REVIEW ARTICLE

How to make a living from anaerobic ammonium oxidation

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Received 26 June 2012; revised 25 October 2012; accepted 21 November 2012.

DOI: 10.1111/1574-6976.12014

Editor: Hauke Hennecke

Keywords
anammox; nitric oxide; hydrazine; anaerobic respiration; Wood–Ljunghdahl pathway; nitrogen cycle.

Abstract

Anaerobic ammonium-oxidizing (anammox) bacteria primarily grow by the oxidation of ammonium coupled to nitrite reduction, using CO_2 as the sole carbon source. Although they were neglected for a long time, anammox bacteria are encountered in an enormous species (micro)diversity in virtually any anoxic environment that contains fixed nitrogen. It has even been estimated that about 50% of all nitrogen gas released into the atmosphere is made by these 'impossible' bacteria. Anammox catabolism most likely resides in a special cell organelle, the anammoxosome, which is surrounded by highly unusual ladder-like (ladderane) lipids. Ammonium oxidation and nitrite reduction proceed in a cyclic electron flow through two intermediates, hydrazine and nitric oxide, resulting in the generation of proton-motive force for ATP synthesis. Reduction reactions associated with CO_2 fixation drain electrons from this cycle, and they are replenished by the oxidation of nitrite to nitrate. Besides ammonium or nitrite, anammox bacteria use a broad range of organic and inorganic compounds as electron donors. An analysis of the metabolic opportunities even suggests alternative chemolithotrophic lifestyles that are independent of these compounds. We note that current concepts are still largely hypothetical and put forward the most intriguing questions that need experimental answers.

Introduction

Thirty-five years ago, Austrian physicochemist and alleged KGB spy Broda (1977) predicted the existence of two groups of missing autotrophs on evolutionary and thermodynamic grounds. These would employ ammonium as the electron donor of their metabolism: (1) phototrophs and (2) chemolithrophs using nitrate or nitrite as oxidants to make dinitrogen gas (N_2) as the end product of their energy metabolism. The first group still remains to be discovered. Broda's prediction regarding the ammonium oxidizers that would reduce nitrate or nitrite in the absence of oxygen was met with a lot of skepticism because it was believed that oxygen would be indispensable to activate the relatively inert ammonium and convert it into hydroxylamine (NH_2OH). Although attempts must have been made to enrich such organisms, the lack of literature suggests that they were not successful due to inadequate culturing methods, a lack of patience, or both. Nevertheless, occasionally oceanographers reported an unexplainable loss of ammonium from anoxic marine basins (Hamm & Thompson, 1941; Richards, 1965). A similar observation in a denitrifying bioreactor prompted the quest by Delft microbiologists to investigate the cause of the ammonium loss (Van de Graaf *et al.*, 1995, 1996). This ammonium loss turned out to be biological. Supported by dedicated culturing techniques, the 'missing lithotroph' was enriched and described as an 'anaerobic ammonium oxidizer' (anammox) (Strous *et al.*, 1999a,b). Since their first description in the mid-1990s, research on these microorganisms developed at a pace and over a reach that very well could be beyond Broda's imagination. For the reader who is interested in these developments over the first decade of anammox research, we would like to refer to a number of reviews (Jetten *et al.*, 2003, 2005, 2009; Kuenen, 2008; Kartal *et al.*, 2012).

The enrichment and characterization of the first anammox bacteria enabled the design of molecular and

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metabolic tools to detect these organisms in their natural habitats: These 'impossible' organisms appeared to be omnipresent in anoxic, fixed nitrogen-containing environments. Presently, anammox bacteria have been detected in soil, groundwater, wastewater treatment plants, freshwater and marine sediments, lakes, estuaries, oxygen minimum zones and continental shelves in the oceans, polar regions, hot springs, and deep-sea hydrothermal vents (Op den Camp *et al.*, 2006; Penton *et al.*, 2006; Schmid *et al.*, 2007; Jetten *et al.*, 2009; Humbert *et al.*, 2010). Anammox bacteria are scarce in some environments, but are metabolically dominant in others, in particular in oxygen minimum zones, which are the major sources of nitrogen release into the atmosphere from the oceans (Lam & Kuypers, 2011). It has even been estimated that about 50% of the annual fixed nitrogen loss could be attributed to anammox activity (Arrigo, 2005; Lam & Kuypers, 2011). Moreover, the anammox process is currently applied as a cost-effective and environment-friendly system for the removal of nitrogen from wastewater (Kartal *et al.*, 2010a).

Anammox bacteria are amazing microorganisms from a microbiological and molecular biological perspective. They are characterized by a complicated cell plan featuring a voluminous intracellular organelle: the anammoxosome (Lindsay *et al.*, 2001; Van Niftrik *et al.*, 2004, 2008a,b). The anammox cell is divided into three different sections, including the central anammoxosome, by three membrane layers. The membranes themselves contain highly unusual linearly concatenated ring structures (Sinninghe Damsté *et al.*, 2002, 2005). Compared to other chemolithoautotrophs, the genetic foundation is laid by a large genome (~4 Mb), which encodes the enzymatic machinery that allows anammox bacteria to make a living from the anaerobic oxidation of ammonium. Here, we present an overview of the recent progress in our understanding of the anammox metabolism, raise the major questions that still need to be answered, and make suggestions for future research.

