Hydroxylamine-dependent Anaerobic Ammonium Oxidation (Anammox) by “*Candidatus Brocadia sinica*”

By

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Summary (178 words)

Although metabolic pathways and associated enzymes of anaerobic ammonium oxidation (anammox) of “*Ca. Kuenenia stuttgartiensis*” have been studied, those of other anammox bacteria are still poorly understood. NO_2^- reduction to NO is considered to be the first step in the anammox metabolism of “*Ca. K. stuttgartiensis*”, however, “*Ca. Brocadia*” lacks the genes that encode canonical NO-forming nitrite reductases (NirS or NirK) in its genome, which is different from “*Ca. K. stuttgartiensis*”. Here, we studied the anammox metabolism of “*Ca. Brocadia sinica*”. ^{15}N -tracer experiments demonstrated that “*Ca. B. sinica*” cells could reduce NO_2^- to NH_2OH , instead of NO, with as yet unidentified nitrite reductase(s). Furthermore, N_2H_4 synthesis, downstream reaction of NO_2^- reduction, was investigated using a purified “*Ca. B. sinica*” hydrazine synthase (Hzs) and intact cells. Both the “*Ca. B. sinica*” Hzs and cells utilized NH_2OH and NH_4^+ , but not NO and NH_4^+ , for N_2H_4 synthesis and further oxidized N_2H_4 to N_2 gas. Taken together, the metabolic pathway of “*Ca. B. sinica*” is NH_2OH -dependent and different from the one of “*Ca. K. stuttgartiensis*”, indicating metabolic diversity of anammox bacteria.

Introduction

Anaerobic ammonium oxidation (anammox) is a microbial process in which NH_4^+ and NO_2^- are directly converted to N_2 gas via N_2H_4 (de Almeida *et al.*, 2011; Kartal *et al.*, 2011). The proposed biochemical pathway of the anammox process is as follows: first, NO_2^- is reduced to nitric oxide (NO) by either cytochrome *cd₁*-type or copper-containing nitrite reductase (NirS and NirK, respectively) (Strous *et al.*, 2006; Hira *et al.*, 2012); then, the produced NO is reduced to hydroxylamine (NH_2OH) and coupled with NH_3 to form hydrazine (N_2H_4) by hydrazine synthase (Hzs) (Dietl *et al.*, 2015; Kartal *et al.*, 2011); and finally, the N_2H_4 is oxidised to N_2 gas by hydrazine dehydrogenase (Hdh) (Shimamura *et al.*, 2007; Kartal *et al.*, 2011). The anammox process has been demonstrated in bacteria affiliated with the order *Brocadiales*. So far, five candidate genera have been proposed: “*Ca. Kuenenia*”, “*Ca. Brocadia*”, “*Ca. Anammoxoglobus*”, “*Ca. Jettenia*” and “*Ca. Scalindua*” (Strous *et al.*, 1999a; Jetten *et al.*, 2009). Anammox bacteria are ubiquitously distributed in natural and engineered anoxic ecosystems including freshwater, brackish water, marine and terrestrial ecosystems, in which the anammox bacteria contribute to significant nitrogen losses (Brandes *et al.*, 2007; Hu *et al.*, 2011; Lam *et al.*, 2009; Long *et al.*, 2013).

Several draft genome sequences have been determined for anammox bacteria affiliated with “*Ca. Kuenenia*” (Strous *et al.*, 2006; Speth *et al.*, 2012), “*Ca. Brocadia*” (Gori *et al.*, 2011; Oshiki *et al.*, 2015), “*Ca. Jettenia*” (Hira *et al.*, 2012; Hu *et al.*, 2012), and “*Ca. Scalindua*” (van de Vossenberg *et al.*, 2013; Speth *et al.*, 2015). Genes responsible for the anammox process have been identified from these genome sequences. Comparative genomic analysis has indicated that core gene sets including *hdh* and *hzs* are conserved in all the anammox bacterial genomes. On the other hand,

genus-specific distribution of genes encoding nitrite reductase has also been described; *i.e.*, “*Ca. Kuenenia*” and “*Ca. Scalindua*” encode *nirS*, whereas “*Ca. Jettenia*” encodes *nirK*. Intriguingly, both *nirS* and *nirK* were not found in the “*Ca. Brocadia sinica*” and “*Ca. Brocadia fulgida*” genomes composed of 3 and 2,786 contigs, respectively (threshold *e* values for blastn and blastp searches were e^{-10} and e^{-15} , respectively) (Gori *et al.*, 2011; Oshiki *et al.*, 2015). The absence of canonical NO-forming nitrite reductases was further confirmed by PCR detection of *nirS* and *nirK* (**Supplementary text**), suggesting the involvement of novel nitrite reductase(s) in NO_2^- reduction by “*Ca. Brocadia*”. Thus, it is speculated that the biochemical pathway for NO_2^- reaction and the downstream reaction (*i.e.*, N_2H_4 synthesis) in “*Ca. Brocadia*” is different from the proposed one in “*Ca. Kuenenia*”.

Here, we investigated the biochemical pathways of anammox process (*i.e.*, NO_2^- reduction, N_2H_4 synthesis and N_2H_4 oxidation) of “*Ca. B. sinica*” using a ^{15}N -tracer technique. The distinct features of “*Ca. B. sinica*” biochemistry were found as follows: 1) $^{15}\text{NO}_2^-$ reduction to $^{15}\text{NH}_2\text{OH}$ and 2) synthesis of $^{14-15}\text{N}_2\text{H}_4$ from $^{15}\text{NH}_2\text{OH}$ and $^{14}\text{NH}_4^+$, but not from ^{15}NO and $^{14}\text{NH}_4^+$. A candidate enzyme involved in the NO_2^- reduction was discussed based on the metagenomic data of “*Ca. B. sinica*” (Oshiki *et al.*, 2015). Furthermore, hydroxylamine disproportionation by “*Ca. B. sinica*” in which $^{15}\text{NH}_2\text{OH}$ was converted to $^{15}\text{NH}_4^+$ and $^{15-15}\text{N}_2$ was discussed because “*Ca. B. sinica*” cells catalyzed this reaction concurrently with anammox process.

