox bacteria because of their long doubling times (10–20 days) and low biomass yields (Strous et al., 1998; Kartal et al., 2007b).

The anammox bacteria form a distinct, deep branching phylogenetic group in the order *Planctomycetales*. So far four genera capable of anaerobic ammonium oxidation have been described and provisionally named as **Candidatus 'Brocadia', Candidatus 'Kuenenia', Candidatus 'Scalindua'** and **Candidatus 'Anammoxoglobus'** (Strous et al., 1999; Schmid et al., 2000, 2003; Kartal et al., 2007b). All
characterized anammox bacteria have a membrane-bound intracytoplasmic compartment, the anammoxosome, and unique ladderane lipids in their cellular membranes (Sinninghe Damsté et al., 2002, 2005; Schmid et al., 2003; Kartal et al., 2007b). Anammox bacteria were assumed to have a completely chemolithoautotrophic lifestyle. However, recent studies have shown that they have a more versatile metabolism. In addition to the anaerobic oxidation of ammonium, they can also use organic acids and iron as an electron donor, and iron and manganese as an electron acceptor (Güven et al., 2005; Strous et al., 2006; Kartal et al., 2007b). Two anammox genera (Candidatus Kuenenia and Candidatus Anamnoxoglobus) were shown to be able to reduce nitrate and/or nitrite to ammonium using organic acids as an electron donor (Kartal et al., 2007a). This trait could give anammox bacteria a competitive advantage in the usually ammonium-limited natural ecosystems (Kartal et al., 2007a).

One particular species, provisionally named Candidatus 'Anamnoxoglobus propionicus', was shown to out-compete other anammox bacteria as well as heterotrophic denitrifiers for propionate as the supplementary electron donor in the presence of ammonium (Kartal et al., 2007b). Interestingly, while oxidizing propionate this anammox species persisted in its autotrophic lifestyle: it did not assimilate propionate directly, but apparently still used CO2 as its only carbon source. The present paper shows that anammox bacteria can also be enriched in the presence of acetate, a more environmentally relevant intermediate in the anaerobic degradation of organic matter than propionate. Feeding of ammonium, nitrate, nitrite and acetate led to the enrichment of Candidatus 'Brocadia fulgida'. The biomass from the enrichment culture was capable of oxidizing other organic compounds such as formate, propionate, monomethylamine and dimethylamine. One other interesting feature of biofilm aggregates of this anammox bacterium is the presence of autofluorescent extracellular substances, never observed before in anammox biofilms.

Materials and methods

Enrichment and cultivation of anammox bacteria

A sequencing batch reactor (SBR, working volume 4 L) was used for enrichment and cultivation of anammox bacteria (Strous et al., 1998). Each SBR cycle consisted of 11 h 45 min of filling, 5 min of biomass settling and 10 min of drawing of the liquid. During each filling period, 1 L of mineral medium (Van de Graaf et al., 1996) containing nitrite, ammonium and nitrate and 500 mL of a sodium acetate solution (concentration specified in ‘Results’) were added continuously to the reactor at flow rates of 1.4 and 0.35 mL min⁻¹, respectively. To maintain anoxic conditions, the reactors and the medium vessels were flushed continuously with Ar/CO2 (95/5%, 10 mL min⁻¹). The SBR was stirred at 200 r.p.m. with a six-bladed turbine stirrer. The temperature of the SBR was maintained at 33°C with a water jacket. CO2 present in the supplied gas was sufficient to buffer the solution and to keep the pH in the SBR between 7.0 and 7.3. The SBR was inoculated with an activated sludge sample originating from the secondary stage of the Dokhaven municipal wastewater treatment plant (Rotterdam, The Netherlands).