Anammox bacteria

Growth

Anammox bacteria are slow-growing, strictly anoxic autotrophic microorganisms that primarily use ammonium and nitrite as substrates for their catabolism. Nitrite is toxic to the cells, but its toxicity is likely to be species related and depends on exposure time (Strous *et al.*, 1999b; Egli *et al.*, 2001). In any case, nitrite concentration should be kept as low as possible. The possibility to grow microorganisms at very low growth rates and very low substrate concentrations is offered by the sequencing batch reactor (SBR) technique (Strous *et al.*, 1998; Kartal *et al.*, 2011a; Oshiki *et al.*, 2011). In SBRs, cells from environmental samples are enriched by continuous cycles of biomass settling, removal of the supernatant, and supplementing the reactor with fresh medium. In this way, cells are essentially kept within the reactors for an infinite amount of time. Reactors are flushed with N_2, helium, or argon to create anoxic conditions and CO_2 to serve as the carbon source. Fresh medium contains ammonium, trace elements, bicarbonate (as a buffer), specific components to be tested, and nitrite at a concentration that will be instantly converted in the reactor. In the start-up phase, nitrate is added to establish oxidative conditions, preventing the growth of other anaerobically respiring microorganisms such as sulfate reducers and methanogens. In response to nitrite consumption, its concentration in the feed is gradually increased. If successful, the biomass slowly turns red, which is a visual indication of the presence of anammox bacteria. Typically, after 180–280 days, the bioreactor reaches its maximal activity and anammox bacteria make up ~80% of the total population. The exponential increase in nitrite conversion infers that the metabolic activity doubles every 7–22 days, which may be taken as an indication of the doubling time of the organisms (Strous *et al.*, 1999b; Tsushima *et al.*, 2007a,b; Van der Star *et al.*, 2008a; Oshiki *et al.*, 2011).

The SBR technique has been fruitfully applied in many laboratories (Op den Camp *et al.*, 2006), although other approaches to culture these organisms have also been developed. Bioreactors such as upflow-anaerobic sludge blanket (UASB) reactors (Strous *et al.*, 1998; Imajo *et al.*, 2004; Schmidt *et al.*, 2004a,b; Ni *et al.*, 2010), rotating biological contactors (Van de Graaf *et al.*, 1996; Egli *et al.*, 2001, 2003; Windey *et al.*, 2005), and even manually fed batch cultivation systems (Sànchez-Melsió *et al.*, 2009; Suneethi & Joseph, 2011; Yasuda *et al.*, 2011) were used. All methods, however, rely on the settling properties of anammox cell aggregates. Within these biofilm aggregates, the microorganisms are associated with a variety of other very diverse species (e.g. Strous *et al.*, 2006; Van de Vossenberg *et al.*, 2012). Forming clusters may reflect the natural growth state of anammox bacteria. Moreover, wastewater treatment engineering certainly benefits from the fast sedimentation velocities of aggregates or granules and the tight metabolic interactions within these (Kartal *et al.*, 2010a,b). From the microbiological, physiological, and biochemical point of view, such biofilms are a challenge. Cells can be difficult to visualize and count (Kartal *et al.*, 2008). Spatial and microbial inhomogeneity impedes the interpretation of the results of whole-cell physiological experiments. The cell paste prepared from the biomass that is rich in extracellular polysaccharides and other sticky compounds resists the

resolution of cellular components for biochemical research (Cirpus *et al.*, 2006). These drawbacks were overcome in membrane bioreactors (MBR) in which freely dispersed cells are contained by the application of a membrane to the medium outflow (Van der Star *et al.*, 2008a; Kartal *et al.*, 2011a). It appears that a small fraction of anammox bacteria occur as free-living, planktonic cells in SBRs. These can be used to inoculate an MBR. Alternatively, an MBR may be inoculated with a small amount (i.e. 4–5%, v/v) of aggregated cells. Without the selective pressure for faster settling cells, aggregates disintegrate in time and the planktonic cells eventually dominate the bioreactor (after ~350–400 days).

Operating under steady state conditions in laboratory-scale bioreactors, ammonium, nitrite, and bicarbonate as the sole carbon source are metabolized according to the following overall Eqn. (1) (Strous *et al.*, 1998):

$$1\ NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \quad (1)$$

$$1\ NH_4^+ + 1\ NO_2^- \rightarrow 1\ N_2 + 2\ H_2O\ (\Delta G^{\circ\prime} = -357\ kJ\ mol^{-1}\ N_2) \quad (2)$$

$$0.27\ NO_2^- + 0.066\ HCO_3^- \rightarrow 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} \quad (3)$$

This overall reaction is the result of two partial processes: the energy-generating reaction, the oxidation of ammonium coupled to nitrite reduction to make N_2 (Eqn. 2), and bicarbonate fixation into cell biomass ($CH_2O_{0.5}N_{0.15}$) (Eqn. 3). It is important to note here that the two nitrogen atoms derive from different sources: ammonium and nitrite. Using one ^{15}N-labeled substrate ($^{15}NH_4^+$ or $^{15}NO_2^-$), $^{14}N^{15}N$ is the specific end product of the anammox reaction. Thus, the analysis of the isotope composition of N_2 ($^{14}N^{14}N$, $^{14}N^{15}N$, $^{15}N^{15}N$) by mass spectrometry (Van de Graaf *et al.*, 1997) allows a clear-cut distinction between anammox bacteria and denitrifiers. In the latter pathway, both nitrogen atoms stem from nitrate or nitrite. One may note that nitrite has a dual role in anammox metabolism: It acts as the electron acceptor in the ammonium-oxidizing reaction (Eqn. 2) and as the ultimate electron donor for the CO_2 reduction to biomass (Eqn. 3). Consequently, growth is always associated with nitrate production, and about four moles of nitrite are oxidized per mol of fixed carbon. A closer look at reaction stoichiometries (Eqn. 1) shows that 0.066 mole of carbon is fixed per mole of oxidized ammonium, which equals to one carbon per 15 catabolic cycles. This biomass yield is very similar to chemolithotrophs that use the Calvin–Benson–Bassham cycle for autotrophic CO_2 fixation (0.07–0.09 mol mol^{-1} C).