Results

NO_2^- reduction to NH_2OH

Intermediates of NO_2^- reduction by “*Ca. B. sinica*” cells were examined by anoxically incubating the cells with $^{15}\text{NO}_2^-$ (2 mM), $^{14}\text{NH}_4^+$ (2 mM), $^{14}\text{NH}_2\text{OH}$ (0.1 mM) and acetylene (50 μM). Acetylene inhibited ammonium oxidation through the anammox pathway (Jensen *et al.*, 2007), resulting in accumulation of an intermediate of NO_2^- reduction (Kartal *et al.*, 2011). $^{14}\text{NH}_2\text{OH}$ was used as a pool substrate to detect newly synthesized $^{15}\text{NH}_2\text{OH}$ from $^{15}\text{NO}_2^-$ reduction because the turnover rate of $^{15}\text{NH}_2\text{OH}$ is very fast. The NH_2OH concentration was determined by gas chromatography mass spectrometry (GC/MS) after derivatisation of NH_2OH to acetoxime ($\text{C}_3\text{H}_7\text{NO}$, MW=73) with acetone (Peng *et al.*, 1999). Two molecular ion peaks were detected at $m/z = 73$ and 74, respectively, for the culture samples (**Fig. 1a**), whereas only a single ion peak was detected at $m/z = 73$ for a pure $^{14}\text{NH}_2\text{OH}$ solution (data not shown). This result indicates that the ion peak at $m/z = 74$ represents the presence of $^{15}\text{NH}_2\text{OH}$.

In the anoxic incubations, acetylene inhibited the production of $^{14-15}\text{N}_2$ (> 90%), resulting in the accumulation of $^{15}\text{NH}_2\text{OH}$ without delay (**Fig. 1b**).

Approximately one-fifth of the reduced $^{15}\text{NO}_2^-$ was detected as $^{15}\text{NH}_2\text{OH}$ during the 30 min of incubation with no accumulation of ^{15}NO (detection limit; < 5 ppm and < 3 nM in gas and liquid phase, respectively). The production of $^{14-15}\text{N}_2\text{H}_4$ and $^{15-15}\text{N}_2\text{H}_4$ was also not observed. $^{15}\text{NH}_2\text{OH}$ accumulation was not observed when the incubation was

repeated 1) with $^{15}\text{NH}_4^+$ instead of $^{15}\text{NO}_2^-$, 2) without biomass, and 3) with autoclaved biomass, indicating the $^{15}\text{NH}_2\text{OH}$ accumulation was a microbial process. The incubation was further repeated with ^{14}NO (10 μM) as a pool substrate instead of $^{14}\text{NH}_2\text{OH}$. Only 2% of the reduced $^{15}\text{NO}_2^-$ was detected as ^{15}NO (**Fig. 1c**).

N_2H_4 synthesis from NH_4^+ and NH_2OH but not from NH_4^+ and NO

Downstream reaction of NO_2^- reduction in anammox process is N_2H_4 synthesis. To examine substrates for N_2H_4 synthesis, “*Ca. B. sinica*” Hzs was purified from cell-free extracts by liquid chromatography, and the purified protein fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Three protein bands appeared on the PAGE gel (*i.e.*, B1, B2 and B3 in **Fig. 2a**), and were identified to be α , β and γ subunits of Hzs by nanoscale liquid chromatography tandem mass chromatography (nanoLC/MS/MS) analysis after in-gel tryptic digestion (**Table S1**).

The purified “*Ca. B. sinica*” Hzs was anoxically incubated with $^{14}\text{NH}_2\text{OH}$ (1 mM), $^{15}\text{NH}_4^+$ (1 mM) and cytochrome *c* (50 μM , equine heart), resulting in significant production of $^{14-15}\text{N}_2$ (**Fig. 2b**). The same incubation was repeated without addition of the cytochrome *c*. As a result, N_2H_4 accumulated up to 0.086 mM at the end of the incubation (data not shown). This result indicated that “*Ca. B. sinica*” Hzs was capable of synthesizing $^{14-15}\text{N}_2\text{H}_4$ from $^{14}\text{NH}_2\text{OH}$ and $^{15}\text{NH}_4^+$ and further oxidizing $^{14-15}\text{N}_2\text{H}_4$ to

$^{14-15}\text{N}_2$ (four-electron oxidation) using the cytochrome *c* as an electron acceptor.

Specific activity of $^{14-15}\text{N}_2$ production by “*Ca. B. sinica*” Hzs was 15 nmol-N mg-protein⁻¹ h⁻¹, which was comparable with that of “*Ca. Kuenenia stuttgartiensis*” Hzs; *i.e.*, 34 nmol-N mg-protein⁻¹ h⁻¹ (Kartal *et al.*, 2011).

The utilization of NO by “*Ca. B. sinica*” Hzs was also examined by supplementing “*Ca. B. sinica*” Hdh (4.7 µg) that oxidizes N_2H_4 to N_2 , $^{14-14}\text{N}_2\text{H}_4$ (5 µM), and the cytochrome *c* to the assay buffer as previously described (Kartal *et al.*, 2011). In this combined assay, electrons required for reduction of NO to NH_2OH (*i.e.*, 3 e^- were required) could be provided from $^{14-14}\text{N}_2\text{H}_4$ oxidation to N_2 catalyzed by Hdh via cytochrome *c*. For the NO assay, “*Ca. B. sinica*” Hdh was partially purified from cell-free extracts by liquid chromatography (**Fig. S1a**). The collected protein fraction gave UV-VIS spectra of anammox bacterial Hdh as previously described (Shimamura *et al.*, 2007) (**Fig. S1b and c**), and oxidized N_2H_4 to N_2 with a specific activity of 0.71 µmol of reduced cytochrome *c* mg-BSA⁻¹ min⁻¹, but not NH_2OH (<0.01 µmol of reduced cytochrome *c* mg-BSA⁻¹ min⁻¹). However, no significant production of $^{14-15}\text{N}_2$ was observed from ^{14}NO and $^{15}\text{NH}_4^+$ in the combined assay supplemented with the Hdh, $^{14-14}\text{N}_2\text{H}_4$, and cytochrome *c* (**Fig. 2b**), which confirmed the substrate of “*Ca. B. sinica*” Hzs was NH_2OH rather than NO.

