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# Anammox—Growth Physiology, Cell Biology, and Metabolism

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## ABSTRACT

Anaerobic ammonium-oxidizing (anammox) bacteria are the last major addition to the nitrogen-cycle (N-cycle). Because of the presumed inert nature of ammonium under anoxic conditions, the organisms were deemed to be nonexistent until about 15 years ago. They, however, appear to be present in virtually any anoxic place where fixed nitrogen (ammonium, nitrate, nitrite) is found. In various marine ecosystems, anammox bacteria are a major or even the only sink for fixed nitrogen. According to current estimates, about 50% of all nitrogen gas released into the atmosphere is made by these bacteria. Besides this, the microorganisms may be very well suited to be applied as an efficient, cost-effective, and environmental-friendly alternative to conventional wastewater treatment for the removal of nitrogen.

So far, nine different anammox species divided over five genera have been enriched, but none of these are in pure culture. This number is only a modest reflection of a continuum of species that is suggested by 16S rRNA analyses of environmental samples. In their environments, anammox bacteria thrive not just by competition, but rather by delicate metabolic interactions with other N-cycle organisms. Anammox bacteria owe their position in the N-cycle to their unique property to oxidize ammonium in the absence of oxygen.

Recent research established that they do so by activating the compound into hydrazine ( $N_2H_4$ ), using the oxidizing power of nitric oxide (NO). NO is produced by the reduction of nitrite, the terminal electron acceptor of the process. The forging of the N—N bond in hydrazine is catalyzed by hydrazine synthase, a fairly slow enzyme and its low activity possibly explaining the slow growth rates and long doubling times of the organisms. The oxidation of hydrazine results in the formation of the end product ( $N_2$ ), and electrons that are invested both in electron-transport phosphorylation and in the regeneration of the catabolic intermediates ( $N_2H_4$ , NO). Next to this, the electrons provide the reducing power for  $CO_2$  fixation. The electron-transport phosphorylation machinery represents another unique characteristic, as it is most likely localized on a special cell organelle, the anammoxosome, which is surrounded by a glycerolipid bilayer of ladder-like (“ladderane”) cyclobutane and cyclohexane ring structures.

The use of ammonium and nitrite as sole substrates might suggest a simple metabolic system, but the contrary seems to be the case. Genome analysis and ongoing biochemical research reveal an only partly understood redundancy in respiratory systems, featuring an unprecedented collection of cytochrome *c* proteins. The presence of the respiratory systems lends anammox bacteria a metabolic versatility that we are just beginning to appreciate. A specialized use of substrates may provide different anammox species their ecological niche.

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## ABBREVIATIONS

16S rRNA	16S ribosomal ribonucleic acid
Anammox	anaerobic ammonium oxidation
DNRA	dissimilatory nitrite reduction to ammonium
HAO	hydroxylamine oxidoreductase

HDH	hydrazine dehydrogenase
HZS	hydrazine synthetase
MBR	membrane bioreactor
Nar	nitrate reductase
NirS	nitrite::nitric oxide oxidoreductase; cytochrome <i>cd<sub>1</sub></i> nitrite reductase
NrfA	dissimilatory nitrite::ammonium oxidoreductase
OMZ	oxygen minimum zone
Pmf	proton-motive force
RET	reversed-electron transport
SBR	sequencing batch reactor

## 1. INTRODUCTION

Our atmosphere consists of 78% of dinitrogen gas. While relatively constant in concentration over geochemical ages,  $N_2$  is continuously taken up and produced at a turnover rate of one billion years<sup>-1</sup>. Nitrogen conversion is balanced by nitrogen fixation, anammox, denitrification, and other processes in the nitrogen-cycle (N-cycle), which is mainly driven by the action of microorganisms. Human activities, however, may disturb the balance not only locally but perhaps also on a global scale ([Galloway \*et al.\*, 2008](#)). Industrial nitrogen fixation by the Haber Bosch process has been of vital importance for modern agriculture. However, the use of fixed nitrogen (ammonium, nitrate) has drawbacks such as toxicity to fish, eutrophication of fresh water ecosystems, emission of the potent greenhouse gas nitrous oxide ( $N_2O$ ), and acid rain. Fertilizer run-off and the drainage of large amounts of organic waste into rivers and estuaries result in the horizontal and vertical expansion of oxygen-depleted zones in the oceans with side effects as diverse as habitat compression of fish and, again, increased  $N_2O$  production ([Duce \*et al.\*, 2008](#); [Stramma \*et al.\*, 2012](#)). The effects are only partly understood due to our limited knowledge of the microbial N-cycle processes. The cycle that was assumed to be fairly well understood for more than a century appears to be much more intricate due to the action of overlooked key players, ignored partial processes, and highly complex microbial interactions. Regardless of the state of this knowledge, wastewater treatment systems are required to prevent the supply of overloads of fixed nitrogen into the environment, especially in densely populated areas.

It now turned out that on the average, every other  $N_2$  molecule in the atmosphere is made by a group of microorganisms that were deemed to be nonexistent until about 15 years ago: anaerobic ammonium-oxidizing (anammox) bacteria (Mulder *et al.*, 1995). Anammox bacteria couple the oxidation of ammonium with nitrite reduction and produce  $N_2$  (Van de Graaf *et al.*, 1995, 1996). The long-time common notion that the activation of the inert ammonium molecule *per se* would require oxygen apparently blindfolded the search for organisms that convert the compound anoxically. Moreover, anammox bacteria appear to be notoriously slow growing and require dedicated enrichment and cultivation techniques, which could be factors that may have prevented their earlier detection. Still, merely based on thermodynamic considerations, their existence was predicted by the Austrian physicist Broda (1977). In fact, oceanographers had already noticed an unexplainable loss of ammonium in stratified anoxic zones in marine environments (Hamm and Thompson, 1941; Richards, 1965). A similar observation in a denitrifying bioreactor prompted the quest for the causative agents. Using specific enrichment techniques and batch tests with inhibitors, the anammox process was established to be microbiological (Van de Graaf *et al.*, 1995). Following its isolation (>99.6% pure) from the enrichment culture by gradient ultracentrifugation, a bacterial species was obtained that specifically produced  $N_2$  from ammonium and nitrite (Strous *et al.*, 1999a). Also, cells were capable of  $CO_2$  fixation, making anammox a chemolithoautotroph. In addition to the unique metabolic properties, it was suggested that the anammox bacteria may be very well suited to be applied as an efficient, cost-effective, and environmental-friendly alternative to conventional wastewater treatment for the removal of nitrogen (Jetten *et al.*, 1997).

Anammox bacteria are quite amazing in a number of aspects. Their ability to oxidize ammonium anaerobically requires a specialized catabolic mechanism. 16S ribosomal ribonucleic acid (16S rRNA) analysis places the organisms within the phylum *Planctomycetes* (Fuerst and Sagulenko, 2011), which together with the *Verrucomicrobia* and *Chlamydiae* forms the PVC superphylum (Wagner and Horn, 2006). From the prokaryotic perspective, *Planctomycetes* are characterized by a highly complicated cell plan featuring intracellular organelles (Lindsay *et al.*, 2001; Fuerst, 2005). In anammox bacteria, the organelle, termed the anammoxosome, comprises 50–70% of the cell volume (Lindsay *et al.*, 2001; Van Niftrik *et al.*, 2004, 2008a,b). Cell membranes surrounding the anammoxosome and outer parts of the cell are composed of lipid structures, called ladderanes, which are truly unique in nature (Sinninghe Damsté *et al.*, 2002; see Section 3). The specific metabolic properties, distinctive proteins,

characteristic 16S rRNA gene sequences, and typical lipid components provide us with a toolbox to detect these organisms in their natural habitats and to evaluate their role in different ecosystems. These aspects and their potential for wastewater treatment has raised broad scientific interest all over the world resulting in about 600 papers in slightly more than a decade (for reviews over this time, see i.e., [Jetten et al., 2003, 2005, 2009; Kuenen, 2008](#)).

At the moment, nearly 2000 16S rRNA gene sequences affiliated with anammox bacteria have been deposited in the GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). These sequences and other biomarkers have been obtained from laboratory enrichments and samples taken from virtually any environment including soil, groundwater, wastewater treatment plants, freshwater and marine sediments, lakes and estuaries, the oceans' oxygen minimum zones (OMZs) and continental shelves, polar regions as well as hot springs and deep-sea hydrothermal vents ([Op den Camp et al., 2006; Penton et al., 2006; Jetten et al., 2009; Humbert et al., 2010](#)). The once-thought "impossible" organisms appear to be ubiquitously present in suboxic, fixed-nitrogen-containing environments. Whereas their numbers may be modest in some systems, the bacteria are metabolically dominant in others, like in the Black Sea ([Kuypers et al., 2003; Lam et al., 2007](#)) and the Benguelan ([Kuypers et al., 2005; Woebken et al., 2007](#)), Arabian Sea ([Jensen et al., 2011](#)), Chilean, and Peruvian ([Lam et al., 2009](#)) OMZs, which are the largest primary production sites in the oceans. Indeed, current estimates attribute about 50% of all dinitrogen gas released into the atmosphere to be derived from anammox activity ([Arrigo, 2005](#)).

So far, nine anammox species have been isolated, albeit none of these in classical pure culture, lending them a *Candidatus* status. Sequence identities of the 16S rRNA gene range between 87% and 99%. Together, deposited 16S rRNA gene sequences cover a spectrum that gives room to a continuum of as yet uncultured species, subspecies, and strains, each having found its specific niche in the enormous variety of habitats where the organisms are found. Known species are divided over five genera ([Jetten et al., 2010](#)), all belonging to the same order *Brocadiales*, which constitutes a monophyletic clade, deeply branching inside the *Planctomycetes* ([Fuerst and Sagulenko, 2011](#)). *Kuenenia*, represented by *K. stuttgartiensis* ([Strous et al., 2006](#)), *Brocadia* (three species: *B. anammoxidans*, *B. fulgida*, and *B. sinica*; [Strous et al., 1999a; Kartal et al., 2008; Oshiki et al., 2011](#)), *Anammoxoglobus* (one species: *A. propionicus*; [Kartal et al., 2007a](#)), and *Jettenia* (one species: *J. asiatica*; [Quan et al., 2008; Hu et al., 2011](#)) have been enriched from activated sludge, *K. stuttgartiensis*, *B. anammoxidans*,

*B. fulgida*, and *A. propionicus* even from the same inoculum. The fifth genus, *Scalindua* (three known species: *S. brodae*, *S. sorokinii*, and *S. wagneri*; Schmid *et al.*, 2003; Woebken *et al.*, 2008), inhabits the marine environment in an unappreciated microdiversity (Schmid *et al.*, 2007; Woebken *et al.*, 2008; Dang *et al.*, 2010; Hong *et al.*, 2011a,b; Li *et al.*, 2011), but it is found in freshwater systems and wastewater treatment systems as well (Schmid *et al.*, 2003; Schubert *et al.*, 2006; Hamersley *et al.*, 2009). The genome of *Candidatus K. stuttgartiensis* was assembled from an environmental metagenome (Strous *et al.*, 2006), while the genomes sequencing projects of several other species are underway (e.g., Gori *et al.*, 2011). The *K. stuttgartiensis* genome and transcriptome revealed one of the most redundant organizations with respect to electron transfer processes, in which no less than 63 different cytochrome *c*-type proteins make up about 30% of their protein complement. In addition, the genome sequencing and concomitant physiological experiments showed anammox bacteria to be more than a lithotrophic specialist and suggested a metabolic versatility, which is neither explored nor exploited. Most and for all, the work provided the conceptual framework for a fundamental understanding of the enigmatic anammox metabolism.

Anammox bacteria are slow-growing microorganisms with a complex cell biology comprising an intracellular organelle surrounded by ladderane lipids that have to deal with an inert substrate (ammonium) under highly competitive and anoxic conditions. Still, the organisms found a firm place on Earth, even in a presumably broad range of species, having a great potential in advanced wastewater treatment technology (Kuenen, 2008; Siegrist *et al.*, 2008; Kartal *et al.*, 2010a). So, the questions are what makes anammox utterly successful and which factors determine species differentiation? Hereafter, we will try to answer the questions from what is known about their growth physiology, and how cell plan and function unite in cell biology and metabolism.

## 2. ANAMMOX GROWTH PHYSIOLOGY

For a long time, culturing anammox bacteria has been a challenge due to their long doubling times. This requires culture techniques in which biomass is retained effectively at low substrate concentration as is found in natural habitats. These demands are met by a sequencing batch reactor (SBR) setup (Strous *et al.*, 1998; Kartal *et al.*, 2011a), which now has been successfully applied in many laboratories (Op den Camp *et al.*, 2006). By

continuous cycles of filling, biomass settling, and withdrawal of the supernatant, cells are selected for their settling properties and essentially are kept in the reactor for an indefinite period of time. For anammox enrichment, reactors inoculated with an environmental sample are fed with ammonium, nitrite, and bicarbonate. Nitrate is added to avoid low-redox potentials that would favor growth of sulfate reducers or methanogens. As anammox bacteria are obligate anaerobes, reactor and feed are sparged with a mixture of argon, helium or nitrogen gas, and CO<sub>2</sub>. Nitrite is initially added at very low concentrations. At concentrations above 10 mM, nitrite impairs the metabolism, whereas growth is completely arrested above 20 mM (Strous *et al.*, 1999b). One may note that, in the literature, different concentrations are reported with respect to the nitrite toxicity (Table 1; Egli *et al.*, 2001; Strous *et al.*, 1999b); possibly the sensitivity depends on the exposure time or is species-dependent. In the course of the enrichment, the influent concentration is gradually increased, taking care that the nitrite inside the reactor remains low. Intermittently, other nutrients are tested for stimulation on growth. If successful, the reactor typically has turned red after 180–280 days, which is characteristic for the presence of anammox bacteria that may comprise by now about 70–80% of the bacterial population. From the population increase and mass balances, an

Table 1 Physiological characteristics of *Brocadia anammoxidans*, *Brocadia sinica*, and *Kuenenia stuttgartiensis*.