Species diversity

So far, ten anammox species have been described, including seven that are available in laboratory enrichment cultures. As none of these were obtained as classical pure cultures, all have the taxonomical status of '*Candidatus*'. Known species are divided over five genera: (1) *Kuenenia*, represented by *Kuenenia stuttgartiensis* (Strous *et al.*, 2006), (2) *Brocadia* (three species: *B. anammoxidans*, *B. fulgida*, and *B. sinica*; Strous *et al.*, 1999a; Kartal *et al.*, 2008; Oshiki *et al.*, 2011), (3) *Anammoxoglobus* (one species: *A. propionicus*; Kartal *et al.*, 2007a), (4) *Jettenia* (one species: *J. asiatica*; Quan *et al.*, 2008; Hu *et al.*, 2011), and (5) *Scalindua* (four species: *S. brodae*, *S. sorokinii*, *S. wagneri*, and *S. profunda*; Schmid *et al.*, 2003; Woebken *et al.*, 2008; Van de Vossenberg *et al.*, 2012). Representatives of the first four genera were enriched from sludge from wastewater treatment plants; *K. stuttgartiensis*, *B. anammoxidans*, *B. fulgida*, and *A. propionicus* were even obtained from the same inoculum. *Scalindua* dominates the marine environment, but it is also present in some freshwater ecosystems and wastewater treatment plants (Schmid *et al.*, 2003; Schubert *et al.*, 2006; Hamersley *et al.*, 2009). Together, these 10 species most probably represent a minute fraction of anammox biodiversity. For instance, currently over 2000 16S rRNA gene sequences affiliated with anammox bacteria have been deposited to the Genbank (http://www.ncbi.nlm.nih.gov/genbank/), reflecting an unappreciated continuum of species, subspecies, and strains, each apparently having found its specific niche in the wide variety of habitats where anammox bacteria are encountered. Species microdiversity is particularly impressive for the marine representative *Scalindua* (Schmid *et al.*, 2007; Woebken *et al.*, 2008; Dang *et al.*, 2010; Hong *et al.*, 2011a,b; Li *et al.*, 2011). A still open question is which environmental factors determine species differentiation for anammox bacteria.

Sequence identities of the deposited anammox 16S rRNA genes range between 87 and 99%, and phylogenetic analysis places them all within the phylum *Planctomycetes* (Fuerst & Sagulenko, 2011), which form the PVC superphylum together with *Verrucomicrobia* and *Chlamydiae* (Wagner & Horn, 2006). Within the *Planctomycetes*, anammox bacteria deeply branch as a monophyletic clade. Their phylogenetic position together with a broad range of specific physiological, cellular, and molecular traits endow anammox bacteria their own order *Brocadiales* (Jetten *et al.*, 2010).

The cell plan

A typical characteristic of the *Planctomycetes* is their complicated cell plan comprising intracellular organelles (Lindsay *et al.*, 2001; Fuerst, 2005; Fuerst & Sagulenko, 2011). Anammox is not an exception to this. Still, anammox bacteria are not very special at first glance. Cells are visualized under the microscope as small coccoid cells with a diameter of about 0.8 μm. The more detailed inspection by different electron microscopy techniques discloses a complex cell plan typical for *Planctomycetes* (Strous *et al.*, 1999a). In essence, the cell is composed of three compartments, each surrounded by a membrane bilayer (Fig. 1a) (Lindsay *et al.*, 2001; Van Niftrik *et al.*, 2008b; for a comprehensive review, see Van Niftrik & Jetten, 2012). The outermost membrane encloses both the cell and the outer compartment, the so-called paryphoplasm. It is not known whether the outer membrane is fully closed, which would make it a cytoplasmic membrane, or gated like the periplasmic membrane of Gram-negative bacteria. The presence of a variety of genes potentially encoding porin-like outer membrane proteins supports the latter possibility. Besides the membrane, the cell wall might also be structured by proteins, peptidoglycan, or both. Fuerst *et al.* (2006) found indications for the presence of an S-layer protein lattice in *K. stuttgartiensis*. In the genome of this organism (Strous *et al.*, 2006), a large cluster containing 19 of 21 peptidoglycan synthesis genes is detected, notably lacking a gene coding for penicillin-binding protein I. It is unknown whether the peptidoglycan synthesis genes are functionally expressed. The second membrane surrounds the so-called riboplasm that harbors the nucleoid and ribosomes (Van Niftrik *et al.*, 2008a). Like in the cytoplasm of common prokaryotes, the transcription, translation, and household machinery are presumed to reside in this compartment. Here, glycogen granules and larger particles resembling polyhydroxyalkanoate bodies are observed (Van Niftrik *et al.*, 2008a,b). The third, innermost, highly curved membrane bounds the central cell structure: the anammoxosome. Detailed analyses show that this structure is fully closed and that it is vertically inherited to the daughter cells during cell division (Van Niftrik *et al.*, 2004, 2008a; Van Niftrik *et al.*, 2008b). These observations define the anammoxosome as a true cell organelle. As described below, a variety of heme *c* proteins, such as hydroxylamine oxidoreductase (HAO)-like proteins and the hydrazine synthase (HZS) complex, are involved in anammox catabolism and are hypothesized to reside in the anammoxosome. The energy released in the anammox process is conserved by a membrane-bound ATP synthase (ATPase). Diaminobenzidine staining revealed that nearly all heme *c* complement was localized in close proximity of the inner rim of the anammoxosome membrane (Van Niftrik *et al.*, 2008a,b). An HAO-like protein (Lindsay *et al.*, 2001; Jetten *et al.*, 2009) (Fig. 1b), HZS (Karlsson