N_2H_4 synthesis was further examined by incubating intact “*Ca. B. sinica*” cells with $^{15}\text{NH}_2\text{OH}$ (1.5 mM) and $^{14}\text{NH}_4^+$ (3 mM). As a result, concurrent production of

$^{14-15}\text{N}_2\text{H}_4$ and $^{14-15}\text{N}_2$ and consumption of $^{15}\text{NH}_2\text{OH}$ were observed without accumulation of ^{15}NO (**Fig. 3a**). Isotopomer of the produced N_2H_4 was analysed by GC/MS after derivatization using pentafluorobenzaldehyde (Preece *et al.*, 1992). The N_2H_4 derivatives in culture samples gave molecular ion peaks at $m/z = 389$ and 390 (**Fig. 3b**), whereas a single peak was observed at $m/z = 388$ when a pure $^{14-14}\text{N}_2\text{H}_4$ solution was analyzed (data not shown). Therefore, the observed molecular ion peaks at $m/z = 389$ and 390 represented $^{14-15}\text{N}_2\text{H}_4$ and $^{15-15}\text{N}_2\text{H}_4$, respectively. According to the isotopomer analyses of N_2H_4 and N_2 , “*Ca. B. sinica*” cells synthesized $^{14-15}\text{N}_2\text{H}_4$ from $^{15}\text{NH}_2\text{OH}$ and $^{14}\text{NH}_4^+$ and further oxidized to $^{14-15}\text{N}_2$. On the other hand, when “*Ca. B. sinica*” cells were incubated with ^{15}NO and $^{14}\text{NH}_4^+$ instead of $^{15}\text{NH}_2\text{OH}$ and $^{14}\text{NH}_4^+$, the cells did not produce $^{14-15}\text{N}_2$ (**Fig. S2**); supporting the substrate specificity of “*Ca. B. sinica*” Hzs as previously described.

Utilization of NO by “*Ca. B. sinica*” was further examined by incubating the cells with $^{15}\text{NH}_4^+$ (2.5 mM), $^{14}\text{NO}_2^-$ (2.5 mM) and a NO-scavenger, 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) (2.5 mM). Carboxy-PTIO did not inhibit $^{14-15}\text{N}_2$ production by “*Ca. B. sinica*” (**Fig. 4a**), but inhibited the $^{14-15}\text{N}_2$ production by “*Ca. Scalindua japonica*” that encodes *nirS* (**Supplementary text 1**) (**Fig. 4b**).

Notably, when “*Ca. B. sinica*” cells were incubated with $^{15}\text{NH}_2\text{OH}$ and $^{14}\text{NH}_4^+$, accumulations of $^{15}\text{NH}_4^+$, $^{15-15}\text{N}_2\text{H}_4$ ($m/z = 390$) and $^{15-15}\text{N}_2$ were detected in addition to

$^{14-15}\text{N}_2\text{H}_4$ and $^{14-15}\text{N}_2$ (**Fig. 3a, b**). The $^{15}\text{NH}_4^+$ and $^{15-15}\text{N}_2$ could be produced through hydroxylamine disproportionation as the following equation (Pacheco *et al.*, 2011), and

$^{15-15}\text{N}_2\text{H}_4$ can be synthesized from the produced $^{15}\text{NH}_4^+$ and $^{15}\text{NH}_2\text{OH}$:

To demonstrate hydroxylamine disproportionation by “*Ca. B. sinica*”, additional incubation was carried out with only $^{15}\text{NH}_2\text{OH}$. In this incubation, $^{15}\text{NH}_4^+$, $^{15-15}\text{N}_2$ and $^{15-15}\text{N}_2\text{H}_4$ were produced, but the $^{15-15}\text{N}_2\text{H}_4$ was produced with little delay (**Fig. 5**). This observation suggested that $^{15-15}\text{N}_2\text{H}_4$ was formed from $^{15}\text{NH}_2\text{OH}$ and the produced $^{15}\text{NH}_4^+$ in hydroxylamine disproportionation. Occurrence of hydroxylamine disproportionation can explain the greater production of $^{15-15}\text{N}_2$ than $^{14-15}\text{N}_2$ when “*Ca. B. sinica*” cells were incubated with $^{15}\text{NH}_2\text{OH}$ and $^{14}\text{NH}_4^+$ (**Fig. 3a**); *i.e.*, $^{15-15}\text{N}_2$ gas was directly produced from hydroxylamine disproportionation without formation of $^{15-15}\text{N}_2\text{H}_4$.

Discussion

Anammox pathway of “*Ca. B. sinica*”

In the present study, the anammox pathway of “*Ca. B. sinica*” that does not possess gene(s) encoding the canonical NO-forming nitrite reductases (neither NirS nor NirK) was investigated by ^{15}N -tracer experiments. The results revealed that “*Ca. B. sinica*” i) reduced NO_2^- to NH_2OH , ii) synthesised N_2H_4 from NH_2OH and NH_4^+ , and iii) oxidized N_2H_4 to N_2 , which was summarized in **Figure 6**. Although a trace amount of ^{15}NO was produced during $^{15}\text{NO}_2^-$ reduction (**Fig. 1c**), NO was not a substrate of “*Ca. B. sinica*” Hzs (**Fig. 2**) and cells (**Fig. S2**). A very small amount of ^{15}NO could be expected from $^{15}\text{NH}_2\text{OH}$ oxidation by anammox bacterial Hao (Shimamura *et al.*, 2007; Maalcke *et al.*, 2014). No inhibition of $^{14-15}\text{N}_2$ production by a NO scavenger, carboxy-PTIO, further supported that “*Ca. B. sinica*” cannot utilize NO for N_2H_4 synthesis.

The substrate of “*Ca. B. sinica*” Hzs was distinct from that of “*Ca. K. stuttgartiensis*” (sequence similarity to “*Ca. B. sinica*” HzsBGA; 79, 86 and 82%, respectively) (Kartal *et al.*, 2011). However, the recent crystallography study of “*Ca. K. stuttgartiensis*” Hzs suggested that N_2H_4 synthesis is a two-step reaction; 1) NO reduction to NH_2OH and 2) subsequent condensation of NH_2OH and NH_3 (Dietl *et al.*, 2015). Taken together, both the “*Ca. B. sinica*” and “*Ca. K. stuttgartiensis*” Hzs could utilize NH_2OH as a substrate for N_2H_4 synthesis, while NH_2OH was supplied in different manners; *i.e.*, “*Ca. B. sinica*” cells directly reduced NO_2^- to NH_2OH , while “*Ca. K. stuttgartiensis*” *nirS* reduced NO_2^- to NO (Kartal *et al.*, 2011) and the formed NO is further reduced to NH_2OH by “*Ca. K. stuttgartiensis*” Hzs (Dietl *et al.*, 2015).