Anammox activity assays

Biomass (5 mL with ~5 mg mL⁻¹ protein) was harvested from the SBR described above. The biomass sample was washed three to five times with mineral medium without substrates until the nitrite, nitrate and ammonium in the sample were < 20 μM. The biomass was then transferred to 30-mL serum bottles. The bottles were sealed with 5-mm butyl rubber stoppers and were made anoxic by alternately applying underpressure and argon seven times. An over-pressure of 1 bar was maintained in the bottles. The soluble substrates were added to the bottles from 100 mM anoxic stock solutions. To measure anaerobic ammonium oxidation activity, final concentrations of 3 mM NO₂⁻ and NH₄⁺ were used. To measure nitrate reduction activity, final concentrations of 5 mM NO₃⁻ and 12.5 mM formate, 3 mM acetate, 2 mM propionate, 6 mM monomethylamine or 6 mM dimethylamine were used. To measure the transient accumulation of hydrazine, final concentrations of 4 mM NH₂OH and 5 mM NH₄⁺ were used. The bottles were incubated at 33°C and were shaken continuously at 300 r.p.m. for 2–5 h.

Analytical methods

Nitrate, nitrite, propionate, acetate and formate were measured with HPLC as described previously (Kartal et al., 2007b). Ammonium was measured colorimetrically after the reaction with ortho-phthalaldehyde as described previously (Kartal et al., 2006). Hydroxylamine, hydrazine and protein were determined as described before (Strous et al., 1998).

DNA extraction, retrieval of 16S rRNA gene sequences and phylogenetic analysis

Biomass was harvested from the SBR by centrifugation of a 1.5-mL sample. DNA was extracted as described (Juretschko et al., 1998). DNA was then suspended in 50 μL ultrapure water, and kept at 4°C for 24 h until further analysis. A primer combination of Pla46F (Escherichia coli positions
(630R, 46–63) forward and universal reverse (630R, E. coli positions 1529–1545) primers was used for the preferential amplification of 16S rRNA genes of the members of the Planctomycetes (Schmid et al., 2003). PCR amplificates were cloned directly using the TOPO TA Cloning kit (Invitrogen, Groningen, The Netherlands) according to the manufacturer’s instructions. Plasmid DNA was isolated with the Flex prep kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Sequencing and retrieval of the cloned 16S rRNA genes and phylogenetic analyses were performed as described in Kartal et al. (2007b).

**Probe design and FISH**

Phylogenetic analysis of the sequences and design of oligonucleotide FISH probes were carried out using ARB software as previously described (Schmid et al., 2003). Biomass (1 mL) was harvested from the enrichment culture fixed in paraformaldehyde, and hybridizations with fluorescent probes were performed as described previously (Schmid et al., 2000). All probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives from Thermo Electron Corporation (Ulm, Germany). The following probes were used to monitor the enrichment of the anammox population as described in Schmid et al. (2003): Amx 368 (S-*Amx-0368-a-A-18, specific for all known anammox genera; Schmid et al., 2003), EUB 338 (S-D-Bact-0338-a-A-18, Amann et al., 1990), EUB 338 II (S-D-Bact-0338-b-A-18), EUB 338 III (S-D-Bact-0338-c-A-18), together with EUB and EUBII specific for most bacteria; Daims et al., 1999), and Pla46 (S-P-Planct-0046-a-A-18, specific for Planctomycetes). Probe Bfu 613 (S-*Bfu-0613-a-A-24) was designed for specific hybridizations with Candidatus 'Brocadia fulgida'.

**Electron microscopy**

Small aggregates of biomass from the SBR were cryofixed and analyzed with transmission electron microscopy (TEM) as described previously (van Niftrik et al. 2007).

**Lipid analysis**

The biomass from the SBR and reactor supernatant were separated using centrifugation and subsequently, the biomass was frozen and freeze dried. Total lipids were extracted from the biomass and were analyzed using GC and GC-MS as described previously (Kartal et al., 2007a, b). Ladderane and other lipids were identified on the basis of published mass spectral data (Sinninghe Damsté et al., 2005). The stable carbon isotopes of lipids were measured using a ThermoFinnigan Delta C isotope ratio monitoring MS GCirn/MS which has been described previously (Schouten et al., 2004). δ13C values were corrected for the additional carbon obtained during derivitization.