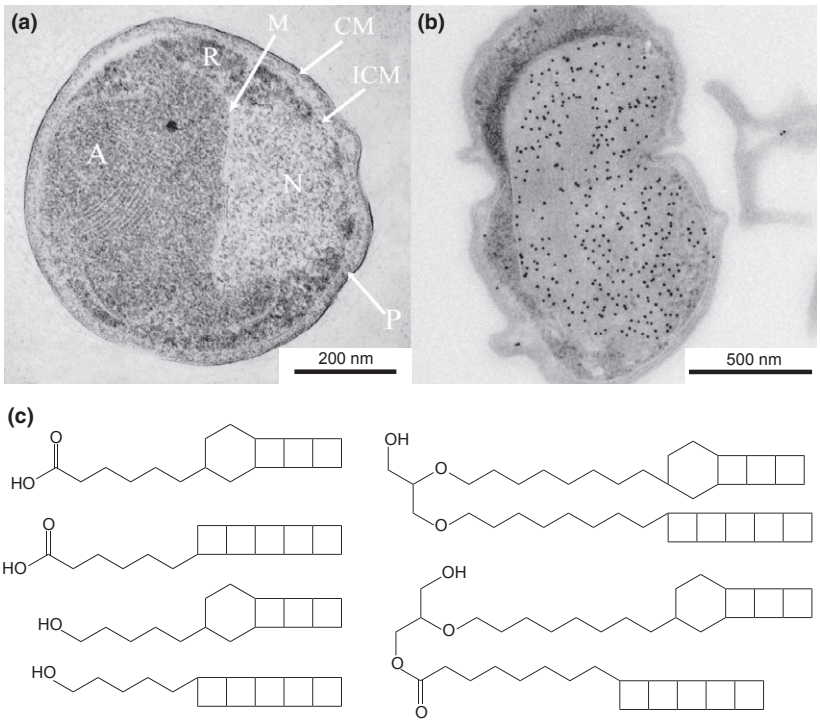

Fig. 1. Unique features of the anammox cell. (a) The anammox cell plan represented by a transmission electron microscopy image of *Anammoxoglobus propionicus*. A, anammoxosome containing tubule-like structures; R, riboplasm containing the nucleoid (N) opposed to the anammoxosome membrane (M); P, paryphoplasm or periplasm separated from the riboplasm by an intracytoplasmic membrane (ICM); CM, cytoplasmic or outer cell membrane. (b) Immunogold localization within the anammoxosome of a major hydroxylamine oxidoreductase (kustc1061). (Courtesy, L. van Niftrik). (c) 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.

et al., 2009) and the major ATPase (Van Niftrik *et al.*, 2010) were specifically located within the anammoxosome by immunogold-labeled antibodies. Together, these findings are consistent with its function of the organelle as the ATP-generator-room of the anammox cell.

As pointed out in the introduction, another unique feature of anammox bacteria is the nature of the cell membrane constituents. Like in all other living organisms, anammox membranes are composed of glycerolipids. These 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. 1c). What makes anammox special is the presence of saturated C17-C20 fatty acids and alcohols that are fused by *cis*-ring junctions to make ladder-like ('ladderane') cyclobutane and cyclohexane ring systems (Sinninghe Damsté *et al.*, 2002, 2005; Kuypers *et al.*, 2003; Schmid *et al.*, 2005). Ladderanes 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. Different species display species-specific variations on the same theme (Boumann *et al.*, 2006; Rattray *et al.*, 2008, 2010). The fatty acids are esterified with methanol or with the glycerol backbone, while the ladderane alcohols are ether-linked with glycerol, all in different combinations (Fig. 1c). At the *sn*-1 position, glycerol is substituted with one of many different hydrophobic tail types, whereas different polar head groups (phosphocholine, phosphoethanolamine, or phosphoglycerol) may be substituted at the *sn*-3 position (Boumann *et al.*, 2006; Rattray *et al.*, 2008).

A property of ladderanes is their dense packing, which makes them highly impermeable for fluorophores that readily pass through common membranes (Sinninghe Damsté *et al.*, 2002; Boumann *et al.*, 2009a,b). C27 hopanoid ketone and bacteriohopanetetrol, both pentacyclic compounds derived from hopane, convey the membranes their functional rigidity and fluidity (Boumann *et al.*, 2009b). In *B. anammoxidans*, ladderanes comprised 34% of the total lipid content, but the content was higher (53%) in a cell fraction that was partially enriched with anammoxosomes (Sinninghe Damsté *et al.*, 2002). Nevertheless, it is still unclear whether or not ladderanes are the specific lipid components of this cell organelle. An answer to this question needs solid protocols for the isolation of anammoxosomes with a high yield.

Anammox genomes

In 2006, the first genome of an anammox bacterium (*K. stuttgartiensis*) became available (Strous *et al.*, 2006). Meanwhile, the partial genome of *B. fulgida* was described (Gori *et al.*, 2011), followed by the recent publication of the genome of the marine species *S. profunda* (Van de Vossenberg *et al.*, 2012). Besides these, the genome of the anammox strain KSU-1 is publicly available (Hira *et al.*, 2012). All four were obtained by metagenomic approaches, and none of them are complete. In the original publication by Strous *et al.* (2006), the *K. stuttgartiensis* genome comprised five large contigs, numbered kustae, covering an estimated >98.5% of the whole genome and missing approximately 60 genes. The recent resequencing of *K. stuttgartiensis* and a closely related strain enriched from a Chinese wastewater treatment plant confirmed the original assembly to great detail (Speth *et al.*, 2012a). The resequencing added 49 new genes, including the only missing tRNA synthase gene (leucyl-tRNA synthase) and permitted the closure between contigs B and E.