It is a subject of great interest to identify an as yet unidentified nitrite

reductase(s) of “*Ca. B. sinica*” that could reduce NO_2^- to NH_2OH . The formation of NH_2OH during NO_2^- reduction might suggest the involvement of a relative of NH_4^+ -forming nitrite reductase(s). Pentahaem cytochrome *c* nitrite reductase (NrfA), a NH_4^+ -forming nitrite reductase, is able to perform a six-electron reduction of NO_2^- to NH_4^+ (Simon, 2002; Corker & Poole, 2003), in which NH_2OH is transiently formed as a result of a four-electron reduction (Berks *et al.*, 1995). On the other hand, a canonical NO-forming nitrite reductase performs a one-electron reduction of NO_2^- to NO (N oxidation state; from +3 to +2), in which NH_2OH (-1) cannot be formed. Notably, NrfA has evolved to an octahaem cytochrome *c* protein (Hao) (Bergmann *et al.*, 2005; Klotz *et al.*, 2008; Klotz & Stein, 2008; Kozłowski *et al.*, 2014; Poret-Peterson *et al.*, 2008), and octahaem cytochrome *c* nitrite reductase (designated as Hao-like protein here) that can reduce NO_2^- to NH_2OH using electrons shuttled from quinone pools by a membrane-anchored cytochrome *c* protein appeared during the evolution (Campbell *et al.*, 2009; Hanson *et al.*, 2013). Thus, Hao-like proteins are the most likely candidate enzymes catalysing NO_2^- reduction to NH_2OH in “*Ca. B. sinica*” as predicted from genomic studies of “*Ca. K. stuttgartiensis*” and “*Ca. S. profunda*” (Kartal *et al.*, 2013; van de Vossenberg *et al.*, 2013). Hao-like protein lacks the tyrosine residue needed for crosslinking of catalytic haem 4 (*i.e.* Y₄₆₇ of *Nitrosomonas europaea* Hao) (Igarashi *et al.*, 1997; Cederval *et al.*, 2013), and the genes encoding Hao-like protein without Y₄₆₇ residue were found from ten copies of the *hao/hdh* located in the “*Ca. B. sinica*” genome (Oshiki *et al.*, 2015); *i.e.*, the brosi_A0131, brosi_A0501, brosi_A3534, and brosi_A3864 genes (**Fig. S3**). Furthermore, a gene cluster encoding the *bc*₁ protein complex including a hexahaem cytochrome *c* protein was located upstream of the brosi_A0501 gene; *i.e.* the brosi_A0502 to brosi_A0506 genes. These proteins could be

involved in electron transfer from the quinone pool to the brosi_A0501 protein. It would be interesting to investigate the function of the brosi_A0501 protein in NO_2^- reduction in “*Ca. B. sinica*” cells.

In conclusion, the present study demonstrated a NH_2OH -mediating anammox process of “*Ca. B. sinica*”, which is distinct from the previously proposed NO-mediating one of “*Ca. K. stuttgartiensis*”. “*Ca. B. sinica*” cells could directly reduce NO_2^- to NH_2OH , while “*Ca. K. stuttgartiensis*” cells reduced NO_2^- to NO (Kartal *et al.*, 2011). The “*Ca. B. sinica*” Hzs could utilize NH_2OH , but not NO, as a substrate for N_2H_4 synthesis. This finding reflects the difference in their genomes showing that “*Ca. B. sinica*” does not have the genes encoding canonical NO-forming nitrite reductases (neither NirS or NirK). Furthermore, “*Ca. B. sinica*” could carry out hydroxylamine disproportionation concurrently with the anammox process. Obviously, further studies are required to identify as yet unidentified nitrite reductase(s) of “*Ca. B. sinica*” responsible for NO_2^- reduction to NH_2OH and a specific function of the brosi_A0501 protein in the NO_2^- reduction. Furthermore, it is of great interest to identify enzyme(s) catalyzing hydroxylamine disproportionation to examine how N=N double bond is formed in the reaction.

Experimental procedures.

Biomass. “*Ca. B. sinica*” cells were cultivated in a membrane bioreactor (MBR) equipped with a hollow fibre membrane module (pore size 0.1 μm , polyethylene) as previously described (Oshiki *et al.*, 2013a). “*Ca. B. sinica*” cells accounted for more than 94% of the total biomass as determined by fluorescence *in-situ* hybridisation (FISH) analysis using the oligonucleotide probes AMX820 (Schmid *et al.*, 2001) and EUBmix composed of equimolar EUB338, EUB338II, and EUB338III (Daims *et al.*, 1999). A monospecies of “*Ca. B. sinica*” in the culture was confirmed by determining the 16S rRNA gene sequence of cells in the enrichment culture.

Preparation of “*Ca. B. sinica*” Hzs and Hdh. “*Ca. B. sinica*” cells (20 g-wet) were suspended in 20 mM phosphate buffer (pH 7) at concentrations of 0.4 g-wet ml^{-1} , and disrupted thrice by bead beading at 5,000 rpm for 1 min (BioSpec Products, Bartlesville, OK, USA). The suspension was centrifuged at 13,400 g and 4 °C for 60 min, and the supernatant was collected as crude cell extracts. The crude extract was purified with Q sepharose XL media (GE healthcare, Little Chalfont, UK) equilibrated with 20 mM Tris-HCl buffer (pH 8). Binding proteins were eluted by increasing NaCl concentration stepwisely in the Tris buffer with 0.1 M increments. Proteins eluted at the concentration of 0.1 M NaCl contained Hzs, which were collected and concentrated by ultrafiltration using a Vivaspinn column (30 kDa MWCO) (GE healthcare, Little Chalfont, UK). Protein fraction containing Hdh was eluted at 0.3 M of NaCl, and concentrated by ultrafiltration (100 kDa MWCO). The Hzs and Hdh were further purified by gel chromatography using a Superdex 200 gel filtration column (GE healthcare, Little Chalfont, UK) equilibrated a 20 mM phosphate buffer (pH7.5) containing 0.15 M NaCl. The fractions containing Hzs or Hdh were pooled and concentrated by the ultrafiltration to be 3.8 and 0.82 mg ml^{-1} of

protein concentration, respectively. Purity of Hzs and Hdh was examined by SDS-PAGE analysis. Proteins were separated on a 9 or 8% SDS-containing polyacrylamide gel, respectively, and stained with CBB protein safe stain (Takarabio, Shiga, Japan) following the instruction manual supplied by manufactures.

Identification of Hzs was conducted by excising protein bands at 85, 45 and 36 kDa from the gel and subjected to nanoLC/MS/MS analysis after in-gel tryptic digestion (Kasahara *et al.*, 2012). Peptide mass fingerprints were analysed using the MASCOT search program version 2.3.01 (Perkins *et al.*, 1999). The amino acid sequences of genes located in the “*Ca. B. sinica*” genome (accession number:

BAFN01000001-BAFN01000003) (Oshiki *et al.*, 2015) were used as the reference database.

Activity of Hdh was examined as previously described (Shimamura *et al.*, 2007). One ml of 100 mM PO₄ buffer (pH8) containing 2.5 µg “*Ca. B. sinica*” Hdh, 50 µM cytochrome *c* ($\epsilon_{550} = 19.6 \text{ mM}^{-1} \text{ cm}^{-1}$), and 25 µM ¹⁴⁻¹⁴N₂H₄ or 500 µM ¹⁴NH₂OH was dispensed into 1.8-ml glass vials, and incubated in the anaerobic chamber at 20°C. After 5 min of the incubation, absorbance at a wavelength of 540 nm was determined.