**Analysis of δ13C of the mineral and acetate media**

The reactor supernatant was analyzed for δ13C values of total dissolved inorganic carbon (DIC) as described previously (Kartal et al., 2007b). δ13C values of the sodium acetate crystals used for reactor medium preparation were determined using Elemental Analysis irmMS as described before (Kartal et al., 2007b).

**Extraction and analysis of fluorescent extracellular polymeric substances**

Biomass (20 mL) was harvested from the SBR and the extracellular polymeric substances (EPS) were extracted by sequential incubation with formaldehyde (40% v/v, 4 °C, 1 h) and NaOH (200 mM, 4 °C, 4 h) (Liu & Fang, 2002). Subsequently, the biomass was separated from the extract with centrifugation (20,000 g, 4 °C, 20 min). The optimal excitation and emission wavelengths for the fluorescent EPS were determined with a fluorescence spectrophotometer (Varian, CA). To determine the approximate molecular weight of the fluorescent compound, the extract was passed through ultrafiltration membranes with different molecular mass cut-offs (300 000, 100 000, 50 000, 10 000 and 3000 Da), and after each filtration step the fluorescence was monitored.

**Results**

In this study we used the same experimental setup (an SBR), the same medium and the same inoculum used previously to enrich the anammox bacteria Candidatus 'Brocadia anamnoxidans', Candidatus 'Kuenenia stuttgartiensis' and Candidatus 'Anammoxoglobus propionicus'. The only difference was the addition of acetate to the reactor as an extra electron donor. The acetate-carbon : ammonium ratio in the medium was ultimately 1 : 6.

In the first 4 months of the enrichment culture the concentrations of nitrite and ammonium in the influent medium were increased from 3 to 45 mM, while the nitrate concentration was maintained at 6 mM. Simultaneously, the acetate concentration was increased from 1 to 30 mM. The concentrations of acetate and nitrite in the effluent were almost always below the detection limit (10 μM). The microbial population of the enrichment culture was monitored continuously with FISH analysis.

In the first 2 months, the anammox population increased to c. 50% and in the following 2 months to 80% of the enrichment culture as determined with FISH analysis. When genera-specific FISH probes were used, it appeared that the only detectable anammox bacteria in the enrichment culture...
belonged to *Candidatus* ‘Brocadia’ genus. After the first 4 months of the enrichment culture, the anammox biomass gradually became autofluorescent. The autofluorescence was prevalent in the same wavelength as fluorescein isothiocyanate (FITC, excitation/emission 490/520) and Cy3 (excitation/emission 550/570); thus, FISH analysis with these dyes became impossible. The fluorescence was observed only in the aggregated anammox cells, not in the single cells, indicating that the source of the fluorescence was the extracellular matrix. The extracted EPS was fluorescent between 390 and 630 nm. There were two excitation maxima, 352 and 442 nm, and two corresponding emission maxima, 464 and 521 nm (Fig. 1). This indicated two different fluorescence sources. Membrane ultrafiltration showed that the masses of the fluorescent polymers were between 3 and 10 kDa.

To determine the precise affiliation of the anammox species present in the enrichment culture we applied a *Planctomycetes* specific full cycle rRNA approach. DNA was extracted from the enrichment culture biomass. 16S rRNA gene sequences of *Planctomycetes* were amplified using PCR with the primers Pla46F and 630R, and cloned. Twenty clones were randomly picked for sequencing. Near-full-length 16S rRNA gene sequences were obtained. All of the 16S rRNA genes had the same sequence and branched within the *Candidatus* ‘Brocadia’ lineage (Fig. 2).

A new oligonucleotide probe S-*.Bfu-613-a-A-24 was constructed to match specifically with the dominant 16S rRNA gene sequence from the enrichment culture. Retrospective analysis of biomass samples showed that upon inoculation, no anammox cells were detected. During the enrichment there was a gradual increase in the population of the anammox species binding with the new probe (Fig. 3). This species became dominant in 2 months, and after 4 months it out-competed all other bacteria and made up 80% of the population.