Genome annotation, analysis, and comparison were surprising. The first surprise was the size of the genomes: Chemolithoautotrophic specialists – as anammox bacteria were presumed to be – encode roughly 2000 genes, while no fewer than ~5000 open reading frames (ORFs) are present in the anammox genomes. Such a large number of ORFs, most of which encode gene products with an unknown function, also made it clear that we still understand only very little of these organisms. For instance, of 4663 ORFs from *K. stuttgartiensis*, 3279 (70.3%) showed significant similarity with genes in databases, but only 1385 genes (29.7%) could be annotated with a function (Strous *et al.*, 2006). Another surprise was the enormous differences in the genomes of species that were considered to be closely related. In the *S. profunda* assembly (4664 ORFs), for instance, only 693 genes had significant BLASTN hits with *K. stuttgartiensis* (Van de Vossenberg *et al.*, 2012). BLASTP had a more positive result (2016 hits), although the averaged identity of the ORFs was only 48.6%. Moreover, 2187 ORFs in the *K. stuttgartiensis* genome did not match with any ORF in the genome of *S. profunda*. Nevertheless, analyses of the genome sequences not only raised questions, but also provided new insights. The presence of genes coding for a variety of metabolic routes and transport systems strongly suggests that anammox bacteria are not just specialists, but might use an unappreciated range of organic and inorganic compounds to sustain their metabolism. The presence of over 200 genes in the *K. stuttgartiensis* genome that code for enzymes putatively involved in respiratory processes is particularly astonishing. Key systems, such as complex I, the bc_1 complex, and ATP synthase, are present as different, redundant copies. The apparent redundancy seems to be fully conserved in *S. profunda*. In *K. stuttgartiensis*, the respiratory proteins comprise 63 heme *c*-type proteins, and this number is as high as 85 in *S. profunda*, outnumbering the range of heme *c*-type

proteins of omnivores such as *Shewanella* and *Geobacter*. More importantly, the detailed analysis of genomes permitted the formulation of experimentally testable hypotheses on how the anammox machinery could work.

Anammox catabolism

Intermediates

The question that remained unanswered for a long time was how anammox bacteria are able to oxidize ammonium anaerobically. Like in each metabolic system, the process would proceed via intermediates. The breakthrough in our understanding was a serendipitous finding by Van de Graaf *et al.* (1997). When these scientists tested the effect of hydroxylamine on anammox cells, they observed the transient accumulation of a compound with a molecular mass of 32 (33 when either $^{15}NH_2OH$ or $^{15}NH_4^+$ was used). The compound was unambiguously identified as hydrazine (N_2H_4) (Fig. 2). Ever since, the experiment shown in Fig. 2 has been repeated many times with different anammox species and always with the same result (e.g. Strous *et al.*, 1999a; Schalk *et al.*, 2000, Kartal *et al.*, 2007a, 2008; Van der Star *et al.*, 2008b). The experiment is even considered diagnostic for the presence of anammox. Moreover, anammox cultures that became inactive for one reason or another could be 'boosted' by the addition of hydroxylamine or hydrazine. On the basis of their observations, Van de Graaf *et al.* (1997) proposed a three-step model with hydroxylamine and hydrazine as intermediates. This model comprised (1) the four-electron reduction of nitrite to hydroxylamine, (2) the subsequent condensation of hydroxylamine and ammonium to make hydrazine, and (3) the four-electron oxidation of hydrazine to yield the end product N_2. These suggestions were consistent with the experimental results, and two of the proposed enzymes had precedence in literature. Nitrite reduction to hydroxylamine is the reverse of the second step of aerobic ammonium oxidation, which is mediated by HAO. Moreover, it was already known that HAO is also capable of oxidizing hydrazine into N_2 *in vitro* (Hooper *et al.*, 1997). Hydrazine synthesis would need a new, unknown enzyme: The compound had never been detected in any biological system and required a special enzyme to synthesize it. Still, the conversions seen in Fig. 2 could be experimental artifacts as the result of hydroxylamine administration. In fact, the turnover of neither hydroxylamine nor hydrazine was demonstrated under physiological conditions. Another complicating factor was that a candidate gene coding for hydroxylamine reductase making nitrite could not be found in the genome of *K. stuttgartiensis* (Strous *et al.*, 2006). Instead, the genome revealed the presence of genes encoding cd_1 nitrite reductase (NirS) and its accessory proteins. NirS catalyzes the reduction of nitrite to nitric oxide (NO). On the basis of genome analyses and results from physiological experiments, Strous *et al.* (2006) put forward an alternative three-reaction process (Eqns 4–6). This new three-reaction system was incorporated into a hypothetical model taking energy conservation into account. The new model (with further modifications) is shown in Fig. 3.

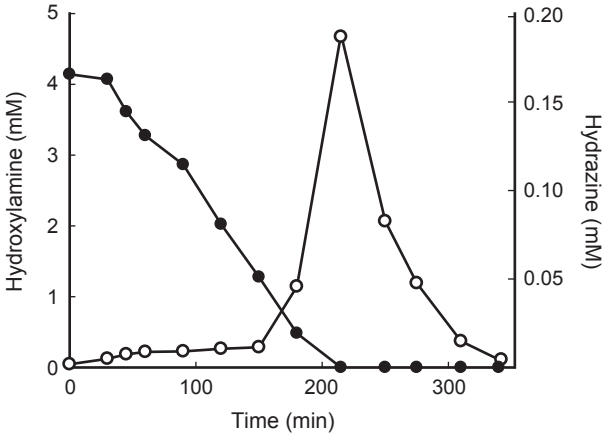

Fig. 2. Production of hydrazine (open circles) by *Brocadia fulgida* after the addition of hydroxylamine (closed circles). (Adapted from Kartal *et al.*, 2008).