Activity tests. Standard anaerobic techniques were employed in an anaerobic chamber where oxygen concentration was maintained at lower than 1 ppm (Oshiki *et al.*, 2013b). Anoxic buffers and stock solutions were prepared by repeatedly vacuuming and purging He gas (>99.99995%) before experiments. Purity of ¹⁵N-labelled compounds (*i.e.* ¹⁵NH₄Cl, Na¹⁵NO₂⁻, ¹⁵NH₂OH·HCl, ¹⁵NO gas, and ¹⁵⁻¹⁵N₂ gas), which were purchased from Cambridge Isotope Laboratories (Andover, MA, USA), was greater than 98%.

Anaerobic incubation using “*Ca. B. sinica*” cells was carried out at pH 7.6

and 37°C. The cells were suspended in inorganic nutrient media without NH_4^+ and NO_2^- (Oshiki *et al.*, 2013a) at concentrations of 0.1–1.0 mg protein ml^{-1} and dispensed into serum glass vials (Nichiden-Rika glass, Tokyo, Japan). After sealing with butyl rubber stoppers and aluminium caps, the headspace was replaced with He gas (>99.99995%), and the vials were incubated for up to 8 h in triplicates. Substrates and chemicals were added with the following combinations; i) $^{14}\text{NH}_4^+$, $^{15}\text{NO}_2^-$ (2 mM each), $^{14}\text{NH}_2\text{OH}$ (0.1 mM) and acetylene (50 μM), ii) $^{14}\text{NH}_4^+$, $^{15}\text{NO}_2^-$ (2 mM each), ^{14}NO (10 μM) and acetylene (50 μM), iii) $^{14}\text{NH}_4^+$ (3 mM) and $^{15}\text{NH}_2\text{OH}$ (1.5 mM), iv) $^{14}\text{NH}_4^+$ (3 mM) and ^{15}NO gas, and v) $^{15}\text{NH}_4^+$, $^{14}\text{NO}_2^-$ (2.5 mM each), carboxy-PTIO (Dojindo, Kumamoto, Japan) (2.5 mM), and vi) $^{15}\text{NH}_2\text{OH}$ (1.5 mM). As for ^{15}NO gas, 2 and 20 ml of the ^{15}NO gas were flushed to 4 ml of headspace in 7-ml glass vial by using a gas-tight glass syringe (VICI AG International, Schenkon, Switzerland). Liquid samples were collected using a 1-ml plastic disposable syringe, immediately filtered using 0.2- μm cellulose acetate filter, and subjected to determination of the concentrations of NH_4^+ , NO_2^- , NO , NH_2OH , and N_2H_4 and isotopomer analyses of NH_2OH and N_2H_4 . Gas samples were collected using a gas-tight glass syringe and immediately injected to a gas chromatograph to determine $^{14-15}\text{N}_2$, $^{15-15}\text{N}_2$, and ^{15}NO concentrations.

Anaerobic incubation of “*Ca. B. sinica*” Hzs was carried out to study utilization potential of NH_2OH and NO for N_2H_4 synthesis as follows. For the NH_2OH assay, 1 ml of 20 mM PO_4 buffer (pH7) containing 2.6 mg “*Ca. B. sinica*” Hzs, 50 μM cytochrome *c* (equine heart) (Wako, Osaka, Japan), 1 mM $^{15}\text{NH}_4^+$ and 1 mM $^{14}\text{NH}_2\text{OH}$ was dispensed in 1.8-ml glass vials (Shimadzu, Kyoto, Japan). The cytochrome *c* was used as an electron acceptor for N_2H_4 oxidation to N_2 . The vial was incubated in the anaerobic chamber at 37°C in triplicates. For the NO assay, 300 μl of ^{14}NO gas instead of

$^{14}\text{NH}_2\text{OH}$ was injected to the vial by using a gas-tight glass syringe, and the vial was shaken to dissolve the gaseous NO to the liquids prior to the incubation. Since the reduction of NO to NH_2OH by Hzs requires 3 electrons, combined assays of “*Ca. B. sinica*” Hzs and Hdh were carried out as previously described (Kartal *et al.*, 2011), in which electrons could be provided from N_2H_4 oxidation to N_2 by Hdh. Therefore, 5 μM $^{14-14}\text{N}_2\text{H}_4$ and 4.7 μg Hdh were supplemented to the assay buffer containing 1 mM $^{15}\text{NH}_4^+$, 2.6 mg “*Ca. B. sinica*” Hzs, 50 μM cytochrome *c* and 300 μl of ^{14}NO . Headspace gas was collected using a gas-tight glass syringe, and analyzed by GC/MS for determination of $^{14-15}\text{N}_2$ gas.

Chemical analysis. Concentrations of NH_4^+ and NO_2^- were determined using an ion chromatograph IC-2010 equipped with a TSKgel SuperIC-Anion HS or TSKgel SuperIC-Cation HS column (Tosoh, Tokyo, Japan) for anion or cation analysis, respectively. In addition, NO_2^- concentration was also determined by the naphthylethylenediamine method (American Public Health Association and American Water Works Association and Water Environment Federation, 2012). Samples were mixed with 4.9 mM naphthylethylenediamine solution, and the absorbance was measured at a wavelength of 540 nm.

NH_2OH concentrations were determined colorimetrically (Frear & Burrell, 1955). Samples were mixed with 0.48% (w/v) trichloroacetic acid, 0.2% (w/v) 8-hydroxyquinoline and 0.2 M Na_2CO_3 , heated at 100°C for 1 min, and the absorbance was measured at a wavelength of 705 nm. $^{15}\text{NH}_2\text{OH}$ concentrations were determined by GC/MS analysis after derivatisation using acetone (Peng *et al.*, 1999). One-hundred microliter of liquid sample was mixed vigorously with 4 μl of acetone, and 2 μl of the

derivatised sample was injected into a gas chromatograph GCMS-QP 2010SE equipped with a CP-Pora BOND Q fused silica capillary column in a splitless mode. NH_2OH was derivatized by acetone to acetoxime $\text{C}_3\text{H}_7\text{NO}$, and the molecular ion peaks were detected at $m/z = 73$ and 74 for $^{14}\text{NH}_2\text{OH}$ and $^{15}\text{NH}_2\text{OH}$, respectively. Standard curve ($1\text{--}30\text{ }\mu\text{M}$ of NH_2OH , $R^2 = 0.998$) was prepared using serially diluted NH_2OH solutions. The $^{15}\text{NH}_2\text{OH}$ determination by GC/MS was not hindered in the presence of NO molecules. Liquid sample containing several hundred micromolar ^{14}NO was derivatised and analysed in the same manner, while no mass peak was detectable at $m/z = 42$, 58 and 73 . The addition of ^{14}NO did not result in significant over- and underestimation of $^{15}\text{NH}_2\text{OH}$ concentration.