Physiological characteristic	<i>B. anammoxidans</i>	<i>B. sinica</i>	<i>K. stuttgartiensis</i>
Growth temperature (°C)	20–43	25–45	25–37
Growth pH	6.7–8.3	7.0–8.8	6.5–9.0
Growth rate (h <sup>-1</sup> )	0.0027	0.0041	0.0026–0.0035
Doubling time (days)	10.7	7	8–11
Biomass yield (mmol C mmol N <sup>-1</sup> )	0.07	0.0063	ND
<i>K<sub>m</sub></i> for ammonia (μM)	<5	28 ± 4	ND
<i>K<sub>m</sub></i> for nitrite (μM)	<5	86 ± 4	0.2–3
Tolerance:			
Nitrite (mM)	7	<16	13, 25
Dissolved oxygen (μM)	<1	<63	0–200
NaCl (mM)	ND	<500	200
References	Strous <i>et al.</i> (1997, 1998, 1999b), Jetten <i>et al.</i> (2005)	Oshiki <i>et al.</i> (2011)	Egli <i>et al.</i> (2001), van der Star <i>et al.</i> (2008a,b)

*K<sub>m</sub>*, apparent half-saturation constant for activity; ND, not determined.



approximate doubling time can be inferred, which ranges between 7 and 20 days, depending on the species (Strous *et al.*, 1999b; Tsushima *et al.*, 2007a,b; Van der Star *et al.*, 2008a; Oshiki *et al.*, 2011).

Figure 1 shows an example of the patience, trial and errors, frustrations, and surprises that go with the work. In their attempt to enrich *Scalindua* species that were known to be present in the sediment of Gullmar Fjord (Sweden, Schmid *et al.*, 2007), Van de Vossenberg *et al.* (2008) inoculated two bioreactors in parallel, one operating at 18 °C (later on at 23 °C) and the other at 15 °C. Initially, no increase in anammox activity could be detected, which did occur when artificial sea water was replaced by Red Sea salt from an aquarium shop. Ultimately, two other components were required for maximal activity, FeSO<sub>4</sub> (9 μM) and phosphate (0.2 mM). (Please note in Fig. 1 the dramatic effect that went with oxygen leakage.) After 14 months, stable enrichments were obtained. Quite remarkably, both bioreactors harbored two different *Scalindua* species in about equal amounts, one genotype closely related and a second, more distantly affiliated with *S. brodae/sorokinii*. For unknown reasons, the latter gradually disappeared from the 23 °C culture, but its population remained stable

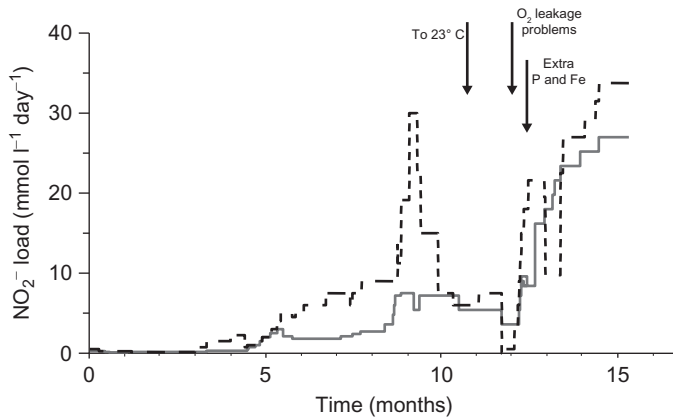


Figure 1 Enrichment of *Scalindua* species from Gullmar Fjord (Sweden) marine sediment in two SBR bioreactors. Dashed black line: 18–23 °C; solid gray, 15 °C. Enrichments were followed by gradually increasing the nitrite concentration of the influent, resulting in a daily nitrite load as indicated. After about 13 months, bioreactors both contained two different species. One of the species gradually disappeared thereafter from the 23 °C culture and could no longer be detected after 18 months. By the arrow indicated, the temperature of the 18 °C culture was increased to 23 °C; extra Fe and P, the FeSO<sub>4</sub> and phosphate concentrations in the medium were increased to 9 μM and 0.2 mM, respectively. (Adapted from van de Vossenberg *et al.*, 2008.)

when maintained at 15 °C. This indicates that temperature could be a selective marker, although other factors, like small differences in reactor design and hydraulic retention times of the nutrients, may have contributed to the outcome of the enrichments. As mentioned before, *Scalindua* is commonly found in saltwater systems and the species has been enriched from those environments by other groups as well (Kawagoshi *et al.*, 2009, 2010; Kindaichi *et al.*, 2011a,b). It is, however, elusive, which specific molecular traits make *Scalindua* a marine organism.

Besides a better fitness to environmental factors such as temperature and pH, the competitive advantage of a microorganism will depend on the affinity (apparent half-saturation constant,  $K_m$ ) for its substrates, metabolic activities ( $V_{max}$ ), and accompanying growth rates as well as sensitivity toward inhibiting substances. In Table 1, a comparison is made in these aspects between three related anammox species, *K. stuttgartiensis*, *B. anammoxidans*, and *B. sinica*. All three species were enriched from activated sludge using comparable conditions as to ammonium and nitrite loads. In addition, the species were able to metabolize their substrates in the same temperature and pH ranges. *B. sinica*, however, showed a growth rate which was 1.5-fold higher than the other two. In contrast,  $K_m$  values for ammonium and nitrite were significantly lower. Thus, it seems that *B. sinica* has maximized its metabolic activity at the expense of substrate affinity. In line herewith, Van der Star *et al.* (2008a) observed a complete population shift from a *B. anammoxidans*-dominated to a *K. stuttgartiensis*-dominated culture when suspended cells of *B. anammoxidans* were cultured as free-living, planktonic cells in a membrane bioreactor (MBR) (see below), operated under nitrite limitation at relative long hydraulic retention times. Similar population shifts were observed in our own lab (B. Kartal, unpublished results). So, *K. stuttgartiensis* may have geared its metabolism toward a low  $K_m$  for nitrite, and perhaps also for ammonia.

There are more factors that determine the competitive outcome. To study the effect of organic compounds on the performance of the anammox bacteria, Kartal *et al.* (2007a, 2008) inoculated two SBRs with the same activated sludge from which previously *B. anammoxidans* and *K. stuttgartiensis* had been enriched. The reactors were operated with surplus ammonium and nitrate, and limiting nitrite. One reactor received propionate, while the other was supplemented with acetate to the extent that effluent concentrations always were below detection ( $<1 \mu\text{M}$ ). Eventually two stable cultures developed in which anammox bacteria made up approximately 80% of the biomass, but with an unexpected result: the bioreactors contained two different species. The propionate-supplemented system enriched for *A. propionicus*, whereas *B. fulgida* was the specifically present in the acetate

bioreactor. Moreover, cells from both reactors were able to couple the oxidation of organic acids to CO<sub>2</sub> with the reduction of nitrate or nitrite to N<sub>2</sub> (Table 2). Specific rates were only 4–6% compared to the ammonium-oxidizing activity (15 μmol min<sup>-1</sup> g<sup>-1</sup> of protein). Also, other anammox bacteria showed this property instantaneously, that is, without induction, but all with slight differences (Table 2). Specific propionate and acetate oxidation rates were highest for *A. propionicus* and *B. fulgida*, respectively, in agreement with the organic source during enrichment. In comparison with ammonium oxidation rates, conversion rates of the organic compounds may seem insignificant and differences among different species are not impressive, but they do have a significant impact. When a bioreactor dominated by *B. anammoxidans* and *A. propionicus* as an only minor species was supplemented with propionate, the latter completely took over in less than 4 months, indicating that the presence of propionate gave *A. propionicus* this specific competitive advantage (Kartal *et al.*, 2007a). *B. anammoxidans*, and also *J. asiatica*, have been detected in many different freshwater ecosystems (Jetten *et al.*, 2003; Jaeschke *et al.*, 2009; Sánchez-Melsió *et al.*, 2009; Hu *et al.*, 2010, 2011; Musat *et al.*, 2010). Both species might rely on the use of a broad spectrum of organic compounds, perhaps none of these very efficiently, yet permitting these species to adapt to changing environments. Another striking observation from these experiments was that heterotrophic denitrifiers did not contribute significantly to substrate conversion in the propionate- and acetate-fed enrichments: denitrification coupled to acetate or propionate oxidation is energetically much more favorable compared to the anammox reaction. Apparently, these anammox species were able to out-compete the denitrifiers on basis of substrate affinities, *viz.*,  $K_m$  values.

Table 2 Oxidation of organic acids and nitrate reduction by anammox bacteria<sup>a</sup>.

Organic acid	Specific activity <sup>b</sup> for:				
	<i>B. anammoxidans</i>	<i>B. fulgida</i>	<i>A. propionicus</i>	<i>K. stuttgartiensis</i>	<i>Scalindua</i> sp.
Formate	6.5	7.6	6.7 (2.8)	5.8 (3.0)	7
Acetate	0.57	0.95	0.79 (0.7)	0.31 (1.5)	0.7
Propionate	0.12	0.31	0.64 (1.0)	0.12 (0.88)	0.3

<sup>a</sup>Data were taken from Kartal *et al.* (2007b, 2008) and van de Vossenberg *et al.* (2008).

<sup>b</sup>Specific activities (μmol min<sup>-1</sup> g<sup>-1</sup> of protein) of organic acid oxidation and nitrate reduction (in parentheses).

The SBR technique has been a most fruitful and established method to enrich anammox bacteria. Meanwhile, other methods have been developed to grow the organisms using upflow-anaerobic sludge blanket digesters (Strous *et al.*, 1997; Imajo *et al.*, 2004; Schmidt *et al.*, 2004b; Ni *et al.*, 2010), rotating contact digesters (van de Graaf *et al.*, 1996; Egli *et al.*, 2001, 2003; Windey *et al.*, 2005), and even standard fed-batch fermenting systems (Sánchez-Melsió *et al.*, 2009; Suneethi and Joseph, 2011; Yasuda *et al.*, 2011). All methods have in common that anammox cells are kept in biofilms or cell aggregates, often associated with a realm of other, mostly minor, microbial species and glued together by extracellular polymeric substances (polysaccharides, DNA, proteins). This conglomeration may very well reflect the organisms' natural status and it certainly is beneficial from an applied, operational point of view. From the microbiological, physiological, and biochemical perspective, such biofilms can be a hindrance and even a nuisance. Cells can be difficult to visualize, for instance by fluorescent *in situ* hybridization, or to count due to autofluorescence (Kartal *et al.*, 2008). The multilayered biofilms represent a variety of physiologically different microstates governed by physical phenomena such as granule dimension, mass transfer, and diffusion limitations resulting in steep nutrient gradients to which the organism have to adapt. Hence, it is always the question whatever physiological property is actually measured using aggregated cells. For example, literature reports significant differences in oxygen tolerance among anammox bacteria and even for the same species (Table 1). It is, however, difficult to decide whether the tolerance is an intrinsic species-specific property or that it is related with the culture type used. Oxygen may be removed by O<sub>2</sub>-respiring community members before it reaches the anammox cell such as in one-step anammox reactors that are specially designed with a layer of aerobic ammonium-oxidizing bacteria on the outer part of the granules (Slikers *et al.*, 2002; Nielsen *et al.*, 2005; Kindaichi *et al.*, 2007; Abma *et al.*, 2010). Furthermore, biofilm cells resist breaking for biochemical experiments, and cell extracts can be so viscous that column separations for protein purification become almost undoable (Cirpus *et al.*, 2006). These problems now have been solved by the introduction of the MBR (Van der Star *et al.*, 2008a; Kartal *et al.*, 2011a). Here, free-living cells are contained within the bioreactor by the application of a membrane microfilter. When cultured in an SBR, a minor fraction appears to be present as planktonic cells that are washed out. These can be collected from the effluent, serving as the inoculum for the MBR. Alternatively, a small amount of aggregated cells (i.e., 0.5 L in 12 L) can be used to inoculate a bioreactor. Without the selective pressure for faster settling cells, aggregates disintegrate in time (~350–400 days) resulting in planktonic cells (Van der Star *et al.*, 2008a; Kartal *et al.*, 2011a).