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4\mathbf{e}\ (E_0' = -0.75\ V) \quad (4)$$

$$NO + NH_4^+ + 2H^+ + 3\mathbf{e} \rightarrow N_2H_4 + H_2O (E_0' = +0.06\ V) \quad (5)$$

$$NO_2^- + 2H^+ + \mathbf{e} \rightarrow NO + H_2O (E_0' = +0.38\ V) \quad (6)$$

As already proposed by Van de Graaf *et al.* (1997), nitrogen formation would be the result of the four-electron oxidation of hydrazine, catalyzed by an HAO-like enzyme, termed hydrazine dehydrogenase (HDH) (Eqn. 4). These four electrons would then drive nitrite reduction to NO (by NirS) (Eqn. 6) and hydrazine synthesis (Eqn. 5). The latter step, the condensation of NO and ammonium together with the input of three electrons, invoked a biochemical novelty: HZS. In 2011, Kartal *et al.* (2011b) were able to substantiate this concept with experimental results. The results came from a series of complementary approaches involving whole-cell transcriptomics and proteomics, and physiological and biochemical experiments. By supplying metabolizing cells with unlabeled (^{14}N) hydrazine and ammonium together with ^{15}N-nitrite, it was shown that hydrazine is turned over during ammonium and nitrite conversion. The role of NO as an intermediate was established by a combination

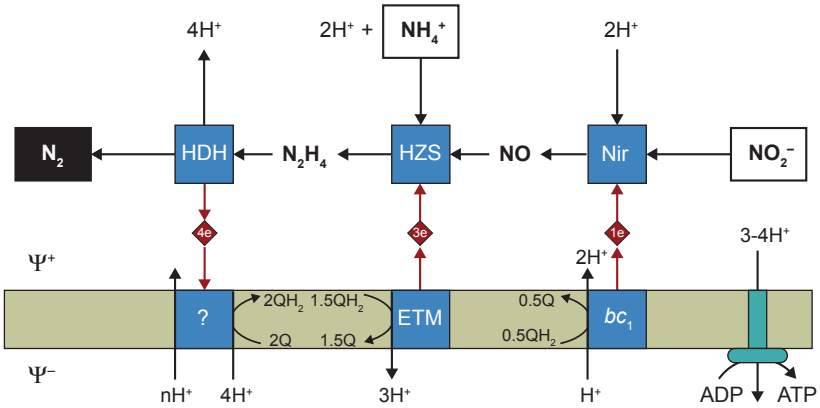

Fig. 3. Proposed process of nitrogen formation and ATP generation from ammonium and nitrite with nitric oxide (NO) and hydrazine (N_2H_2) as intermediates. Diamonds represent putative cytochrome *c* proteins and the number of electrons that are transferred in between the related reactions. The hypothetical reduced cytochrome:quinone (Q) oxidoreductase that feeds electrons from hydrazine oxidation into the Q-pool is indicated by the question mark. bc_1: quinol:cytochrome *c* oxidoreductase (bc_1, complex III), ETM: electron transfer module providing hydrazine synthase (HZS) with reductant, HDH: hydrazine dehydrogenase, Nir: nitrite reductase. Ψ^+, Ψ^-, the positive and negative sides of the electrochemical gradient compartments, which most likely are the anammoxosome and cytoplasm (riboplasm), respectively.

of inhibition studies and fluorescent labeling of the cells. Moreover, NO and ammonium served as the direct substrates for hydrazine synthesis. Apparently, anammox bacteria take advantage of the oxidizing power of NO to activate the relatively inert ammonium. Lastly, by direct purification, Kartal and coworkers (Kartal *et al.*, 2011b) identified the enzymes catalyzing hydrazine oxidation and synthesis (Eqns 4 and 5).

Because they are chemolithotrophs, the only means by which anammox bacteria can conserve energy is by a chemiosmotic mechanism. Such a mechanism would imply the net translocation of protons across a semipermeable membrane system in concert with redox reactions (Eqns 4–6). This results in the creation of proton-motive force (*pmf*) to drive ATP synthesis by a membrane-bound ATP synthase. All these reactions are present in the model shown in Fig. 3. In this model, intermediary electron transfers are carried out by a series of currently unknown soluble cytochrome *c* proteins and quinone derivatives present in the membranes. Strous *et al.* (2006) assigned the central role in proton translocation to quinol:cytochrome *c* oxidoreductase (complex III, bc_1) and, in direct connection, to the proton-motive Q-cycle. We note that the *K. stuttgartiensis* genome codes for three different bc_1 complexes and four different ATPases. Moreover, the scheme presented in Fig. 3 involves cyclic electron flow. The immediate implication is that each electron that is withdrawn from the cycle has to be replenished by the oxidation of nitrite or some other organic or inorganic electron donor. The loss of electrons not only arises from reduction reactions for cell carbon synthesis but also from the loss of electron-storing intermediates (NO, N_2H_4). The latter aspect is not trivial. The mere fact that hydrazine and NO have a stimulatory effect on anammox activity in whole cells and that they can be detected outside the cells implies that they can escape the cell. This diffusible character also applies to hydroxylamine, although the present understanding would leave no apparent role for hydroxylamine as a free intermediate in the anammox metabolism.

HAO-like proteins and nitrogen formation

As stated above, HAO from aerobic ammonium oxidizers is able to catalyze the four-electron oxidation of hydrazine to N_2 artificially. Its physiological role is the oxidation of hydroxylamine to nitrite (Eqn. 7). HAO is structurally and functionally well characterized (Igarashi *et al.*, 1997; Arp *et al.*, 2002; Mowat & Chapman, 2005; Klotz *et al.*, 2008). The enzyme is composed of three identical subunits, each containing eight heme *c* molecules, numbered 1–8. Heme *c* is bound to the protein backbone via two neighboring cysteines and a histidine constituting the proximal ligand to the heme iron. This feature is easily observed in protein sequences from the CXXCH motif. Catalysis takes place at heme-4, termed P_{460} after a characteristic absorbance band at 460 nm in the fully reduced protein. The other *c*-type hemes mediate electron transfer to the external electron acceptor, which is a cytochrome *c*-type protein. The catalytic heme is covalently linked to a tyrosine present in the C-terminal part from a neighboring subunit. This linkage somehow lends the enzyme an oxidative role (Klotz *et al.*, 2008). However, HAO is also able to catalyze the reduction of

nitrite and hydroxylamine to a range of nitrogen compounds with reduced viologen dyes as powerful reductants (Kostera *et al.*, 2008, 2010; Pacheco *et al.*, 2011).