N_2H_4 concentrations were determined colorimetrically (Watt & Chrisp, 1952). Samples were mixed with 0.12 M 4-dimethylaminobenzaldehyde, and the absorbance was measured at a wavelength of 460 nm . Stable isotopic composition of N_2H_4 was examined by GC/MS after derivatisation using pentafluorobenzaldehyde (Preece *et al.*, 1992). Two-hundred microliters of liquid sample was mixed with $2\text{ }\mu\text{L}$ of thiosulfate (1 mg ml^{-1}), $0.2\text{ }\mu\text{l}$ of 14.8 M phosphoric acid and $20\text{ }\mu\text{l}$ of pentafluorobenzaldehyde (1% , w/v), and then incubated for 16 h at room temperature. After hexane extraction, organic phase ($2\text{ }\mu\text{l}$) was injected into a gas chromatograph GCMS-QP 2010SE equipped with a HP-5MS capillary column (Agilent Technologies, Santa Clara, CA, USA) in a splitless mode. Mass spectra were scanned from 10 to $400\text{ }m/z$.

NO concentration in liquid phase was determined by myoglobin absorbance measurement (detection limit; $20\text{ }\mu\text{M}$) (Hogg & Kalyanaraman, 1998) and by using a NO-sensitive microsensor (NO-100, detection limit; 3 nM) (Unisense science, Aarhus, Denmark). Determination of NO concentration by myoglobin absorbance measurement

was performed as previously described (Hogg & Kalyanaraman, 1998). Briefly, myoglobin from equine heart (Sigma-Aldrich, St. Louis, MO, USA) reduced with sodium dithionite was titrated with 25 μ l of the sample in a sealable 3-ml glass cuvette.

Absorbance was determined at a wavelength of 580 nm and NO concentration was calculated by using a titration curve prepared by using an NO-saturated aqueous solution and 10,300 M⁻¹ cm⁻¹ of molecular extinction coefficient. Conditioning, calibration, and measurement using the NO-sensitive microsensor were performed according to the manufacturer's instruction. The vials were uncapped in the anaerobic chamber and tip of the microsensor was dipped into the liquid culture.

Concentrations of ¹⁴⁻¹⁵N₂, ¹⁵⁻¹⁵N₂ and ¹⁵NO gas were determined by GC/MS analysis (Isobe *et al.*, 2011a). Fifty microliters of headspace gas were injected into a GCMS-QP2010SE gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a CP-Pora BOND Q fused silica capillary column (Agilent Technologies, Santa Clara, CA, USA) or Agilent 5975C gas chromatograph equipped with a Porapak Q column (Agilent Technologies, Santa Clara, CA, USA). A standard curve for ¹⁴⁻¹⁵N₂ and ¹⁵⁻¹⁵N₂ gas quantification was prepared with ¹⁵⁻¹⁵N₂ gas. A standard curve for ¹⁵NO gas quantification was prepared with a ¹⁴NO (4,000 ppm) gas.

¹⁵N/¹⁴N ratio of NH₄⁺ was determined by GC/MS analysis after conversion of NH₄⁺ to N₂O gas as previously described by Isobe *et al.* (2011b) with minor modification. Briefly, NH₄⁺ in liquid samples was collected by distillation, oxidized to NO₃⁻ by potassium persulfate, and reduced to N₂O gas by the denitrifier method using *Pseudomonas chlororaphis* subsp. *aureofaciens* ATCC13985^T. The formed N₂O gas was injected into the GCMS-QP2010SE gas chromatograph equipped with a CP-Pora BOND Q fused silica capillary column.

Bradford assay with Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) was performed to determine protein concentrations according to the manufacturer's instructions. Bovine serum albumin was used as a protein standard.

Thermodynamic calculations. The Gibbs energy of the reaction (ΔG_R^0) was calculated from the Gibbs free energies of formation (ΔG_f^0). The calculation was performed under the following conditions; 298K, 0.1 MPa, pH 7, and concentrations of products and reactants are 1 M. ΔG_f^0 values for NH_4^+ , NO_2^- , NO, N_2 , and H_2O were taken from the *Chemical Handbook* (The Chemical Society of Japan, 2005), and those for NH_2OH and N_2H_4 were taken from the previous literature (van der Star *et al.*, 2008).

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Figure legends

Fig. 1. Reduction of $^{15}\text{NO}_2^-$ to $^{15}\text{NH}_2\text{OH}$ and ^{15}NO : (a) Mass chromatogram of NH_2OH derivatives. Liquid sample collected during the anoxic incubation shown in the panel (b) was derivatised using acetone for gas chromatography mass spectrometry (GC/MS) analysis. $^{14}\text{NH}_2\text{OH}$ derivative ($\text{C}_3\text{H}_7^{14}\text{NO}$, molecular weight =73) showed mass peaks at $m/z = 42, 58$ and 73 . Occurrence of peak shifts with $m/z = 1$ interval (*i.e.* $m/z = 43, 59$, and 74) indicated $^{15}\text{NH}_2\text{OH}$ formation. (b) $^{15}\text{NO}_2^-$ reduction to $^{15}\text{NH}_2\text{OH}$. The cells were anoxically incubated in 12.5-ml glass vials with addition of $^{14}\text{NH}_4^+$ (2 mM), $^{15}\text{NO}_2^-$ (2 mM), $^{14}\text{NH}_2\text{OH}$ (0.1 mM), and acetylene (50 μM). $^{15}\text{NO}_2^-$ (triangle) and $^{15}\text{NH}_2\text{OH}$ (circle) concentrations and $^{15}\text{N}/^{14}\text{N}$ isotopic ratio of NH_2OH (square) were determined in time course. Error bars represent standard deviations obtained from triplicate vials. Both the $^{15}\text{NO}_2^-$ reduction and $^{15}\text{NH}_2\text{OH}$ accumulation were not detectable in vials without biomass. ^{15}NO accumulation was not detectable during the incubation. (c) $^{15}\text{NO}_2^-$ reduction to ^{15}NO by “*Candidatus Brocadia sinica*” cells. The anoxic incubation was repeated with ^{14}NO (10 μM) as a pool substrate instead of $^{14}\text{NH}_2\text{OH}$. A trace amount of ^{15}NO gas production was determined in time course. Error bars represent standard deviations obtained from triplicate vials.