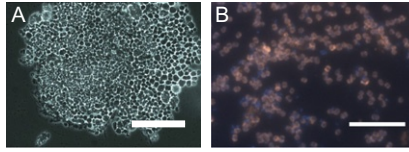


Figure 2 *Kuenenia stuttgartiensis* grown as (A) biofilm and (B) as planktonic single cells. Scale bars: (A) 50  $\mu\text{m}$ , (B) 5  $\mu\text{m}$ .

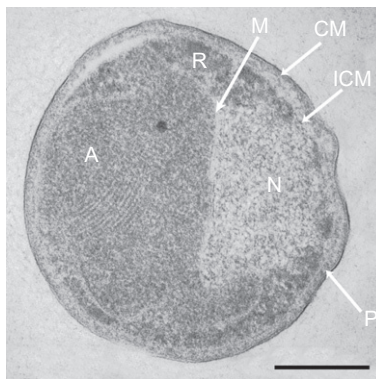
Like many other microorganisms, anammox bacteria can have both a sessile (aggregated) and a planktonic lifestyle (Fig. 2). An as yet unaddressed question is which stimulus triggers the transition from one state to the other. Next, the transition will require the tight expression control over many proteins, for instance, involved in the synthesis of (otherwise unknown) extracellular polymers in sessile cells. Again, the regulatory mechanisms remain to be elucidated. Interestingly, free-living cells show a much higher growth rate. Whereas *K. stuttgartiensis* doubles every 11–20 days in an SBR, the doubling time is “only” 7 days in an MBR. The decrease likely has to do with facilitated substrate transfer and with the fact that no energy has to be invested in the biosynthesis of biofilm matrix components. Still, also single cells tend to cluster (Fig. 2), suggesting that the aggregated lifestyle is in some way advantageous.

As mentioned, anammox bacteria presumably occur as multiple species of which only a handful is known by now, each species having found its specific niche and each one having to adapt to a changing environment. The examples outlined above indicate that organisms thrive in their natural environment by tuning their activity to temperature, either maximizing their activity (*R* strategist) or gearing their metabolism toward high substrate affinity (*K* strategist). Furthermore, anammox species may derive a competitive advantage by the (supplementary) use of specific organic and perhaps also inorganic compounds as electron donors to sustain their metabolism (see Section 4.4).

### 3. ANAMMOX CELL BIOLOGY

Under the microscope, anammox bacteria are observed as simple small coccoid cells with a diameter of about 0.8  $\mu\text{m}$ . However, a more detailed inspection using different electron microscopic techniques reveals quite a complex cell plan typical for *Planctomycetes* (Strous *et al.*, 1999a; Fuerst,

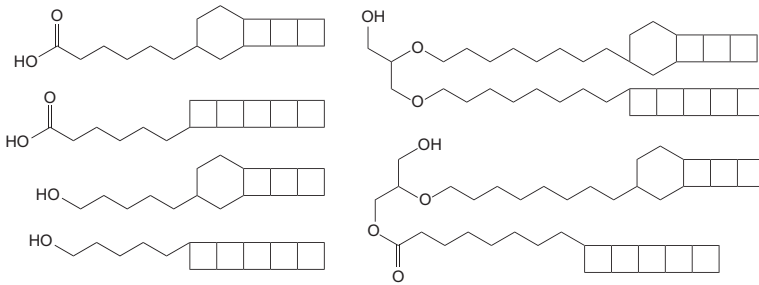
2005; Fuerst and Sagulenko, 2011). The cell is essentially composed of three compartments, each surrounded by a membrane bilayer (Fig. 3) (Lindsay *et al.*, 2001; Van Niftrik *et al.*, 2008b). Together with a thin cell wall, the outermost membrane encloses both the cell and the outer compartment, termed the paryphoplasm. Occasionally pili-like appendages are observed (Van de Vossenberg *et al.*, 2008). It is yet unknown whether the cell wall is structured by proteins, peptidoglycan, or the combination of both. There are some indications for the presence of an S-layer protein lattice in *K. stuttgartiensis* (Fuerst *et al.*, 2006). Although in the genome of this organism a large cluster is present, which contains 19 out of 21 genes coding for peptidoglycan synthesis enzymes (Strous *et al.*, 2006), it is unknown whether the genes are functionally expressed. Furthermore, it remains to be established if the outer membrane is chemiosmotically closed—making it the cytoplasm membrane—or gated by porin proteins, such as the periplasmic membrane of Gram-negative bacteria. The second membrane surrounds the riboplasm where the nucleoid and ribosomes are located (Van Niftrik *et al.*, 2008a). Here, the transcription, translation, and household machinery are presumed to reside. In addition, glycogen granules can be observed in the ribosomal compartment. *B. fulgida* and *A. propionicus* store larger particles in their riboplasm remarkably resembling polyhydroxyalkanoate bodies (Van Niftrik *et al.*, 2008a,b). The third, innermost membrane is highly curved,



*Figure 3* Transmission electron microscopy of *Anammoxoglobus propionicus*. A, anammoxosome containing tubule-like structures; R, riboplasm containing the nucleoid (N) opposed to the anammoxosome membrane (M); P, paryphoplasm separated from the riboplasm by an intracytoplasmic membrane (ICM); CM, cytoplasmic membrane. Scale bar, 200 nm.

yet fully closed, that is, it never contacts the riboplasmic membrane. It bounds a central vacuole, the anammoxosome. The latter is vertically inherited to the daughter cell during cell division, highly suggesting it to be a true cell organelle (Van Niftrik *et al.*, 2004, 2008a,b). If so, intact anammoxosomes and their protein complement should be amenable to isolation; preliminary studies, indeed, show this to be the case (Sinninghe Damsté *et al.*, 2002; Neumann *et al.*, 2011). Still, progress in this field awaits protocols for a reproducible purification with a high yield. This is especially relevant regarding the often proposed, but never conclusively proven role of the anammoxosome in the central energy metabolism. As described in Section 4.1, anammox bacteria rely on a specific set of cytochrome *c*-type proteins, in particular hydroxylamine oxidoreductase-like octaheme proteins (HAOs) and hydrazine synthase (HZS), for their catabolism. Being, chemolithotrophs, energy should be conserved by a chemiosmotic mechanism featuring a membrane-bound ATP synthase (ATPase) (see Section 4.2). Diaminobenzidine staining showed that the full cytochrome *c* complement was localized in close proximity to the anammoxosome membrane, notably at the inner side (Van Niftrik *et al.*, 2008a,b). Furthermore, immunogold-labeled antibodies raised against a dominant HAO (Lindsay *et al.*, 2001; Jetten *et al.*, 2009) or against parts of the central catabolic enzymes (Karlsson *et al.*, 2009) specifically located in these proteins within the cell organelle. By the same approach, the major ATPase was demonstrated to be mainly associated with the organelle's membrane (Van Niftrik *et al.*, 2010). Together, the findings strongly support the function of the anammoxosome as the bioenergetic heart of the anammox cell.

The anammox cell is compartmentalized by three lipid membrane layers. Membranes are nature's great invention to physically and chemically separate the inner cell from the outer world. Semi-permeable membranes enable the maintenance of concentration differences of (charged) compounds and create gradients that can be used for energy conservation. Nature shows an astonishing variation on the theme by the synthesis of an almost endless number of, often species-specific, lipid molecules with different chemical and physical properties and combinations thereof employed to optimize membrane fluidity and stiffness. Anammox bacteria have added something new to this: ladderane molecules. As in all other living organisms, anammox membranes are composed of glycerolipid bilayers. The lipids contain a combination of ester-linked (typical of the Bacteria and Eukarya) fatty acids or ether-linked (typical of the Archaea) long-chain alcohols (Fig. 4). What makes anammox special is the presence of saturated C17-C20 fatty acids and alcohols that are fused by *cis*-ring junctions to make



**Figure 4** C17–C20 ladderane lipids from anammox bacteria containing three linearly concatenated cyclobutane rings and one cyclohexane or five cyclobutane rings. Fatty acids are esterified with methanol or the glycerol backbone, and the ladderane alcohols are ether-linked with glycerol, all in different combinations.

ladder-like (“ladderane”) cyclobutane and cyclohexane ring structures (Sinninghe Damsté *et al.*, 2002, 2005; Kuypers *et al.*, 2003; Schmid *et al.*, 2005). Ladderanes mostly occur as two-ring systems: a saturated C6–C8 carboxylic acid or C5–C8 alcohol chain connected to either five linearly concatenated cyclobutane moieties or three cyclobutanes and one cyclohexane. The fatty acids are esterified with methanol or the glycerol backbone, while the ladderane alcohols are ether-linked with glycerol, all in different combinations (Fig. 4). A typical property of ladderanes is their dense packing making them, for instance, highly impermeable for fluorophores that readily pass through common membranes (Sinninghe Damsté *et al.*, 2002; Boumann *et al.*, 2009a,b). In *B. anammoxidans*, ladderanes comprise 34% of the total lipid content, whereas an anammoxosome-enriched cell fraction showed a significantly higher content (53%). The analysis of highly pure anammoxosomes, however, has to show if ladderanes are the specific lipid components of this cell organelle (Sinninghe Damsté *et al.*, 2002). At the C1 position, the glycerol backbone is *sn*-1 phospho-esterified with one of many different hydrophobic tail types, whereas different polar headgroups (phosphocholine, phosphoethanolamine, or phosphoglycerol) may be substituted at the *sn*-3 position (Boumann *et al.*, 2006; Rattray *et al.*, 2008). A comparative analysis of four different anammox species (*A. propionicus*, *B. fulgida*, *Scalindua* spp., and *K. stuttgartiensis*) detected additional ladderane species, notably C18 and C20 fatty acids with either three or five cyclobutane rings and a C20 alcohol with three of these concatenated ring structures (Boumann *et al.*, 2006; Rattray *et al.*, 2008, 2010). Moreover, two new C22 ladderane fatty acids were found that were

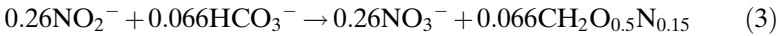
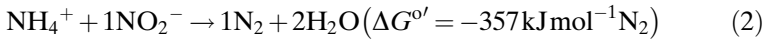
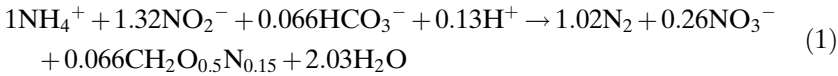


specifically present in *A. propionicus*. Besides ladderanes, all four species contained a C27 hopanoid ketone and bacteriohopanetetrol, both being hopane-derived pentacyclic compounds that convey bacterial membranes the requested rigidity and fluidity (Boumann *et al.*, 2009b). As mentioned, ladderanes are unique to anammox bacteria, which make the compounds highly specific taxonomic markers in present-day ecological and in paleobiological research (Kuypers *et al.*, 2003; Schmid *et al.*, 2005; Jaeschke *et al.*, 2007, 2009, 2010).

Anammox bacteria multiply by binary fission (Van Niftrik *et al.*, 2008a, 2010). Cell division in simple prokaryotes is already a complex event in which multiple interacting protein complexes run a precisely tuned program (Goehring and Beckwith, 2005; Goehring *et al.*, 2006; Vicente and Rico, 2006; Pilhofer *et al.*, 2008; Rachel, 2009). Followed under the electron microscope, the first hint at anammox cell division is the appearance of a division ring in the midcell paryphoplasm (Van Niftrik *et al.*, 2008a, 2010). Hereafter, the cell wall invaginates slightly, the cell including the anammoxosome, start to elongate thereby doubling in size, and constriction continues until the two cells get separated. The common key player in prokaryotic cell division is the FtsZ protein. It constitutes the division ring and recruits the other protein components. However, the *K. stuttgartiensis* genome lacks the genes coding for FtsZ and associated “divisome” proteins. (The same holds for other members of the *Planctomycetes* and *Chlamydiae* (Pilhofer *et al.*, 2008).) In contrast, the full repertoire of genes encoding the so-called FtsA-independent divisional complex is present in *K. stuttgartiensis*, but the role of the complex in cell division is not fully understood (Goehring *et al.*, 2006; Vicente and Rico, 2006). Given the absence of FtsZ, the division ring seen under the microscope should be derived from some other protein for which a candidate was suggested (kustd1438) (Van Niftrik *et al.*, 2008a, 2010). So, it seems that anammox bacteria, and in the broader context, the *Planctomycetes*, have developed their own way to divide, which makes sense considering their highly complex cell architecture (Rachel, 2009).