Octaheme HAO seems to have evolved from the fusion between pentaheme nitrite reductase (NrfA) and a triheme moiety (Einsle *et al.*, 1999; Klotz *et al.*, 2008). Indeed, the spatial arrangements of hemes 1–5 in NrfA fully match those of 4–8 in HAO. In NrfA, heme-1 is the catalytic part, which is devoid of a covalent link with another subunit. The latter enzyme catalyzes the six-electron reduction of nitrite into ammonium (Eqn. 8), a key reaction in assimilatory and dissimilatory nitrite reduction (see also below) (Simon, 2002; Mohan *et al.*, 2004; Smith *et al.*, 2007; Kern & Simon, 2009). Thus, the presence or absence of a C-terminal tyrosine is indicative for oxidative or reductive catalysis, respectively (Klotz *et al.*, 2008).

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4\mathbf{e} \quad (7)$$

$$NO_2^- + 8H^+ + 6\mathbf{e} \rightarrow NH_4^+ + 2H_2O \quad (8)$$

$$NH_2OH \rightarrow NO + 3H^+ + 3\mathbf{e} \quad (9)$$

Nowadays, many hundreds of HAO-like proteins are found in protein databases. The *K. stuttgartiensis* genome alone codes for 10 different paralogues, six of which are highly expressed at the transcriptional and protein levels, lending the cells their typical red color (Fig. 4) (de Almeida *et al.*, 2011; Kartal *et al.*, 2011b). Nine of these 10 are conserved in *S. profunda*. This organism does not have the kuste2457 orthologue (Van de Vossenberg *et al.*, 2012). The genome of strain KSU-1 contains eight of the ten. It lacks the kustc0458 and kustd2021 orthologues and the second copy of the kustc0694 gene (kustd1340), but it contains an additional HAO-like protein that highly resembles the one found in methane-oxidizing bacteria (Campbell *et al.*, 2011). A detailed sequence analysis places the HAO-like anammox proteins into different families (Klotz *et al.*, 2008; Schmid *et al.*, 2008). Some of the anammox proteins display a high degree of sequence similarity to one and another, suggesting a similar metabolic function (Fig. 4). Unfortunately,

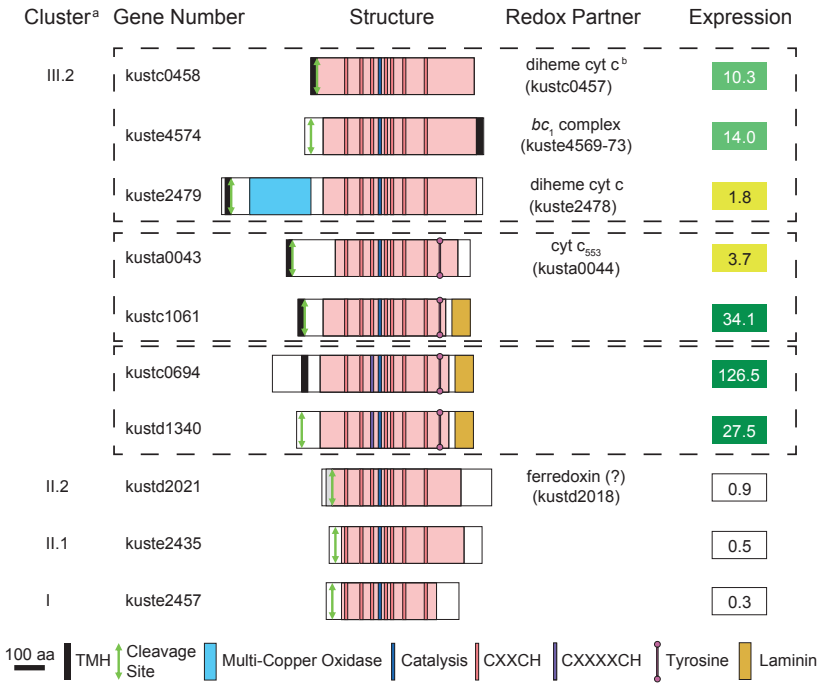

Fig. 4. Ten hydroxylamine oxidoreductase (HAO)-related octaheme proteins in the *Kuenenia stuttgartiensis* genome. The gene products are ordered according to their cluster position in the phylogenetic tree; highly homologous HAO-like proteins are boxed by dashed lines. Lengths of the polypeptides are 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 gene cluster. Expression values are expressed as n–fold coverage of Solexa deep RNA sequencing of the *K. stuttgartiensis* transcriptome (Strous *et al.*, 2006; Kartal *et al.*, 2011b). Structural motifs: TMH: transmembrane-spanning helix, cleavage site: N-terminal cleavage site, multicopper oxidase: multicopper oxidase domain, catalysis: catalytic heme, CXXCH: heme c-binding motif, CXXXXCH: unusual heme c-binding motif in kustc0694 and kustc1340, Tyrosine: tyrosine that covalently links the subunits, laminin: laminin sequence. [a]Classification according to (Klotz *et al.*, 2008). [b]This gene cluster also contains a gene encoding a cupredoxin-like blue copper protein (kustc0456).

such analyses do not answer important questions such as (1) 'Which one is the genuine HDH?' (2) 'What is the role of the others?' and (3) 'How is each protein tuned to a specific function?'.