This 80% enriched anammox biomass had a specific activity of 15 μmol NH₄⁺ g protein⁻¹ min⁻¹. When the
biomass from the enrichment culture was incubated in the absence of acetate, per mole of ammonium 1.5 mol of NO\textsubscript{2}/C\textsubscript{0} was converted, similar to the previously reported values (Strous et al., 1998). When the biomass was incubated with hydroxylamine, there was a transient accumulation of 0.19 mM hydrazine, a phenomenon unique to anammox bacteria (Fig. 4). The nitrate reducing activity of the enrichment culture with small organic compounds was $3 \pm 0.22 \text{NO}_3 \text{ mmol g protein}^{-1} \text{ min}^{-1}$ for formate, $1.5 \pm 0.06$ for acetate, $0.88 \pm 0.02$ for propionate, and $1 \pm 0.09$ for monomethylamine and dimethylamine. In the incubations with acetate and propionate, there was a transient nitrite accumulation of $0.8 \pm 0.06$ and $0.3 \pm 0.02$ mM, respectively. There was no detectable transient nitrite accumulation in the incubations with monomethylamine, dimethylamine, and formate. The formate and acetate oxidation rates with the current biomass were notably higher than rates observed with Candidatus ‘Brocadia anammoxidans’, Candidatus ‘Kuenenia stuttgartiensis’ and Candidatus ‘Anammoxoglobus propionicus’ (Table 1). The propionate oxidation rate of the biomass was three times higher than that of Candidatus ‘Brocadia anammoxidans’ and Candidatus ‘Kuenenia stuttgartiensis’, but half that of Candidatus ‘Anammoxoglobus propionicus’.

The presence of ladderane lipids, another unique characteristic of all the anammox bacteria, in the enrichment culture was also investigated. The most abundant ladderane lipids in the total lipid extract from the SBR were the anammox specific ladderane fatty acids b and c (Fig. 5). Large amounts of C16 fatty acids were also present. Although these straight chain fatty acids are present in many other organisms, they were also shown to be incorporated into intact membrane ladderane lipids, thus confirming their presence in anammox bacteria (Boumann et al., 2006).

To determine whether the anammox bacteria in the enrichment culture used CO\textsubscript{2} or acetate for the synthesis of the ladderane lipids (i.e. as carbon source) their stable carbon isotope compositions were measured. Both potential carbon sources had similar $\delta^{13}$C values (Table 2), but only for CO\textsubscript{2} a significant isotopic fractionation would be expected (Schouten et al., 2004; Londry & Des Marais, 2003). The $\delta^{13}$C values were variable and ranged from $-32.8\%$ (nonspecific C\textsubscript{16:1} fatty acid) to $-84.2\%$.

---

**Fig. 3.** Monitoring the enrichment of Candidatus ‘Brocadia fulgida’ from sludge from inoculum over 2 months with fluorescent in situ hybridization. Candidatus ‘Brocadia fulgida’ is double hybridized with Pla 46 (cy5, blue) and Bfu613 (cy3, red) and depicted in pink. Other bacteria are hybridized with FLUOS-labeled EUB mix probes and are depicted in green. Candidatus ‘Brocadia fulgida’ was enriched from < 1% (a) to 80% in 60 days (b). The scale bar is 20 \textmu m.

**Fig. 4.** Production of hydrazine (empty circles) in the presence of hydroxylamine (filled circles) under anoxic conditions.

---

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved
(ladderane lipid c), indicating that CO$_2$ was the most likely main carbon source for the lipids.

A typical characteristic of the previously described anammox organisms is the presence of a membrane-bound intracytoplasmic compartment known as the anammoxosome (Lindsay et al., 2001). TEM was performed on thin sections prepared from the enriched biomass containing the new anammox organism. The new anammox species displayed typical ultrastructural features of anammox bacteria: a single membrane bound anammoxosome containing tubule like structures, and riboplasm with ribosome-like particles separated from paryphoplasm at the cell rim by an intracytoplasmic membrane (Fig. 6).