#### 4. ANAMMOX METABOLISM

Anammox bacteria grow on the conversion of ammonium and nitrite with CO<sub>2</sub>/bicarbonate as the sole carbon source. Operating under steady-state conditions in lab-scale bioreactors, the compounds are metabolized according to this overall equation (1) (Strous *et al.*, 1998):



The overall reaction is the net sum of two partial reactions: the energy-generating process, *viz.*, the oxidation of ammonium coupled to nitrite reduction to make dinitrogen gas (Eq. 2), and bicarbonate fixation into cell biomass ( $\text{CH}_2\text{O}_{0.5}\text{N}_{0.15}$ ) (Eq. 3). One may note that both nitrogen atoms derive from two different substrates, ammonium and nitrite. This provides us with an important property for physiological and biochemical experiments, or to detect anammox activity in natural systems. Using  $^{15}\text{N}$ -labeled substrates ( $^{15}\text{NH}_4^+$  or  $^{15}\text{NO}_2^-$ ),  $^{29}\text{N}_2$  is the specific end product of the anammox reaction. The isotope composition of dinitrogen gas formed ( $^{14}\text{N}^{14}\text{N}$ ,  $^{14}\text{N}^{15}\text{N}$ ,  $^{15}\text{N}^{15}\text{N}$ ) can be analyzed by mass spectroscopy (Van de Graaf *et al.*, 1997). Next, nitrite plays a dual role: it acts as the electron acceptor in the ammonium-oxidizing reaction (Eq. 2) and as electron donor for the  $\text{CO}_2$  reduction to biomass (Eq. 3). In the latter case, nitrite is anaerobically oxidized to nitrate and, as a consequence, growth is always associated with nitrate production. From the above reaction stoichiometries (Eqs. 1–3), it is inferred that 1 mol of carbon is bound per 15 catabolic cycles. Similarly, about 4 mol of nitrite are oxidized per mol fixed carbon.

Hereafter, we will give an overview of what is presently known about the catabolic pathway of the anammox process, the way energy is conserved from the reaction, and the  $\text{CO}_2$  fixation route. It also has become clear that anammox bacteria are not just specialized chemolithotrophs. Besides ammonium and nitrite, they may use a (presumably broad) range of alternative organic and inorganic compounds to sustain their metabolism. This topic will be discussed in Section 4.4.

## 4.1. The Central Nitrogen Metabolism

Ammonium is an inert molecule that usually needs the oxidative power of oxygen to get activated, notably to hydroxylamine ( $\text{NH}_2\text{OH}$ ). Therefore, the immediate question after the first detection of anammox was: How are these organisms capable of doing this in the absence of  $\text{O}_2$ ? Like in every other biochemical pathway, this would involve a number of intermediates and partial reactions. The first clue about the intermediates came from a

chance finding (Van de Graaf *et al.*, 1997). When culturing the bacteria or working with cell suspensions, cells may become inactive for one reason or another. It turned out that activity could be restored by adding catalytic amounts of hydroxylamine. Following the conversion of  $^{15}\text{NH}_2\text{OH}$  over time, the intermediary formation of another nitrogenous compound was observed with a molecular mass of 33, which was unambiguously identified as  $^{14}\text{N}^{15}\text{N}$ -hydrazine ( $\text{N}_2\text{H}_4$ ) (Van de Graaf *et al.*, 1997; Schalk *et al.*, 1998). Ever since, the experiment has been repeated many times with different anammox species and with the same result (Fig. 5) (e.g., Strous *et al.*, 1999a; Kartal *et al.*, 2007a, 2008; Van der Star *et al.*, 2008b). Like hydroxylamine, hydrazine was also able to “boost” inactive anammox cells (Strous *et al.*, 1999b). Van de Graaf *et al.* (1997) concluded that both hydroxylamine and hydrazine were intermediates. They proposed at the time, by all means, a reasonable three-step pathway involving (1) a four-electron reduction of nitrite, that is, the reversed reaction as is done by HAO in aerobic ammonium oxidation, followed (2) by the condensation of hydroxylamine and ammonium to make hydrazine, and (3) the subsequent oxidation of hydrazine to yield the end product dinitrogen gas and four electrons to drive nitrite reduction. The latter reaction is also performed by HAO, yet artificially (Hooper *et al.*, 1997). However, neither hydrazine nor  $\text{NH}_2\text{OH}$  had ever been demonstrated in anammox cells under physiological relevant conditions, but concentrations could be too low to detect with available methods. In addition, genome analysis favored a modified scheme.

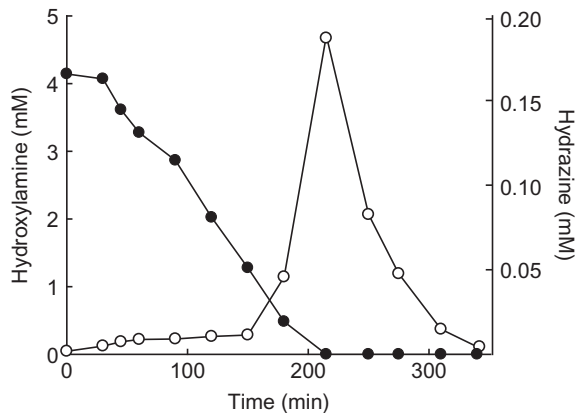
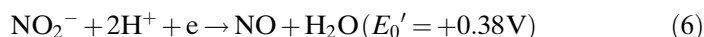
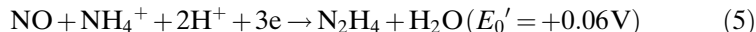
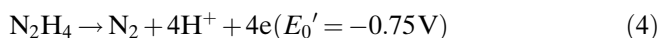
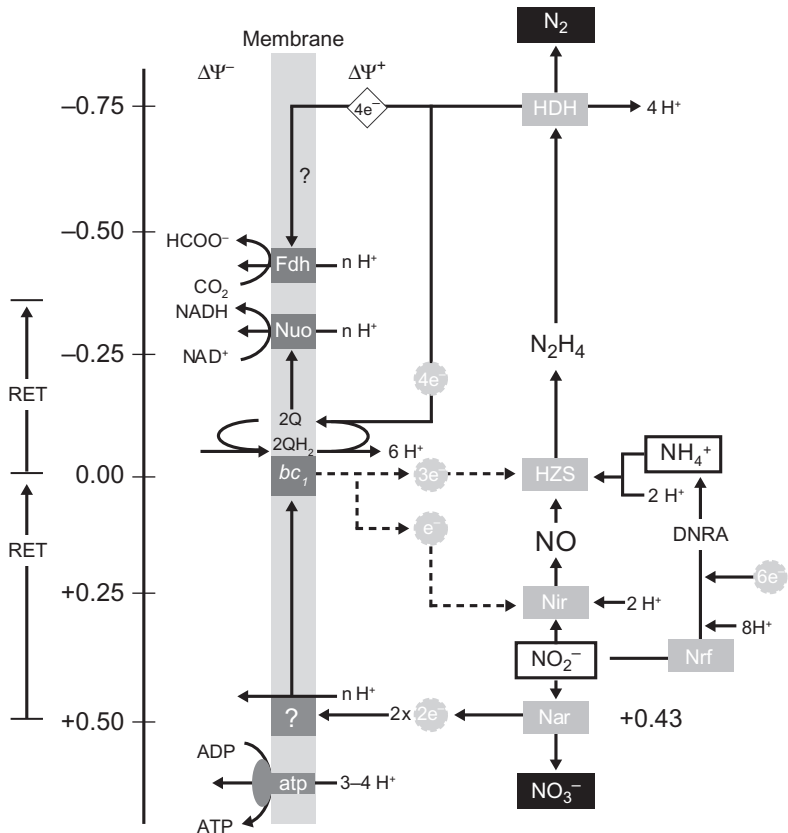


Figure 5 Production of hydrazine (○) by *Brocadia fulgida* after the addition hydroxylamine (●). (Adapted from Kartal *et al.*, 2008.)

In 2006, the almost complete genome of *K. stuttgartiensis* (4.2 megabases) became available as five large contigs (Strous *et al.*, 2006). Together, the contigs, numbered kusta-e, coded for 4663 open reading frames and covered an estimated >98.5% of the whole genome, missing an estimated 60 genes. In all, 3279 genes (70.3%) showed significant similarity with genes in other databases, but only 1385 genes (29.7%) have been annotated with a function. Considering that genomes of lithotrophic specialists harbor roughly 2000 genes, the size of the *K. stuttgartiensis* genome and the number of encoded proteins are astonishing. Furthermore, over 200 genes are related with catabolism and respiration, including 63 *c*-type proteins, which exceed the number of established omnivores like *Shewanella* and *Geobacter*. However, the genome lacked one conspicuous candidate, nitrite::hydroxylamine oxidoreductase. Instead, a gene cluster coding for nitrite::nitric oxide oxidoreductase (cytochrome *cd<sub>1</sub>* nitrite reductase, NirS) and accessory proteins was present, which suggested nitric oxide (NO) to be an intermediate. On the basis of the genome data and scarce physiological evidence, Strous *et al.* (2006) proposed that the following three reactions (Eqs. 4–6) might account for the central anammox metabolism:



The four-electron oxidation of hydrazine by an HAO-like protein called hydrazine dehydrogenase (HDH) would make dinitrogen gas (Eq. 4) and provide the electrons for nitrite reduction by NirS (Eq. 6) and hydrazine synthesis (Eq. 5), the latter reaction catalyzed by the putative HZS representing a true biochemical novelty. In addition, the presence of a quinol::cytochrome *c* oxidoreductase (*bc<sub>1</sub>*, complex III) and an ATPase complex in the genome of *K. stuttgartiensis* permitted the proposal of a chemiosmotic mechanism by which the energy derived from the anammox reaction is conserved as ATP (Fig. 6). The electrons derived from hydrazine oxidation, nature's most powerful reductant ( $E_0' = -0.75\text{V}$ ), are transferred via quinone to the *bc<sub>1</sub>* complex. The latter serves two functions: (a) it distributes the electrons toward nitrite reduction (Eq. 6) and hydrazine synthesis (Eq. 5). (b) In concert with the proton-motive Q cycle, protons are translocated across a membrane system to create a proton-motive force (pmf) driving ATP synthesis. Intermediary electron transfer had to be accomplished by a set of cytochrome *c*-type proteins. We may note that the *K. stuttgartiensis* genome actually codes for three different *bc<sub>1</sub>* complexes and no less than four ATPases (see Section 4.2).

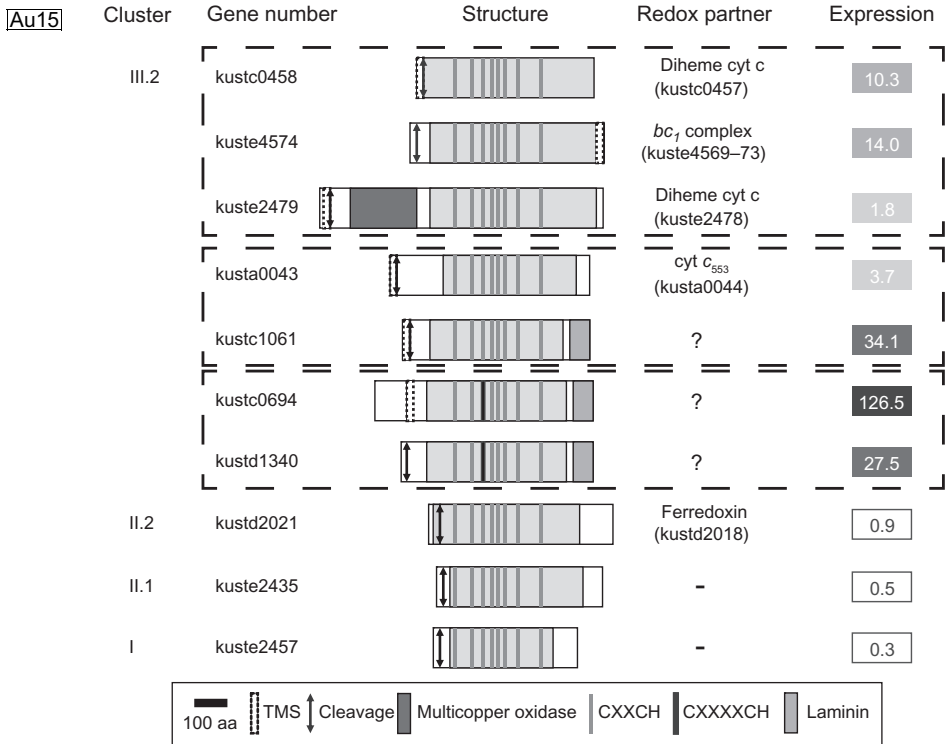


**Figure 6** Central metabolism of anammox bacteria. Circles denote cytochrome *c* proteins; the diamond represents an electron carrier putatively involved in electron transfer to low-redox potential reduction reactions in cell carbon synthesis. atp, ATP synthase; *bc*<sub>1</sub>, quinol:cytochrome *c* oxidoreductase (*bc*<sub>1</sub>, complex III); Fdh, formate dehydrogenase; HDH, hydrazine dehydrogenase; HZS, hydrazine synthase; Nar, nitrite:nitrate oxidoreductase; Nir, nitrite:nitric oxide oxidoreductase; nrf, nitrite:ammonium oxidoreductase; Nuo, NADH:quinone oxidoreductase; DNRA, dissimilatory nitrite reduction to ammonium; RET, reversed-electron transport. Enzymes are ordered according to the midpoint redox potentials (scale bar at the left; numbers in Volt) of the reactions they catalyze.

Furthermore, the scheme presented in Fig. 6 involves a cyclic electron flow, which has the implication that each intermediary molecule (NO, N<sub>2</sub>H<sub>4</sub>) that gets lost and each electron that is taken from the cycle has to be replenished somehow.