Fig. 2. Purification and characterization of “*Candidatus Brocadia sinica*” hydrazine synthase (Hzs): (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of a protein fraction of “*Ca. B. sinica*” Hzs purified by gel chromatography (lane S) with molecular size standard markers (lane M, unit: kDa). Three prominent protein bands (*i.e.*, B1, B2 and B3) appeared at 85, 45 and 36 kDa, respectively. The protein bands were excised and subjected to peptide mass fingerprint

analyses, and identified as α , β and γ subunits of “*Ca. B. sinica*” Hzs, respectively (See **Table S1** for the outcomes of peptide mass finger print analyses). **(b)** Substrate utilization potential of the purified “*Ca. B. sinica*” Hzs. Hzs was anoxically incubated for 12 h in 1.8-ml glass vials containing 1 ml of assay buffer containing 20 mM PO_4 buffer (pH7), 2.6 mg Hzs, 50 μM cytochrome *c* (cyt), 1 mM $^{15}\text{NH}_4^+$ and 1 mM $^{14}\text{NH}_2\text{OH}$, and $^{14-15}\text{N}_2$ gas production was examined by gas chromatography mass spectrometry (GC/MS). Significant $^{14-15}\text{N}_2$ was produced in time course with a rate of 15 nmol-N mg-protein $^{-1}$ h $^{-1}$ (closed triangle), while $^{14-15}\text{N}_2$ production was not found when incubated without Hzs or both Hzs and cyt (open triangle or square, respectively). The same anoxic incubation was repeated with ^{14}NO instead of $^{14}\text{NH}_2\text{OH}$ (injection of 300 μl ^{14}NO gas into headspace) (closed grey circle) and with addition of 4.7 μg “*Ca. B. sinica*” hydrazine dehydrogenase (Hdh) and 5 μM $^{14-14}\text{N}_2\text{H}_4$ (closed black circle) (see Experimental procedures for the details). However, no significant $^{14-15}\text{N}_2$ production was detected as compared with the incubation without Hzs (open circle). All the incubations were performed in triplicate, and error bars represent the standard deviation.

Fig. 3. Consumption of $^{15}\text{NH}_2\text{OH}$ by “*Candidatus Brocadia sinica*” cells: The 30-ml cell suspension containing $^{15}\text{NH}_2\text{OH}$ (1.5 mM) and $^{14}\text{NH}_4^+$ (3 mM) was anoxically incubated in 120-ml glass vials at 37°C. **a)** Time-course monitoring of N_2H_4 (open circle), NH_2OH (circle, black), NH_4^+ (circle, grey), $^{14-15}\text{N}_2$ (square, red) and $^{15-15}\text{N}_2$ (square, blue) concentrations. Bottom panel shows $^{15}\text{N}/^{14}\text{N}$ ratio of NH_4^+ , and increase of the ratio indicates accumulation of $^{15}\text{NH}_4^+$. Error bars represent standard deviations derived from triplicate vials. ^{15}NO accumulation was not detectable, and thus not shown here. **b)** Mass chromatogram of N_2H_4 derivatives (molecular ion peaks at $m/z = 389$ and 390, which

correspond to $^{14-15}\text{N}_2\text{H}_4$ and $^{15-15}\text{N}_2\text{H}_4$ derivatives, respectively). Liquid sample after 90 min of incubation was derivatised using pentafluorobenzaldehyde and analysed by gas chromatography mass spectrometry (GC/MS).

Fig. 4. Influences of a NO-scavenger, carboxy-PTIO, on $^{14-15}\text{N}_2$ gas production by “*Candidatus Brocadia sinica*” (a) and “*Ca. Scalindua japonica*” cells (b): The cells were anoxically incubated in 7-ml glass vials containing 2-ml cell suspension in the presence of $^{15}\text{NH}_4^+$ and $^{14}\text{NO}_2^-$ (each 2.5 mM). The incubations were performed with or without 2.5 mM carboxy-PTIO (circle or square, respectively). Specific activities of $^{14-15}\text{N}_2$ gas production by “*Ca. Brocadia sinica*” cells with and without the carboxy PTIO were 15 ± 2.1 (mean $\pm$ standard deviation) and 14 ± 3.0 amol cell $^{-1}$ h $^{-1}$, respectively. Draft genome sequence of “*Ca. Scalindua japonica*” has been determined recently, and a gene encoding cytochrome *cd_I*-type NO-forming nitrite reductase (*nirS*) was found in the determined genome (*e.g.*, M. Oshiki, unpublished)

Fig. 5. Hydroxylamine disproportionation by “*Candidatus Brocadia sinica*” cells: The 30-ml cell suspension containing $^{15}\text{NH}_2\text{OH}$ (1.5 mM) was anoxically incubated in 120-ml glass vials at 37°C, and concentrations of $^{15}\text{NH}_2\text{OH}$ (circle, black), $^{15-15}\text{N}_2\text{H}_4$ (opened circle), $^{15}\text{NH}_4^+$ (circle, grey), $^{14-15}\text{N}_2$ (square, red) and $^{15-15}\text{N}_2$ (square, blue) were monitored. Accumulations of $^{15}\text{NH}_4^+$ and $^{15-15}\text{N}_2$ were observed immediately after the incubation was started, while $^{15-15}\text{N}_2\text{H}_4$ accumulation occurred after 2 h of incubation. Productions of $^{14-15}\text{N}_2$ and $^{15-15}\text{N}_2$ were not observed when the incubation was repeated without “*Ca. B. sinica*” cells, indicating that hydroxylamine disproportionation is an enzymatically catalyzed reaction.

Fig. 6. Anaerobic ammonium oxidation pathway by “*Candidatus Brocadia sinica*”:

Reactions indicated with solid arrows were confirmed in the present study by ^{15}N tracer experiments. A dashed arrow indicates a possible NH_2OH oxidation to NO , which was not found to occur in the present study. $\Delta G_R^{\theta'}$; Gibbs energy of the reaction.

Supporting Information.

Additional supplementary information for the manuscript can be found in the online version of this article on the publisher's website. The supplementary information includes 1 MS-word text files, 3 supplemental figures, and 2 supplemental table files.

Descriptions of supporting MS-word text files

Supplementary text: PCR detection of *nirS* and *nirK* from “*Candidatus Brocadia sinica*” genome.

Supporting figure legends

Fig. S1. Purification and characterization of “*Candidatus Brocadia sinica*”

hydrazine dehydrogenase (Hdh): a) SDS-PAGE analysis of the purified Hdh.