**Table 1.** Organic acid oxidation rates ($\mu$mol gprot$^{-1}$ min$^{-1}$) of different anammox species

<table>
<thead>
<tr>
<th></th>
<th>Candidatus 'Brocadia anammoxidans*'</th>
<th>Candidatus ‘Kuenenia stuttgartiensis'</th>
<th>Candidatus ‘Anammoxglobus propionicus'</th>
<th>Candidatus ‘Brocadia fulgida'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formate</td>
<td>6.5 ± 0.6</td>
<td>5.8 ± 0.6</td>
<td>6.7 ± 0.6</td>
<td>7.6 ± 0.6</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.57 ± 0.05</td>
<td>0.31 ± 0.03</td>
<td>0.79 ± 0.07</td>
<td>0.95 ± 0.04</td>
</tr>
<tr>
<td>Propionate</td>
<td>0.12 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.64 ± 0.05</td>
<td>0.31 ± 0.007</td>
</tr>
</tbody>
</table>

*These rates are taken from Kartal et al. (2007).

**Discussion**

Compared with aerobic ammonium oxidizers, anammox bacteria have a more divergent 16S rRNA gene phylogeny. Such a considerable degree of evolution could indicate that anammox species are quite different from each other and each might occupy a distinct ecological niche (while belonging to the same ecological guild). The characterization of Candidatus ‘B. fulgida’, the fifth species of anammox bacterium currently described, indicates that the anammoxosome,

---

**Table 2.** $\delta^{13}$C isotopic depletion of selected lipids and substrates ($^{13}$C% vs. VPDB (Vienna Pee-Dee Belemnite))

<table>
<thead>
<tr>
<th>Lipid/medium</th>
<th>$\delta^{13}$C% vs. VPDB</th>
</tr>
</thead>
<tbody>
<tr>
<td>i16:0</td>
<td>−67.0</td>
</tr>
<tr>
<td>16:1</td>
<td>−32.8</td>
</tr>
<tr>
<td>16:0</td>
<td>−42.1</td>
</tr>
<tr>
<td>10MeC15</td>
<td>−60.8</td>
</tr>
<tr>
<td>Ladderane b</td>
<td>−72.5</td>
</tr>
<tr>
<td>Ladderane c</td>
<td>−84.2</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>−28.4</td>
</tr>
<tr>
<td>Medium DIC</td>
<td>−27.6</td>
</tr>
</tbody>
</table>
the ladderane lipids and the capability of producing molecular hydrazine are shared among all anammox bacteria and define the guild of anaerobic ammonium oxidizers as a whole. On the other hand, the presence of organic acids in the medium seems to define some degree of niche differentiation. Previously, Candidatus ‘Anammoxoglobus propionicus’ was shown to out-compete other anammox bacteria for propionate (Kartal et al., 2007b). In the present study, in the presence of acetate Candidatus ‘Brocadia fulgida’ was shown to be a more successful competitor than other anammox bacteria. It should be noted that both these species were previously also transiently observed in enrichment cultures without supplemented organic acids. These previous studies showed that the inoculum used in the present study potentially contains all known wastewater anammox species.

Altogether these anammox species constitute an almost undetectable minority of the complete population of the inoculum. It is interesting that none of the dominant bacteria could compete successfully for nitrite with a chemolithoautotrophic anammox bacterium under denitrifying conditions. Thermodynamic maximal biomass yield calculations based on the Gibbs free energy changes associated with the oxidation of acetate and ammonium showed that if the denitrifiers would have used all the available acetate, a coculture of a heterotrophic acetate-oxidizing denitrifier with Candidatus ‘B. fulgida’ would be expected. According to these calculations the anammox population would only make up 40% of the total biomass. But apparently this did not happen: despite the fact that a sufficient quantity of organic material (acetate) was supplied, heterotrophic bacteria never became the dominant organisms in the enrichment culture. Instead, Candidatus ‘B. fulgida’ was shown to constitute 70–80% of the total population by FISH. Based on the same yield calculations, if all the remaining 20–30% were denitrifiers they would be responsible for 20–40% of acetate consumption. Thus it is possible that the side population could contribute to the acetate oxidation in the continuous culture and the batch incubations.