Taking advantage of the single cell MBR technique for *K. stuttgartiensis*, we were recently able to experimentally verify the proposed mechanism to some extent (Kartal *et al.*, 2011b). Using unlabeled hydrazine and labeled nitrite, it was shown that the compound is actually turned over during ammonium and nitrite conversion. The role of NO as an intermediate was established by a combination of inhibition studies and fluorescent labeling of the cells. Moreover, NO together with ammonium served as the direct substrate for hydrazine synthesis and nitrogen formation. NO being a substrate for N<sub>2</sub> formation, in fact, already had been seen before when studying the possibility to remove this toxic compound via the anammox process (Kartal *et al.*, 2010b). (It had no inhibitory effect in concentrations as high as 3500 ppm.) Apparently, the bacteria use the oxidative power of NO to activate ammonium. So what about the catabolic enzymes? Here, the situation becomes more complicated, and more fascinating, than anticipated.

As mentioned, the oxidation of hydrazine to dinitrogen gas (Eq. 4) may be catalyzed artificially by HAO from aerobic ammonium-oxidizing bacteria. The physiological function of HAO in these organisms is the four-electron oxidation of hydroxylamine to nitrite (Eq. 8), a key reaction in their metabolism (Hooper *et al.*, 1997). HAO is an intricate protein composed of three identical, covalently bound subunits of which each one harbors eight *c*-type hemes, one as the catalytic center and the other seven acting in electron transfer. Genes coding for such proteins were detected after the analyses of the *K. stuttgartiensis* genome (Strous *et al.*, 2006; de Almeida *et al.*, 2011; Kartal *et al.*, 2011b). The complicating thing is that the genome harbors no less than 10 paralogues, 5 of these being highly expressed as found by transcriptomics and proteomics (Fig. 7). Five are linked in the genome with other cytochrome *c* proteins, or ferredoxin, which might assist in electron transfer. Protein and genome databases show a multiplicity of HAO-like proteins that can be classified phylogenetically into a number of clusters and subclusters (Klotz *et al.*, 2008; Schmid *et al.*, 2008). The 10 *K. stuttgartiensis* HAO-like proteins come with different subclusters and some show high sequence identity, indicating a similar function in the organism (Fig. 7). Unfortunately, phylogenetic analysis does not give a clue about the reaction that is performed by a certain species. By direct purification, we were able to prove that kustc0694 is the physiological HDH (Kartal *et al.*, 2011b). The enzyme catalyzes the predicted four-electron oxidation of hydrazine to N<sub>2</sub> (Eq. 4) with high activity and affinity. Remarkably, NO and hydroxylamine are strong competitive inhibitors of the reaction. The *K. stuttgartiensis* genome encodes a second protein, kustd1340, which is nearly identical to kustc0694 (>97%

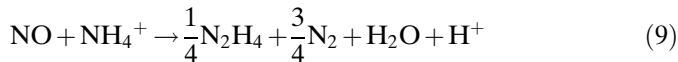
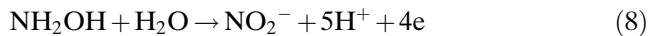
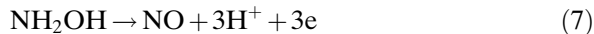


**Figure 7** The 10 hydroxylamine oxidoreductase (HAO)-related octaheme proteins in the *Kuenenia stuttgartiensis* genome. The gene products are ordered according to their cluster position in the phylogenetic tree (Klotz *et al.*, 2008); highly homologous HAO-like proteins are boxed by dashed lines. Lengths of the polypeptides are drawn to scale (aa, amino acids) and homologous cytochrome *c*-rich parts are vertically aligned. Redox partners represent (potential) electron transfer subunits found in the same subunit. Expression values are expressed as *n*-fold coverage of Illumina deep RNA sequencing of the *K. stuttgartiensis* transcriptome (Kartal *et al.*, 2011b). Structural motifs: TMS, transmembrane-spanning region; cleavage, N-terminal cleavage site; multicopper oxidase, multicopper oxidase domain; CXXCH, cytochrome *c*-binding motif; CXXXXCH, unusual cytochrome *c*-binding motif in kustc0694 and kustd1340; laminin, laminin sequence.

both at the amino acid and nucleotide levels), but the presence of the gene product remains to be established. Furthermore, a HAO-like HDH strongly related to both kustc0694 and kustd1340 was described before

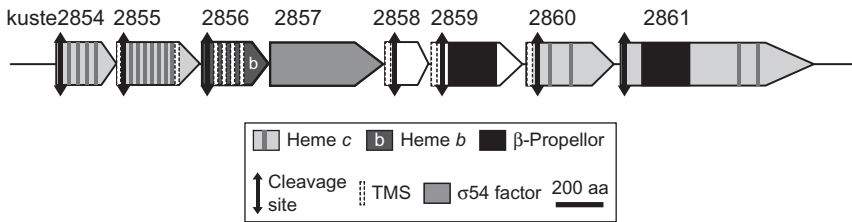
for anammox strain KSU-1 (Shimamura *et al.*, 2007), but its physiological role remained unresolved at the time.

The most abundant HAO-like protein in *K. stuttgartiensis* is kustc1061 (Kartal *et al.*, 2011b). It is the protein that previously was shown to be specifically present inside the anammoxosome (Lindsay *et al.*, 2001; L. van Niftrik and C. Ferousi, unpublished results). Also of this protein close homologs had been previously purified from *B. anammoxidans* and KSU-1 (Schalk *et al.*, 2000; Shimamura *et al.*, 2008). Kustc1061 is capable of hydrazine oxidation, albeit relatively slowly and with low affinity. Its main activity is the oxidation of hydroxylamine to NO (Eq. 7), but not to nitrite as in AOB (Eq. 8) (Kartal *et al.*, 2011b). As hydroxylamine is no longer considered an intermediate in the anammox process and hitherto no reactions have been found to make the compound, the role of kust1061 is puzzling. In hindsight, the presence of HDH kustc0694 and kustc1061 may explain the sequence of events following the administration of hydroxylamine to anammox cells (Fig. 5). Upon its addition, kustc0694 gets inhibited and hydroxylamine is removed by kustc1061, producing NO for hydrazine synthesis. When  $\text{NH}_2\text{OH}$  and NO are consumed, kustc0694 can start oxidizing the accumulated  $\text{N}_2\text{H}_4$ .



Hydrazine synthesis is one of the most intriguing properties of anammox bacteria. In the view of Strous *et al.* (2006), it would proceed by the reaction of NO and ammonium together with the input of three electrons, catalyzed by a novel enzyme, HZS (Eq. 5). On the basis of genome analysis and preliminary genomic data, the authors proposed that HZS might be encoded by one or more genes in the cluster kuste2854–2861 (Fig. 8). Besides a sigma transcriptional regulator, the cluster codes for a number of membrane-bound *b*- and *c*-type proteins (kuste2854–2856) that might constitute an electron-transfer module, and one or two *c*-type proteins potentially involved in catalysis. Our recent work proved the hypothesis (Kartal *et al.*, 2011b). Besides NO reductase, HZS is the only enzyme capable of forging an N–N bond. The purification of the complex demonstrated it to be a heterotrimeric protein composed of kuste2859–2860. Kuste2859 is entirely structured by beta propeller strands. This type of structures usually provides a rigid platform for the catalytic part of an





**Figure 8** Gene-cluster organization of the hydrazine synthase system and its accessory proteins in the *Kuenenia stuttgartiensis* genome. Hydrazine synthase is represented by the kuste2859–2861 gene products. Lengths of the polypeptides are drawn to scale (aa, amino acids). Structural motifs are as specified in the figure. Cleavage site, N-terminal cleavage site; TMS, transmembrane-spanning region.

enzyme. Also, kuste2861 mainly consists of beta propeller sheets, but it contains two *c*-type cytochromes at the C-terminal part, which might act in electron transfer. Kuste2860, again, harbors two *c*-type cytochromes. The protein displays significant sequence homology both with cytochrome *c* peroxidase (Atack and Kelly, 2007; Poulos, 2010) and the mauG protein involved in tryptophan tryptophylquinone biosynthesis, the protein-derived catalytic cofactor of methylamine dehydrogenase from *Paracoccus denitrificans* (Wilmot and Davidson, 2009). This suggests kuste2860 to be the catalytic moiety of the machinery. Remarkably, in the marine species *Scalindua*, kuste2859 and 2860 are fused (M.S.M. Jetten and J. van de Vossenberg, unpublished results). In *K. stuttgartiensis*, HZS makes up about 20% of the protein complement. As isolated, the enzyme complex is not very active, and appreciable activity ( $20 \text{ nmol h}^{-1} \text{ mg}^{-1}$  of protein) is only obtained when the hydrazine synthesis and hydrazine oxidation are coupled (Eqs. 4 and 5), notably by the addition of excess NO-insensitive kustc1061. This activity is only about 1% of the *in vivo* rate, but even when fully active in the living cell, HZS would be a very slow enzyme, possibly explaining the slow growth rate of anammox bacteria. The activity loss already results from just by breaking the cells, indicating that this is due to the disintegration of a tightly coupled multicomponent system (Kartal *et al.*, 2011b). HZS carries out one other reaction. It can make dinitrogen gas from NO and ammonium by its own, *viz.*, in a disproportionation reaction described by Eq. 9. The reason for this is that the enzyme can oxidize its product, hydrazine to  $\text{N}_2$  ( $34 \text{ nmol min}^{-1} \text{ mg}^{-1}$  of protein), which is at a 100-fold higher rate than the forward physiological reaction. From the energetic point of view, such reaction is unproductive: hydrazine is the ultimate energy source, requiring a dedicated enzyme

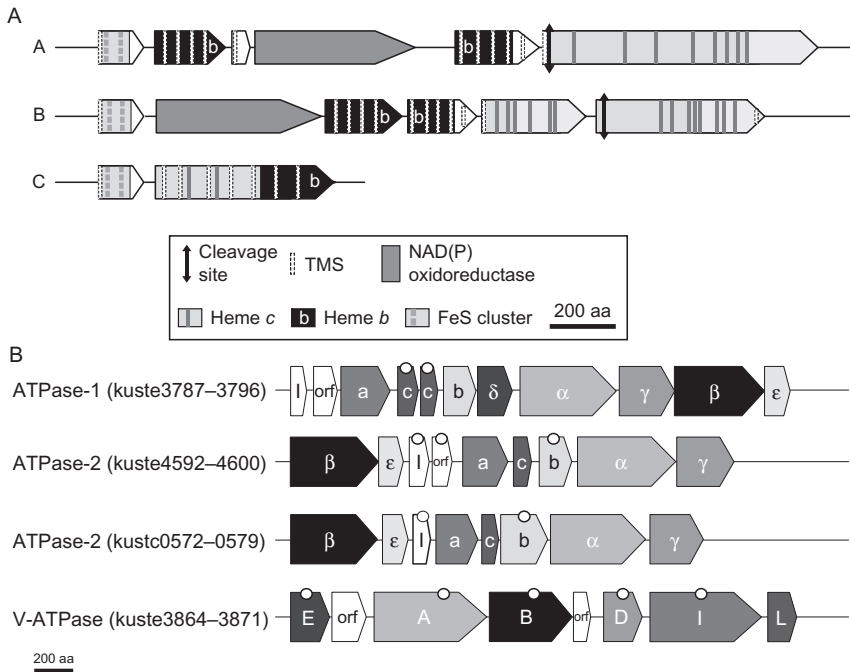
(kustc0694) to feed the electron transport chain for pmf generation. A major question is the catalytic mechanism underlying hydrazine synthesis. It is conceivable that HZS depends for this on the presence HAO-like proteins as backup systems for the removal of toxic intermediates (e.g., hydroxylamine) (Kartal *et al.*, 2011b).

The enzyme catalyzing nitrite reduction (Eq. 6) is an issue. As mentioned, the *K. stuttgartiensis* genome contains all genes coding for cytochrome *cd<sub>1</sub>* nitrite reductase (NirS) and accessory proteins (kuste4136–4140) (Strous *et al.*, 2006). In the organism, however, the genes are hardly expressed at the transcriptional level and are barely detectable in the proteome (Kartal *et al.*, 2011b). In striking contrast, NirS is one of the most abundant proteins in *Scalindua* (M.S.M. Jetten and J. van de Vossenberg, unpublished results). Thus, the question is whether in *K. stuttgartiensis* the even low NirS is sufficient to account for nitrite reduction or that the organism relies on another enzyme. *K. stuttgartiensis* and most likely other anammox bacteria express a collection of HAO-like proteins (Fig. 7), each likely being tuned to a specific function in N-metabolism. Together, the bright red heme *c* proteins make up about 30% of the protein complement, giving anammox bacteria their characteristic color that gradually comes to the fore during enrichment.

## 4.2. Energy Conservation

Whereas we are just becoming to appreciate the ingenuity of the anammox process at the molecular level, the understanding of the way energy is conserved still largely resides on close genome reading and hypotheses that were deduced from this. In the current view, two respiratory complexes play the key role, *bc<sub>1</sub>* (complex III) and membrane-bound ATPase (Fig. 6), and here genome analysis also comes with surprises.