Cell-free extracts of “*Ca. B. sinica*” was subjected to anion-exchange and gel filtration chromatography to purify “*Ca. B. sinica*” Hdh. Proteins in the collected protein fraction (specific activities of N_2H_4 and NH_2OH oxidation were 0.71 and <0.01 μmol of reduced cytochrome *c* $\text{mg-BSA}^{-1} \text{ min}^{-1}$, respectively.) were separated in 8% polyacrylamide gel.

Lane 1 and 2; the collected protein fractions (3.2 μg), and lane M; molecular weight marker (kDa). Aggregates of anammox bacterial hydroxylamine oxidoreductase (Hao) and Hdh can not be denatured by SDS (Schalk et al., 2000; Shimamura et al., 2007); therefore an intense band corresponding to “*Ca. B. sinica*” Hdh appeared at top of the gel as indicated with an arrow. **b) UV-VIS absorption spectra of Hdh.** Solid lines with open and closed circles indicate Hdh (60 $\mu\text{g-BSA ml}^{-1}$) oxidized with air and reduced with sodium dithionite, respectively. **c) Magnified spectra shown in panel b are in the**

range of 440–580 nm. The purified protein reduced with sodium dithionite produced

Soret, β -band, and α -band peaks at 420, 524, and 553 nm, respectively, and an additional peak at 472 nm. These peaks were previously reported for “*Ca. Jettenia caeni*” Hdh by Shimamura *et al.* (2007).

Reference:

Schalk, J., de Vries, S., Gijs Kuenen, J. and Jetten, M.S.M. 2000. Involvement of a novel hydroxylamine oxidoreductase in anaerobic ammonium oxidation. *Biochemistry* **39**: 5405-5412.

Shimamura, M., Nishiyama, T., Shigetomo, H., Toyomoto, T., Kawahara, Y., Furukawa, K. and Fujii, T. 2007. Isolation of a multiheme protein with features of a hydrazine-oxidizing enzyme from an anaerobic ammonium-oxidizing enrichment culture. *Appl. Environ. Microbiol.* **73**: 1065-1072.

Fig. S2. Consumption of ^{15}NO by “*Candidatus Brocadia sinica*” cells: The cells were anoxically incubated in the presence of $^{14}\text{NH}_4^+$ (3 mM) and ^{15}NO . Twenty millilitre of ^{15}NO gas was flushed into 7-ml glass vials containing 3-ml cell suspension, and then the vials were vigorously shaken to dissolve gaseous ^{15}NO to the culture liquids. $^{14}\text{NH}_4^+$ and ^{15}NO were added at a time point indicated with an arrow. Concentrations of NO in the liquid phase (diamond) and $^{14-15}\text{N}_2$ gas in the headspace (circle) were monitored by myoglobin absorbance measurement and gas chromatography mass spectrometry (GC/MS), respectively. Error bar represents the standard deviations of NO concentrations derived from triplicate measurements. Control experiments without the cells were performed in parallel, which were indicated with open symbols. Abiotic production of $^{14-15}\text{N}$ gas occurred due to high reactivity of NO. During 6 h of incubation,

the vials with and without the cells produced $^{14-15}\text{N}_2$ gas at rates of 0.123 ± 0.0045 (mean $\pm$ standard deviation) and $0.12 \pm 0.019 \mu\text{M h}^{-1} \text{ vial}^{-1}$, respectively, indicating no significant difference between the vials ($p = 0.05$, t -test). The same incubation was repeated in the presence of $^{14}\text{NH}_4^+$ (3 mM) and 2 ml of ^{15}NO , where the initial NO concentration in liquid phase was 33 μM . Even in this incubation, no difference in the $^{14-15}\text{N}_2$ gas production was detected between vials with and without the cells.

Fig. S3. Multiple copies of hydroxylamine dehydrogenase/hydrazine dehydrogenase (*hao/hdh*) genes found in the “*Candidatus Brocadia sinica*” genome: Arrow-shaped boxes depict “*Ca. B. sinica*” Hao/Hdh, which were aligned based on the position of catalytic haem 4 indicated with a dotted line. Rectangular boxes filled in black, brown, green and sky blue indicate transmembrane helix (TMH) predicted by the TMHMM program (version 2.0), canonical haem *c*-binding motif “CxxCH”, atypical haem *c*-binding motif “CxxxxCH”, and tyrosine residue needed for crosslinking of catalytic haem 4, respectively. The brosi_A2345 and brosi_A2680 genes contain the haem *c*-binding motif “CxxxxCH” at haem 3, which is a genetic feature of anammox bacterial *hdh* (Shimamura *et al.*, 2007; Kartal *et al.*, 2011). The brosi_A2677 gene is an orthologue of the kustc1061 and KSU1_D0438 genes. The enzymes encoded by these genes catalyse the oxidation of NH_2OH to NO (Schalk *et al.*, 2001; Shimamura *et al.*, 2008; Kartal *et al.*, 2011). Arrows indicate cleavage sites of signal peptides predicted by the SignalP program (version 4.1). Scale represents 100 amino acid (AA) residues.

Supporting Table legends

Table S1. Protein identification by nanoscale liquid chromatography tandem mass chromatography (nanoLC/MS/MS) analysis: Proteins in the protein bands B1, B2 and B3 (**Fig. 2a**) were identified by nanoLC/MS/MS analysis after in-gel tryptic digestion.

Score: the sum of the scores of the individual peptides, Coverage; percentage of the specific protein sequence covered by identified peptides, # Unique Peptides; number of peptides that are unique to a protein group, MW; expected molecular weight without signal peptide sequences that were predicted by the SignalP 4.1 server. The “*Candidatus* Brocadia sinica” genome encodes two copies of *hzsBGA* (*i.e.*, the brosi_A0629 to brosi_A0631 and the brosi_A2674 to brosi_A2676 genes). Because protein sequences of the two copies of *hzsBGA* were identical, those could not be distinguished by peptide mass fingerprinting.

Table S2. Oligonucleotide sequences of primers targeting *nirS* and *nirK* of anammox bacteria and/or denitrifiers.

Fig.1 (Oshiki *et al.*,2016)

Fig.2 (Oshiki *et al.*,2016)

Figure 2
96x55mm (300 x 300 DPI)

Accepted

Fig.3 (Oshiki *et al.*, 2016)

a) “*Ca. Brocadia sinica*”

b) “*Ca. Scalindua japonica*”

Fig. 4 (Oshiki *et al.*, 2016)

Fig. 5 (Oshiki *et al.*, 2016)

Figure 5
129x128mm (300 x 300 DPI)

Fig. 6 (Oshiki *et al.*, 2016)

Figure 6
164x208mm (300 x 300 DPI)