Previously, the organic acid-dependent nitrate reduction pathway of the anammox bacterium Kuenenia stuttgartiensis was elucidated, and it was shown that this anammox bacterium was capable of dissimilatory nitrate reduction to ammonium (Kartal et al., 2007a). Previous studies and the results of the present study indicate that the organic acid oxidation could be a common trait among anammox species. It is conceivable that Candidatus ‘B. fulgida’ performs nitrate reduction through the same pathway. This hypothesis will be investigated in the future with 15N-labeled nitrogen tracer studies. In the present study, Candidatus ‘B. fulgida’ was characterized as a typical anammox bacterium with one exception: the presence of an autofluorescent extracellular substance around its cells.

In SBRs anammox bacteria grow in biofilm aggregates because this type of bioreactor selects for the well-settling biomass. In this case, these biofilm aggregates became fluorescent after several months of enrichment. The fluorescence was prevalent in a wide wavelength range (390–630 nm), making FISH studies with FLUOS and Cy3-labeled probes impossible after 4 months of enrichment. Interestingly, the single cells of anammox bacteria in the enrichment culture were not fluorescent, indicating that the source of the fluorescence was the EPS that form the matrix of the aggregates. When these polymeric substances were extracted from the biomass, it was clear that they were indeed autofluorescent. When analyzed with a fluorescence spectrophotometer, it was determined that there were two distinct excitation and emission maxima. This suggested that there were at least two different compounds or moieties contributing to the autofluorescence of the biofilm aggregates. Via ultrafiltration we also determined that the fluorescent substance had a mass higher than 3 kDa. All described anammox enrichment cultures form EPS as a matrix to build biofilm aggregates, but none are autofluorescent. Hence it was highly likely that the enriched anammox bacterium was the source of the autofluorescence. Based on this feature we propose to provisionally name this anammox species as Candidatus ‘Brocadia fulgida’. The molecular structure and detailed properties of this compound remain unknown, but will be investigated in the future with mass spectrometry.
The ladderane lipids unique to anammox bacteria were the most abundant lipid type in the Candidatus ‘Brocadia fulgida’ enrichment culture. These lipids were depleted in $^{13}$C up to 56.6% relative to the medium DIC. This strong depletion is characteristic for anammox bacteria and is consistent with carbon fixation via the acetyl-CoA pathway (Schouten et al., 2004). The $^{13}$C depletion in the ladderane lipids of Candidatus ‘Brocadia fulgida’ was similar to other described anammox species (depleted 47–51% relative to DIC), suggesting that they use a similar carbon acquisition pathway. The other possible carbon source, sodium acetate, had a $\delta^{13}$C of $-28.4\pm 0.2\%$ relative to Vienna Pee-Dee Belemnite (VPDB), suggesting that this was not the direct source of carbon for the ladderane lipids. Most of the carbon fractionation occurs during carboxylation; thus, if Candidatus ‘Brocadia fulgida’ incorporated acetate directly into its biomass, much less carbon fractionation would have been expected in the lipids (Londry & Des Marais, 2003). This is striking because instead of directly converting acetate into acetyl-CoA, Candidatus ‘Brocadia fulgida’ apparently chooses the hard way; it degrades acetate to CO$_2$ and then ‘re-fixes’ this carbon via acetyl-CoA. The genome analysis of the anammox bacterium Kuenenia stuttgartiensis revealed at least three different acetate-CoA ligases. One might speculate that anammox bacteria oxidize acetate in the periplasm via an unknown mechanism, for example via oxalate. It is also possible that the heterotrophic bacteria dissipate some of the acetate to CO$_2$ and the produced CO$_2$ is then used as carbon source for the anammox bacteria. The mechanism of the acetate oxidation by Candidatus ‘Brocadia fulgida’ enrichment culture will be a topic for future research.