The canonical *bc<sub>1</sub>* complex is composed of three components, membrane-bound quinol-binding cytochrome *b*, the Rieske 2Fe-2S iron-sulfur protein, and cytochrome *c* protein (Crofts, 2004; Osyczka *et al.*, 2005; Crofts *et al.*, 2006; Mulkidjanian, 2010). The complex couples the oxidation of reduced quinone to the reduction of *c*-type cytochromes using a bifurcation mechanism, three protons are translocated across the cell membrane per oxidized quinol (“proton-motive Q cycle”). Anammox seems to have invented variations on the common theme. First, the *K. stuttgartiensis* genome encodes three different *bc<sub>1</sub>* complexes (Fig. 9A) (Strous *et al.*, 2006; de Almeida *et al.*, 2011). In the first one (kuste3096–3097), the heme *b* and *c* parts are fused. The other two are more complicated, which may



**Figure 9** Redundancy of (A)  $bc_1$  and (B) ATPase complexes in the *Kuenenia stuttgartiensis* genome. (A) The three  $bc_1$  complexes encoded by the (A) kustd1480–1485, (B) kuste4569–4575, and (C) kuste3096–3097 gene clusters. Structural motifs are as specified in the figure. FeS cluster, Rieske 2Fe-2S iron-sulfur cluster; Cleavage site, N-terminal cleavage site; TMS, transmembrane-spanning region. (B) The four ATPases. Gene products are labeled according to the homologous subunits in related ATPases. Genes that have been annotated in the *K. stuttgartiensis* genome as unknown proteins or that were erroneously annotated are indicated with white circles on top (van Niftrik *et al.*, 2010). Lengths of the polypeptides are drawn to scale (aa, amino acids).

have to do with the dual role proposed in proton translocation and electron distribution. Rather than a cytochrome *c* with one heme, the kustd1480–1485 complex contains an octaheme *c*-type protein (kustd1485). In the kuste4570–4575 complex, two multiheme proteins are present, a hexaheme *c*-type protein (kuste4573) and the HAO-like octaheme protein kuste4574. The latter is closely related to kustc0458 (Fig. 7). Strikingly, both kustd1480–1485 and kuste4570–4575 go along with a protein with a high sequence similarity to the catalytic NAD(P)- and FMN-binding

subunit of NAD(P) oxidoreductase. mRNA deep sequencing indicates all three complexes to be expressed at the transcriptional and protein levels, albeit in different amounts and *kuste4570–4575* being the major species (Kartal *et al.*, 2011b). The question is what do the NAD(P)-binding and multiheme parts do? It is tempting to speculate that the particular anammox *bc*<sub>1</sub> complexes couple the oxidation of quinol by electron bifurcation with the reduction of both a high-redox potential heme *c* (or in *kuste4575* with a nitrogenous species) and low-redox-potential NAD(P). This would solve the problem of NAD(P)H generation in an elegant way. A conclusive answer on the structure and function of the anammox *bc*<sub>1</sub> complexes has to come from experimental work, *viz.*, the isolation and characterization of the complexes, as do answers to straightforward questions regarding a possible different cellular localization and expression.

As in all respiratory systems, the pmf generated by proton translocation should be conserved as ATP by the action of the nanomotor ATPase complex. The analysis of the *K. stuttgartiensis* genome reveals the presence of four different ATPases: one typical F<sub>1</sub>F<sub>0</sub> ATPase (ATPase-1; *kuste3787–3796*), two closely related atypical F-ATPases lacking the delta subunit (ATPase-2 and ATPase-3, *kuste4592–4600* and *kustc0572–0579*, respectively), and a prokaryotic V-type ATPase (V-ATPase-4, *kuste3864–3871*) (Strous *et al.*, 2006; Van Niftrik *et al.*, 2010) (Fig. 9B). ATPase-1 is the major species. Immunogold labeling with antibodies raised against its catalytic subunits showed ATPase-1 to be localized both in the anammoxosome and outermost (paraphoplasm) membranes (Van Niftrik *et al.*, 2010). ATPase-2 and -3 belong to a new sodium-dependent type, which has been suggested to be implemented in sodium extrusion (Dibrova *et al.*, 2010). As above, the redundancy of the ATPases loudly calls for an experimental answer as to their localization and putative differential expression.

The generation of the pmf derives from proton translocation across a closed semi-permeable membrane system. The localization of the anammox key enzymes (HAO, HDH, HZS, ATPase-1, and the cytochrome *c*-type complement) in the anammoxosome (see above Section 3) leaves little doubt that the chemiosmotic processes reside at this cell organelle. However, definite proof for this only comes from future experiments demonstrating whether or not anammox metabolism by isolated intact anammoxosomes results in a pmf. Apart from all other questions, there is one more left: how are the electrons from hydrazine oxidation shuttled into the quinone pool (Fig. 6)? Considering that hydrazine is the most powerful reductant ( $E_0' = -0.75$  V) and that a pmf of  $-0.12$  to  $-0.25$  V, as is commonly found in respiring organisms,

is sufficient to drive ATP synthesis, the redox potential drop ( $\Delta E=0.74$  V) associated with hydrazine:quinone oxidoreduction leaves ample room for one or more additional  $H^+$ -translocating coupling site(s).

The localization of the anammox key catabolic enzymes inside the anammoxosome implies that, following their biosynthesis in the cytoplasmic compartment, the proteins have to be targeted into the cell organelle. In agreement herewith, these enzymes contain an N-terminal cleavage site (Figs. 7–9). Moreover, the inner site of the cell organelle would comply with the positive (p-site) of the chemiosmotic machinery. In common prokaryotes, the p-site is taken by the periplasmic space, where most of the respiratory enzymes involved, for example, in N-metabolism are found. But why a containment within the anammoxosome?

As outlined above, the gaseous NO and neutral  $N_2H_4$  are the intermediates in anammox metabolism. Addition of these (and of hydroxylamine) stimulates the activity of whole cells. Moreover, the compounds can be measured in the medium, indicating that they can diffuse out of or into the cell. However, each of these molecules that leave the anammox cycle has to be replenished at the cost of energy by reversed-electron transport-driven nitrite oxidation (see Section 4.3). The solution to minimize such losses is to keep the catabolic processes inside a special cell compartment enclosed by highly impermeable membranes with the help of the ladderanes. By flocking together in biofilms or in clusters of planktonic cells (Fig. 3), residual losses of NO and hydrazine can be shared for the benefit of companions. The ladderanes might offer a second advantage. Membranes constitute a barrier for the passage of charged molecules, though not a perfect one. Protons can passively diffuse through the membrane at a certain rate, independent of the metabolic activity, thereby dissipating the pmf. In high-speed mitochondria, such slippage accounts for an estimated 10% energy loss (Haines, 2001). Obviously, this would be detrimental for the slowly metabolizing anammox bacteria. By their dense-packed nature, ladderanes might limit proton leakage to a minimum, as, for instance, has been found for the caldarchaeols in thermophilic archaea (Van de Vossenberg *et al.*, 1999).

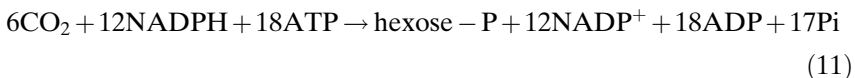
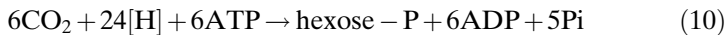
Regardless of the localization of the metabolic processes, charged substrates and supplementary nutrients have to pass one or more membrane systems. It then comes with no surprise that the *K. stuttgartiensis* genome encodes quite a few putative transport systems (Strous *et al.*, 2006). Regarding substrate trafficking, these include five AmtB-type ammonium transporters (kustc0381, kustc1009, kustc1012, kustc1015, kuste3690), four FocA-type formate/nitrite transporters (kusta0004, kustd1720, kustd1721, kuste4324), and two NarK-type nitrite:nitrate antiporters (kuste2308, kuste2335). Once more, future research has to show to which level and

under which conditions they are expressed, and where the transporters are localized in the cell.

### 4.3. Cell Carbon Fixation and Nitrite Oxidation

Anammox bacteria are autotrophic organisms when growing on ammonium, nitrite, and bicarbonate (Strous *et al.*, 1999a). Following the sequencing of *K. stuttgartiensis* genome, all genes were detected encoding the enzymes of the reductive acetyl-CoA (Wood–Ljungdahl pathway), indicating that anammox bacteria employ this route for CO<sub>2</sub> fixation (Strous *et al.*, 2006). Moreover, activity of its key enzyme, acetyl-CoA synthase/CO dehydrogenase was demonstrated (Strous *et al.*, 2006), and isotope ratio mass spectroscopic analysis of anammox biomass and lipid biomarkers showed that these were highly <sup>13</sup>C depleted (~47‰ vs. CO<sub>2</sub>), in agreement with this pathway (Schouten *et al.*, 2004). The presence of the full repertoire of gluconeogenesis and tricarboxylic acid enzymes (except citrate lyase) in the genome readily accounts for the intermediary anabolism.

Energetically, the acetyl-CoA pathway is quite efficient at first impression. If calculated per hexose-6-phosphate made from CO<sub>2</sub>, the Wood–Ljungdahl pathway (Eq. 10) is less ATP-demanding compared to, for instance, the Calvin–Benson–Bassham cycle (Eq. 11).



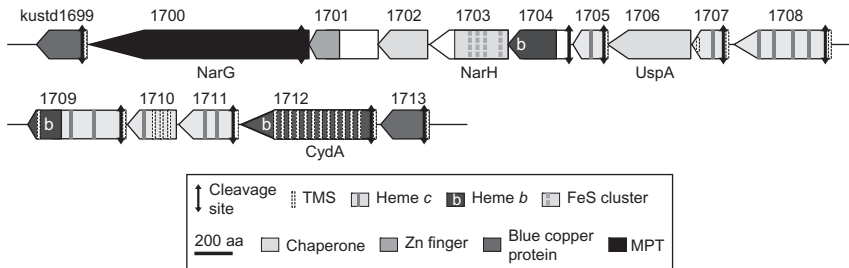
However, acetyl-CoA synthesis comprises a number of low-redox-potential reactions that require strong reductants ([H]), *viz.*, CO<sub>2</sub> reduction to formate ( $E_0' = -0.43$  V), CO<sub>2</sub> reduction in acetyl-CoA synthesis ( $E_0' = -0.50$  V), as well as the reductive carboxylation of the latter to make pyruvate ( $E_0' = -0.47$  V). Furthermore, NADPH ( $E_0' = -0.32$  V) is required as the electron donor in a variety of other anabolic reactions. The redox reactions are catalyzed by intricate membrane-bound protein complexes, encoded in the *K. stuttgartiensis* genome within large gene clusters. The formate::quinone oxidoreductase complex and accessory proteins are located on kustc0821–0842, acetyl-CoA dehydrogenase on kustd1538–1552, and the pyruvate dehydrogenase is found on kustc1054–1056. These enzymes are extremely oxygen-labile, which could be a reason for the sensitivity of anammox for the compound. In addition, two NADH::quinone

oxidoreductases (complex I) are available, an  $H^+$ -dependent one (NuoA-N, kuste2660–2672) and, remarkably, a sodium-translocating species (NqrA-E, kuste3325–3329). The activity of all of these membrane-bound enzyme complexes is known to be associated with ion ( $H^+$ ,  $Na^+$ ) translocation.

Taking the reducing power of hydrazine into account, these aforementioned reductions would not be a big deal, but they are. Electrons for anabolic reduction reactions derive from hydrazine oxidation, but it is unknown whether these electrons follow some low-redox potential route or are supplied via quinol-dependent reversed-electron transport (Fig. 6). In any case, the electrons are drained from the anammox cycle and have to be replenished. With  $CO_2$  as carbon source and ammonium and nitrite as catabolic substrates, the only way to do so is nitrite oxidation to nitrate. This is the very reason that growth of the bacteria under such conditions is connected with nitrate production (Eq. 3). However, nitrite is a relatively poor electron donor ( $E_0' = +0.43$  V) and electrons have to be pumped “uphill,” at least to the  $bc_1$ /quinone level ( $E_0' \sim 0$  V), which represents of a tough case of RET.

The prototype enzyme for nitrate:nitrite oxidoreduction is NarGHI (Einsle and Kroneck, 2004; Moura *et al.*, 2004; González *et al.*, 2006). In denitrifying bacteria, the enzyme catalyzes the quinol-dependent reduction of nitrate to nitrite. The molybdopterin-containing subunit NarG is the catalytic part, the iron-sulfur protein NarH mediates in electron transfer, and the membrane-bound heme *b*-containing subunit withdraws the electrons from quinol. Nitrate reductase activity was measured before (van de Graaf *et al.*, 1997; Schalk *et al.*, 2000; Güven *et al.*, 2005; Kartal *et al.*, 2007b). In the *K. stuttgartiensis* genome, the genes coding for NarG (kustd1700) and NarH (kustd1703) are observed as part of a large gene cluster (kustd1699–1713), however lacking a clear homolog of NarI (Fig. 10). Instead, a colorful collection of cytochrome *c*-type multiheme proteins, a putative heme *b* membrane protein, as well as two polypeptides potentially representing a new family of (cupredoxin-like) blue copper proteins (de Almeida *et al.*, 2011) is present. Together, the kustd1699–1713 proteins almost cover nature's full repertoire of redox proteins.

Returning to our starting point (Eqs. 1–3), a couple of things come to mind. As noted before, about 4 mol of nitrite are oxidized per mol fixed carbon, which equals 8 mol of reducing equivalents [H] (Eq. 3). However, only about 4 mol [H], that is, 2 mol of nitrite, are actually needed per fixed  $CO_2$  (Eq. 10). Where do the remaining 50% [H] rest? One cause is overflow and excretion of organic compounds, including extrapolymeric substances that have not been taken into account in the determination of carbon biomass. Another reason is NO and/or hydrazine loss. Moreover,



**Figure 10** Gene-cluster organization of the nitrite:nitrate oxidoreductase system kustd1699–1713 in *Kuenenia stuttgartiensis*. Lengths of the polypeptides are drawn to scale (aa, amino acids). Structural motifs are as specified in the figure. Cleavage site, N-terminal cleavage site; TMS, transmembrane-spanning region; MPT, molybdopterin-containing subunit; CydA, subunit showing significant sequence homology to subunit 1 of the alternative cytochrome *bd* quinol oxidase.

only 1 mol of carbon is bound per 15 catabolic cycles. Assuming a proton-translocation stoichiometry of  $3-4\text{H}^+$  per ATP synthesized by the ATPase, each catabolic cycle would yield 1.5–2 mol of ATP (Fig. 6). The yield could be even higher if hydrazine oxidation encompasses one or more additional proton-translocating coupling sites. By not doing so, a large amount of energy derived from hydrazine oxidation is dissipated as heat. In any case, per fixed  $\text{CO}_2$  only 1 ATP is needed (Eq. 10), whereas at least a certain amount—either as ATP or its  $\text{H}^+$ -translocation equivalents—is required to drive RET. (The mechanism behind RET still needs to be elucidated. Energy required for “electron pumping” can be obtained from ATP hydrolysis or is released at the expense of proton/ion translocation during their passage across the cell membrane.) Nevertheless, the growth yield is exceedingly low ( $\sim 0.066 \text{ mol C mol}^{-1}$  per  $\text{NH}_4^+$  oxidized; Eq. 1), indicating the catabolism and anabolism are highly uncoupled. Again, the reason for the uncoupling is not known, but it may well have to do with proton slippage and the leakage of intermediates during long doubling times.

The invention to convert ammonium anoxically, however slowly, provided the anammox bacteria their specific niche. The problems to overcome were to metabolize at such high rate that proton extrusion exceeded the backpressure as the result of passive proton slippage (and loss of intermediates), permitting the building up of a pmf of sufficient magnitude to drive ATP synthesis. By the presence of the slow HZS, this only could only be done by expressing this protein in high amounts, finding a compromise between the space occupied by the (membrane-bound) enzymatic machinery and the leaky surface. Herewith, the maximization

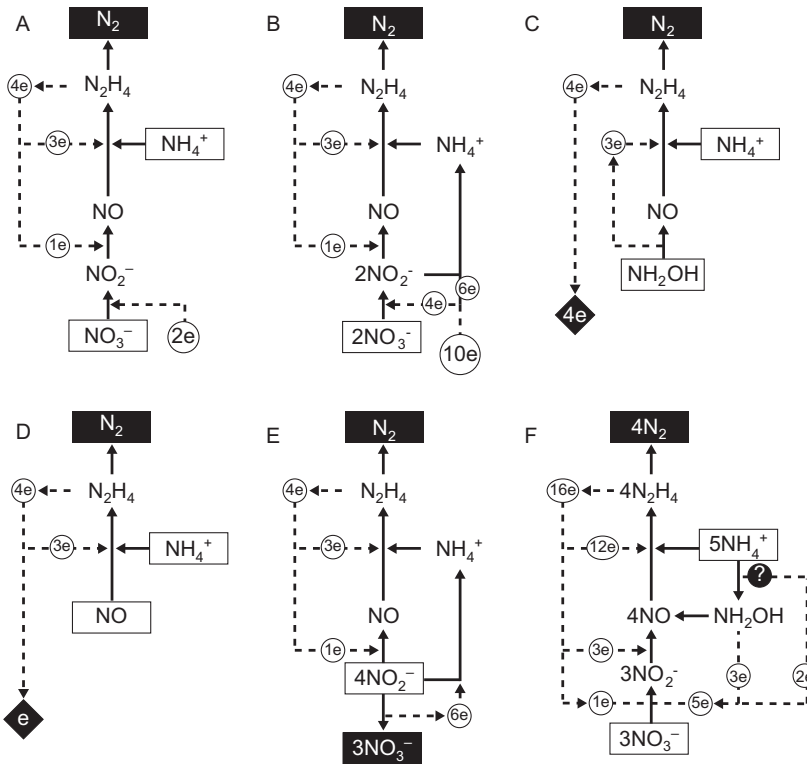


of the catabolic rate could go at the cost of more efficient energy conservation, again finding an optimum: it is pointless to make more ATP than is needed for growth and cell maintenance. Next, substrates should be taken up with such high affinity as to out-compete other rivals in the field or to thrive on the scraps that are left by these rivals. Clearly, anammox bacteria succeeded to overcome these challenges.

#### 4.4. Metabolic Versatility

As outlined above, anammox bacteria can consume organic compounds to sustain their metabolism (see [Section 2](#); [Tables 2 and 3](#)). Species even seem to have developed a certain specialization in this. Formate, acetate, and propionate are completely oxidized to CO<sub>2</sub> ([Kartal et al., 2007b](#)). The former two can employ already available acetyl-CoA pathway enzymes for CO<sub>2</sub> fixation. This is somewhat puzzling since the pathway has to operate in two directions at the same time, which is possible if forward and backward processes proceed at low rate under thermodynamic equilibrium. The presence in the *K. stuttgartiensis* genome of methylmalonyl-CoA decarboxylase (kustd2060–2061) and methylmalonyl-CoA epimerase (kuste4266), the two key enzymes of propionate metabolism, complies with the ability of this organism to utilize propionate ([Table 2](#)). Still, the genome leaves room for other, as yet not investigated, fermentation products from the environment to serve as electron donors for anammox bacteria. The organisms can also use mono- and dimethylamine ([Kartal et al., 2008](#)) and even methanol (B. Kartal and B. de Wild, unpublished results) for this purpose. Nevertheless, the enzymes catalyzing the conversion of the methylated compounds remain to be identified. In a previous study, methanol was found to inhibit anammox activity ([Güven et al., 2005](#)), but this may only hold for the aggregated species tested and organisms may adapt to it.

The consumption of organic reductants is attractive in a number of respects. First, more energy is derived from this process as compared to ammonium and nitrite conversion alone (see [Eqs. \(2\) and \(12\)](#) where the Gibbs free energy change was calculated with formate as the electron donor). Next, by the oxidation of the carbon compounds, electrons drained from the anammox cycle can be replenished without the energy investment related with nitrite oxidation. Lastly, it enables the microorganisms to use nitrate, which is almost always more abundant than nitrite. Following nitrate reduction by the Nar system, nitrite is fed into the catabolic cycle ([Fig. 11A](#)). In fact, the use of organic or inorganic reductants enables



**Figure 11** Schematic representation of the metabolic versatility of anammox bacteria. (A) Ammonium and nitrate metabolism supported by an external organic or inorganic electron donor. (B) "Disguised denitrification" by the reduction of nitrate to  $\text{N}_2$  supported by an external (in)organic electron donor. Stimulation of the anammox metabolism by (C) hydroxylamine and (D) nitric oxide. (E) Nitrite disproportionation. (F) Anammox process from ammonium and nitrate alone. Substrate and electron flows are represented by solid and dashed lines, respectively. Substrates are boxed in white, products in black, electrons derived from external sources are marked by the larger circles at the base, and surplus electrons by black diamonds. The latter can be used for reductive reactions related with supplementation of the anammox cycle intermediates or cell carbon fixation.

anammox to adopt a "disguised" denitrifying lifestyle (Kartal *et al.*, 2007b) (Fig. 11B). In this process, nitrate is first converted into nitrite and half of it is reduced into ammonium. Hereafter, ammonium and nitrite are combined to yield dinitrogen gas by the anammox cycle. In *K. stuttgartiensis*, the ammonium-producing reaction seems to be rate-limiting, frequently

Table 3 Alternative organic and inorganic electron donors and acceptors in anammox metabolism.

	Evidence (reference)
Electron donors and reactions	
$\text{H}_2 \rightarrow 2\text{H}^+ + 2e$	Genome (Strous <i>et al.</i> , 2006)
$\text{HCOO}^- \rightarrow \text{H}^+ + \text{CO}_2 + 2e$	Experimental (Kartal <i>et al.</i> , 2007b)
$\text{CH}_3\text{COO}^- + 2\text{H}_2\text{O} \rightarrow 7\text{H}^+ + 2\text{CO}_2 + 8e$	Experimental (Kartal <i>et al.</i> , 2007a, 2008)
$\text{CH}_3\text{CH}_2\text{COO}^- + 4\text{H}_2\text{O} \rightarrow 13\text{H}^+ + 3\text{CO}_2 + 14e$	Experimental (Kartal <i>et al.</i> , 2007a,b)
$\text{CH}_3\text{OH} + \text{H}_2\text{O} \rightarrow 6\text{H}^+ + \text{CO}_2 + 6e$	Experimental (B. Kartal and B. de Wilt, unpublished results)
$\text{CH}_3\text{NH}_2 + 2\text{H}_2\text{O} \rightarrow 5\text{H}^+ + \text{CO}_2 + \text{NH}_4^+ + 6e$	Experimental (Kartal <i>et al.</i> , 2008)
$(\text{CH}_3)_2\text{NH} + 4\text{H}_2\text{O} \rightarrow 11\text{H}^+ + 2\text{CO}_2 + \text{NH}_4^+ + 12e$	Experimental (Kartal <i>et al.</i> , 2008)
Electron acceptors and reactions	
$2\text{NO}_3^- + 12\text{H}^+ + 10e \rightarrow \text{N}_2 + 6\text{H}_2\text{O}$	“Disguised denitrification” (Kartal <i>et al.</i> , 2007b)
$\text{Fe}^{3+} + e \rightarrow \text{Fe}^{2+}$	Experimental (Strous <i>et al.</i> , 2006)
$\text{FeOOH} + 3\text{H}^+ + e \rightarrow \text{Fe}^{2+} + 2\text{H}_2\text{O}$	Experimental (Strous <i>et al.</i> , 2006)
$\text{MnO}_2 + 4\text{H}^+ + 2e \rightarrow \text{Mn}^{2+} + 2\text{H}_2\text{O}$	Experimental (Strous <i>et al.</i> , 2006)
$\text{O}_2 + 4\text{H}^+ + 4e \rightarrow 2\text{H}_2\text{O}$	Genome (Strous <i>et al.</i> , 2006)

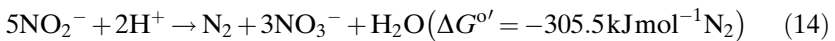
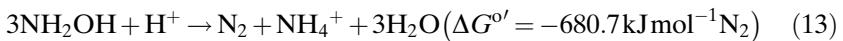
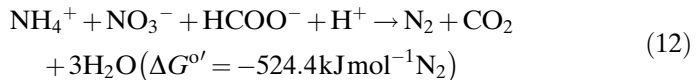
resulting in the intermediary accumulation of nitrite. However, in *B. fulgida* and *A. propionicus*, no such nitrite formation could be detected. The six-electron reduction of nitrite into ammonium resembles the Dissimilatory Nitrate/Nitrite Reduction to Ammonium (DNRA) mechanism. In DNRA bacteria, the reaction is performed without intermediates by the dissimilatory nitrite::ammonium oxidoreductase NrfA, a calcium-containing pentaheme protein (Simon, 2002; Mohan *et al.*, 2004; Smith *et al.*, 2007; Kern and Simon, 2009). Other microorganisms employ different octaheme c-type proteins for this purpose (Atkinson *et al.*, 2007; Polyakov *et al.*, 2009). *B. anammoxidans* and *K. stuttgartiensis* cell extracts show significant  $\text{Ca}^{2+}$ -dependent nitrite reductase activity producing ammonium, and the activity could be enriched to quite a degree (Kartal *et al.*, 2007b). However,

the enzyme still remains to be identified and the genome shows no clear orthologue to known NrfAs. Also in this respect, anammox bacteria must have invented another variation on the theme, either by a novel enzyme (Kartal *et al.*, 2007b) or by tuning one of the HAO-like enzymes (Fig. 7) to this function. The identification of the particular anammox nitrite reductase is relevant, not only from the biochemical point of view, but also for the application as a biomarker. Unlike the standard anammox process, both nitrogen atoms in  $N_2$  stem from nitrate, which will make it hard or even impossible to decide in labeling studies if the gas is made by “true” or “disguised” denitrifiers.