It is possible that acetate oxidation occurs only for the conservation of energy. This would lead to a higher biomass yield for the anammox bacteria per mole of oxidized ammonium. Theoretical yield calculations showed that a 55% increase in the biomass yield would be expected compared with anammox bacteria grown in the absence of acetate. This increase would in theory lead to shorter startup periods for anammox bioreactors. In the case of the Candidatus ‘Brocadia fulgida’ enrichment culture there was no significant difference in the startup time compared to the previous enrichment cultures. However, it is very difficult to follow such an increase precisely during an anammox enrichment because knowledge on the viability and initial cell count of anammox bacteria present in the inoculum is lacking and physical factors such as diffusion limitation into the aggregates could also be important. An alternative explanation for the lack an increase in the yield of anammox bacteria could be the possibility that most of the acetate is oxidized by the 20–30% side population, common in all anammox enrichment cultures (Strous et al., 1999; Strous et al., 2006; Kartal et al., 2006, 2007b). However, as mentioned before, such a high acetate consumption would not have resulted in a highly (70–80%) enriched anammox culture.

Biomass from the Candidatus ‘Brocadia fulgida’ enrichment culture was also able to oxidize methylamines with nitrate and/or nitrite as electron acceptors. The end product of the oxidation of methylamine with nitrate or nitrite is ammonium, and Kuenenia stuttgartiensis reduces nitrate and nitrite to ammonium. In many natural ecosystems, such as the water column of the oceans, anammox bacteria are usually limited by ammonium, and methylamines are available in freshwater and marine ecosystems (Wang & Lee, 1990; Fitzsimons et al., 2006). Hence, the oxidation of methylamines to ammonium could be a valuable survival strategy for anammox bacteria in nature, in the absence of ammonium, because the end product for the oxidation of methylamines is ammonium.

In wastewater treatment the anammox process is used for the removal of ammonium. Application of anammox is dependent on a source of nitrite. The co-oxidation of organic acids could increase the potential for the anammox process in wastewater treatment as follows: (1) anammox bacteria are still enriched to a very high density in the presence of organic acids and ammonium so the process could also be applied to wastewater containing both organic compounds and ammonium; (2) sometimes nitrate is available as an electron acceptor, and nitrate could be used directly by anammox bacteria (or other heterotrophs present in the reactor biomass) with organic acids as a cosubstrate; (3) currently the theoretical maximum ammonia removal efficiency with anammox is 90%. By supplying organic acids as cosubstrate, ammonia removal can become close to 100% efficient.

**Description of Candidatus ‘Brocadia fulgida’**

Brocadia fulgida (Brocadia L.fem.n referring to the place of discovery of the first anammox species; fulgida L. fem. adj. of shining, referring to the strong autofluorescence of the biofilm aggregates).

Anaerobic chemolithoautotrophic cocccoid cells have diameters ranging between 0.7 and 1 µm. Cells oxidize ammonium with nitrite as the electron acceptor and with CO$_2$ as the main carbon source. Cells oxidize propionate, acetate, formate, monomethylamine, and dimethylamine with nitrate and/or nitrite as the electron acceptor. Cells produce hydrazine transiently in the presence of hydroxylamine. Cells possess a membrane-bound intracytoplasmic compartment containing tubule-like structures, known as the anammoxosome. Cells contain ladderane lipids. 16S rRNA gene sequence similarity to the closest relative (Candidatus ‘Brocadia anamoxidans’) is 94%. Accession number of the 16S rRNA gene is DQ459989.
Acknowledgements

This research was financially supported by the Foundation of Applied Sciences (STW, Project NPC 5987). We would like to thank J.W. Mulder for supplying the inoculum from the wastewater treatment plant at Rotterdam and Michiel V.M. Kienhuis (NIOZ) for technical assistance with isotopic analysis. We would like to thank Mark van Loosdrecht and Michiel van der Star for fruitful discussions. We would also like to thank E.G. van Donselaar and B.M. Humbel for assistance with electron microscopy.

References