Besides the organic compounds mentioned, inorganic compounds can serve as electron donors for *K. stuttgartiensis*, notably  $Fe^{2+}$  (Strous *et al.*, 2006) (Table 3). Next, the presence of the complete set of genes coding for Ni-Fe hydrogenase and accessory enzymes (kustd1773–1779) in its genome hints at hydrogen as a potential electron source. On the other hand,  $Fe^{3+}$ , insoluble  $FeOOH$ , and  $MnO_2$  serve as electron acceptors (Strous *et al.*, 2006). Whether this represents a physiological reaction is not yet clear: the opposite oxidative direction would be more beneficial for an organism that is in need of reducing power. Likewise, it is conceivable that the organisms are able to derive electrons from the oxidation of metal-containing solid materials. The conversion of this type of compounds requires dedicated multiheme proteins contacting substrates outside the cell (reviewed in Shi *et al.*, 2007). Indeed, various multiheme proteins are present in the genome that might fulfill such a role. This also holds for molybdopterin-containing enzymes, potentially mediating redox processes related with the conversion of extracellular compounds. Remarkably for an obligate anaerobe, the gene cluster kustc0425–0430 encodes the components of a proton-translocating *cbb*<sub>3</sub>-type terminal oxidase, which are expressed to a certain extent. The question is if this oxidase contributes to the energy metabolism or merely acts in oxygen detoxification.

In essence, anammox bacteria are autotrophic microorganisms primarily growing on ammonium and nitrite. Overlooking their metabolic potentials, a number of other autotrophic lifestyles can be envisaged, which have not, or only partly, been investigated experimentally. As mentioned before, NO and hydroxylamine can stimulate anammox activity. One may note that the compounds are found in natural habitats as free intermediates during imbalanced growth both of aerobic ammonium- and nitrite-oxidizing bacteria (Schmidt *et al.*, 2004a,b; Kampschreur *et al.*, 2008, 2009; Schmidt, 2008; Yu and Chandran, 2010) and of anaerobic denitrifiers (Betlach and Tiedje, 1981; Baumann *et al.*, 1996, 1997; Otte *et al.*, 1996; Saleh-Lakha *et al.*, 2009). Within the N-cycle, these three guilds are natural partners and

competitors for anammox bacteria. Hydroxylamine and NO can be disproportioned into ammonium and nitrite by available routes. In this way, the compounds might support growth, even at very low (submicromolar) concentrations. In fact, hydroxylamine disproportionation (Eq. 13) may have been seen transiently in hydrazine production experiments (Van der Star *et al.*, 2008b). Although not primary substrates, NO and hydroxylamine, as well as hydrazine, may represent a useful supplement to a frugal diet of ammonium and nitrite (Fig. 11C,D). Another intriguing possibility is the disproportionation of nitrite into nitrate and N<sub>2</sub> permitting growth in the absence of ammonium or external electron donors (Eq. 14). This would imply a completely novel type of chemolithotrophy, although the possibility has been ruled out for good reasons, at least for denitrifying organisms (Strohm *et al.*, 2007). Still, nitrite disproportionation is compatible with the known anammox route, and the hypothetical pathway is depicted in Fig. 11E. However, the disproportionation would support growth only if the energy demand for RET-driven oxidation of three nitrite molecules is less than the amount conserved with one anammox cycle. A last possibility does not concur with anammox metabolism as far as we know it,



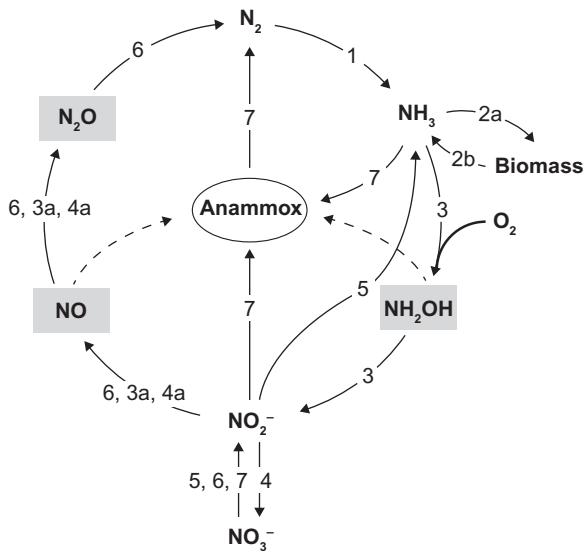
notably one of two reactions predicted by Broda (1977), the anaerobic oxidation of ammonium coupled to the reduction of nitrate to make N<sub>2</sub> (Eq. 15). The problem with this is that it would require the activation of an ammonium molecule into an NO-containing species, such as hydroxylamine (Fig. 11F), which seems to be an exclusive property of aerobic ammonium oxidizers. By the same argumentation, alternative chemolithotrophic processes involving anaerobic ammonium oxidation to N<sub>2</sub> ( $E_0' = -0.278 \text{ V}$ ) coupled with the exergonic reduction of inorganic electron acceptors of more positive redox potential (other than oxygen or nitrogen oxides) are not very likely, unless they include a mechanism for ammonium activation. But still, such reactions are energy yielding, and as we know, “never say never in nature.” The occurrence of a microorganism making its own oxygen, notably from nitrite, is not without precedent (Ettwig *et al.*, 2010).

All in all, anammox bacteria display a metabolic versatility that is only partly appreciated, lending the organisms the opportunity to thrive under conditions where the key substrates, ammonium and nitrite, are limiting. Moreover, it may provide different species their specific ecological niches.

## 5. ANAMMOX AND THE N-CYCLE: A NEW PLAYER WITH AMPLE QUESTIONS

Anammox bacteria are the last major addition to the N-cycle (Fig. 12). For more than a century, it was believed that with the fixation of nitrogen into ammonium, aerobic oxidation of the latter into nitrate (nitrification), and the subsequent reduction under anaerobic conditions of nitrate and nitrite back to nitrogen (denitrification), the case was closed. By now, anammox has been given a central place in the N-cycle. The bacteria have been detected in virtually any fixed-nitrogen-containing anoxic environment. In various systems, they are a major or even the only sink for fixed nitrogen (Thamdrup *et al.*, 2004; Arrigo, 2005; Dalsgaard *et al.*, 2005; Francis *et al.*, 2007; Lam and Kuypers, 2011). Wherever they are present, anammox bacteria have to acquire their substrates in close cooperation and competition with other N-cycle organisms, as there are nitrifiers, denitrifiers, DNRA bacteria, and ammonifiers involved in mineralization (Fig. 12).

Obviously, anammox bacteria owe their position in the N-cycle to their unique property to oxidize ammonium under anoxic conditions. Herein, they are supported by a highly specific catabolic system organized as proton-motive machinery to conserve energy, which most likely is localized in a special cell organelle: the anammoxosome. Recent research reviewed above (see Section 4.1) has established the basics of the catabolism (Fig. 6). The central intermediates, hydrazine and NO, have been identified, as well as the key enzymes, HDH and HZS (Kartal *et al.*, 2011b). A remaining unanswered question is which enzyme catalyzes nitrite reduction to NO. It is conceivable that different anammox species utilize different enzymes (or enzyme combinations) for the reaction. Still, the picture shown in Fig. 6 is probably only a simplistic one. As outlined before (Section 4.1), *K. stuttgartiensis* genome, for example, codes for no less than 10 HAO-like proteins, only one have been given a physiological function (HDH, kustc0694) and one (kustc1061) known to oxidize hydroxylamine into NO (Fig. 7). The physiological role of the latter as well as of the other highly expressed HAO-like proteins is elusive, but these might somehow



*Figure 12* Position of anammox bacteria in the nitrogen-cycle. Partial processes: (1), nitrogen fixation; (2a), ammonium assimilation; (2b), ammonification/mineralization, (3), aerobic ammonium oxidation; (4), nitrite oxidation; (3a) and (4b), anaerobic nitrification–denitrification by ammonium- and nitrite-oxidizing nitrifiers, respectively; (5), dissimilatory nitrite reduction to ammonium (DNRA); (6), anaerobic denitrification; (7), anammox. Bound intermediates in the pathways are marked by light gray boxes. The putative exchange of intermediates between different processes is represented by dashed lines.

serve as backup systems for the central reaction, hydrazine synthesis. The detailed answers to these questions only will come from the step-by-step resolution of the individual reactions, the identification of the intermediary electron carriers and possibly new electron-transfer membrane-bound protein complexes, and the elucidation of the biochemical reactions and catalytic mechanisms of the different enzymes, permitting an insight how each protein is tuned to its specific function. All this holds for HZS in particular.

Concerning the way energy is conserved as ATP in the anammox process, the state of the art is mainly hypothetical. Taking into account the redundancy of ATPases and  $bc_1$  complexes, the scheme presented in Fig. 6, again, is a simple view at best. Here also, only the purification, characterization, and localization of the individual enzyme systems as well as membrane-bound substrate transport systems may give a clue for their

role. The development of a reproducible, solid protocol for the isolation in high yield of intact anammoxosomes is crucial in these respects.

Irrespective of whether the key substrates (ammonium and nitrite) are obtained from metabolic partners or have to be obtained in competition with other N-cycle bacteria, the compounds have to be taken up with the best affinity possible. This also holds for organic and inorganic electron donors that sustain anammox metabolism. By the differential expression of uptake systems and metabolic enzyme systems,  $K_m$  and  $V_{max}$  values can be adapted to prevailing environmental conditions. The conditions differ enormously among the very many habitats where anammox bacteria are found. Deposited 16S rRNA gene sequences reflect a spectrum of as yet uncultured species, subspecies, and strains, each apparently having found its specific niche in its specific habitat. So far, only nine anammox species have been described to a more or lesser degree. For none of these species, it is known at the molecular level what determines their niche specialization. This issue is particularly intriguing for *Scalindua*, which seems to be the pre-eminent marine species. Genome sequencing and comparative genome analyses may give partial answers on species differentiation and separation. However, the presence (or absence) of certain genes not necessarily explain species differentiation. Small differences in enzyme expression levels may provide species a significant competitive advantage, as illustrated in the *B. fulgida* and *A. propionicus* cases (see [Section 2](#)). Consequently, anammox genomics has extended to whole-cell transcriptomics and proteomics performed on cell cultures grown under carefully controlled conditions, together offering a haystack of information, which may hide tiny needles. The “omics” approaches also may give a first insight into adaptive responses toward environmental changes, their underlying regulatory systems being a long chapter with only white pages. A specific question here is which enzyme catalyzes nitrite reduction to ammonium. The presence of the particular enzyme different from the common NrfAs offers anammox bacteria the opportunity to grow with limited or even without ammonium, presenting itself as a “disguised denitrifier.” This manifestation makes it difficult to distinguish between nitrogen formation by anammox bacteria and “genuine” denitrifiers.

As mentioned, anammox bacteria receive their substrates in cooperation and competition with other N-cycle microorganisms. Again, metabolic interactions may turn out to be much more complex and delicate than anticipated. This is, for instance, reflected in the relationship between anammox and aerobic ammonium oxidizers, two guilds that at first glance would be mutually exclusive because of their different lifestyles (anaerobic



vs. aerobic). However, the cooperation between both has laid the foundation for new wastewater treatment technology (Schmidt *et al.*, 2002, 2003; Kuenen, 2008; Kartal *et al.*, 2011a). In the OMZs of the Black Sea, world's largest anoxic basin, and in the Bengualan upwelling system, all dinitrogen gas released can be attributed to the cooperative action between anammox and ammonium oxidizers, both the bacterial and archaeal representatives (Kuypers *et al.*, 2003, 2005; Lam *et al.*, 2007; Woebken *et al.*, 2007). This raises interesting questions as to how the aerobic partners operate under the suboxic conditions. Also in the Omani Gulf, anammox is the major or even only sink for fixed nitrogen, albeit in a different fashion. At this site, anammox receives its substrates from DNRA bacteria (Jensen *et al.*, 2011). Next to these, denitrifiers are not necessarily only competitors. By partial denitrification of nitrates, the organisms also could provide anammox with nitrite. A striking example of this is the recent elucidation of a "cryptic" sulfur cycle in the Chilean upwelling system where anammox, sulfide-oxidizing denitrifiers, and sulfate reducers may be reducing nitrate to N<sub>2</sub> in concert (Canfield *et al.*, 2010). The discovery of overlooked N-cycle microorganisms, such as ammonium-oxidizing archaea that were just referred to (Karner *et al.*, 2001; Martens-Habbena *et al.*, 2009; Schleper and Nicol, 2010), nitrite-oxidizing phototrophs (Griffin *et al.*, 2007), nitrate-reducing foraminifera (Risgaard-Petersen *et al.*, 2006), hyperthermophilic N<sub>2</sub>-fixing methanogens (Mehta and Baross, 2006), and anaerobic nitrite-dependent methane oxidizers (Raghoebarsing *et al.*, 2006; Ettwig *et al.*, 2010) in recent years, has caused a paradigm shift in our understanding of N-cycle processes. Long-time known, recently detected, and most probably a host of still unknown species together permit a multitude of metabolic interactions, not only via established compounds, but perhaps also via intermediates (NO and hydroxylamine). The outcome of the interactive processes may differ in time and place, as found for instance in the Peruvian upwelling system (Lam *et al.*, 2009). For example, rather than an N-cycle, the whole system might represent a network, a web of metabolic interactions. In the middle of this web, anammox has found a place with wide range of questions regarding its physiology.

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