By direct purification, kustc0694 was proven to be the physiological HDH (Kartal *et al.*, 2011b). Its *S. profunda* equivalent is scal03295. A highly related HAO-like HDH was also purified from anammox strain KSU-1 (Shimamura *et al.*, 2007), but its role remained unresolved. Kustc0694 catalyzes the four-electron oxidation of hydrazine to N_2 (Eqn. 4) with high activity and affinity. Importantly, 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% both at the nucleotide and amino acid levels over full-length kustd1340), although the presence of the kustd1340 gene product remains to be established. Both proteins share a CXXXXCH motif for heme-3 (Fig. 4), but the structural and functional significance of the unusual heme binding is not understood. The main difference between kustc0694 and kustd1340 is the 75-amino-acid-longer N-terminal part in the former followed by a transmembrane-spanning helix (TMH). This difference most likely derives from an erroneously interpreted start codon. The proper translation start is immediately before the TMH, which probably represents a signal sequence for protein translocation (W. Maalcke, unpublished result). In fact, all 10 anammox HAOs are predicted to have an N-terminal cleavage site, indicating all are targeted to another cell compartment (Fig. 4).

Hydrazine is nature's most powerful reductant ($E_0' = -0.75$ V). Therefore, it is highly likely that the electrons derived from its oxidation are exploited to promote the generation of *pmf* (Fig. 3). In aerobic ammonium oxidizers, the electrons from hydroxylamine oxidation are thought to be transferred via cytochrome cyt c_{554} (CycA), which is the direct electron acceptor of HAO, toward a membrane-anchored tetraheme c_{m552} (CycB), which is a ubiquinone reductase encoded in the same gene cluster as *hao* and *cycA* (Kim *et al.*, 2008). N-cycle bacteria possess a variety of modules to shuttle electrons to or from the quinone/quinol (Q) pool, and anammox bacteria likely employ a similar mechanism (Klotz & Stein, 2008; Simon & Klotz, 2012). Indeed, cell free extracts of *K. stuttgartiensis* show a high (~1 μmol min^{-1} mg^{-1} of protein) hydrazine-dependent quinone reductase activity (B. Kartal, unpublished result), but the responsible proteins remain to be identified. The heterodimeric NaxLS purified from KSU-1 would be a good candidate as an electron carrier (Ukita *et al.*, 2010). The complex is composed of two monoheme *c*-type proteins with unusually low redox potentials. The homologues of NaxLS in *K. stuttgartiensis* are kusta0087 and kusta0088, and they represent two of the major proteome components (data from Kartal *et al.*, 2011b). As mentioned, the nature of the quinone-reducing moiety is elusive. Its identification is most relevant in direct connection with the question which side of the membrane the protons for quinone reduction would be taken up and to what extent *pmf* generation would derive from scalar and vectorial (active) proton translocation. The drop in redox potential in the four-electron oxidation of hydrazine coupled to (mena)quinone reduction ($\Delta E_0' \sim -0.7$ V) is more than sufficient to translocate protons not only in a scalar way, but leaves ample room for additional vectorial proton pumping as well.

Kustc1061 is the most abundant HAO-like protein in the *K. stuttgartiensis* proteome (Kartal *et al.*, 2011b). Close homologues of this protein were purified before from *B. anammoxidans* and KSU-1 (Schalk *et al.*, 2000; Shimamura *et al.*, 2008), and the *S. profunda* orthologue is scal01317. This particular protein was previously shown to be specifically present inside the anammoxosome (Fig. 1b) (Lindsay *et al.*, 2001; L. Van Niftrik and C. Ferousi, unpublished results). Kustc1061 is capable of hydrazine oxidation, albeit slowly and with low affinity. Its main activity is the oxidation of hydroxylamine, not to nitrite as by HAO (Eqn. 7), but to NO (Eqn. 9) (Kartal *et al.*, 2011b). As hydroxylamine is no longer considered a free intermediate and so far no reactions have been found to produce the compound, the role of kust1061 is enigmatic. On the hindsight, the presence of kustc0694 (HDH) and kustc1061 may well explain the sequence of events following hydroxylamine administration to the cells (Fig. 2). Initially, kustc0694 is inhibited, and while hydroxylamine is removed by kustc1061, NO is made for hydrazine synthesis. So, when NH_2OH and NO are depleted below inhibitory concentrations, kustc0694 starts oxidizing the accumulated N_2H_4.

Just like for kust1061, the function of the other HAO-like proteins is unknown. The finding that they are present and are expressed (Fig. 4) suggests an important physiological role for each one. The highly homologous set kustc0458, kuste4574, and kuste2479 is particularly interesting. In this set, the crosslinking tyrosine in the C-terminus is absent. Its absence is indicative of reductive catalysis, possibly of nitrite. Kustc0458 is linked to genes encoding a diheme cyt *c* (kustc0457) and a novel type-1 blue copper-containing cupredoxin (kustc0456), both of which might serve as redox partners. Whereas the kuste4574 gene product forms part of a remarkable bc_1 complex, kuste2479 is fused to a multicopper oxidase that is related to the large family of oxygen-utilizing laccases (Claus, 2003; Giardina *et al.*, 2010; Di Gennaro *et al.*, 2011) and to the two-domain small laccase in particular (Machczynski *et al.*, 2004; Skálová *et al.*, 2009). Its neighbor kuste2478 is also a diheme cyt *c* with a linocyn

structure at the C-terminal part. Linocyns assemble into icosahedral nanocompartments that are excreted from the cell, thereby encapsulating oxidative stress response proteins in their interior (Sutter *et al.*, 2008). Thus, the kuste2478-2479 complex might very well act in the extracellular removal of oxidative or nitrosative stress compounds. Yet, the presence of kuste2479-containing extracellular nanobodies remains to be established.

Hydrazine synthesis

Arguably, one of the most intriguing properties of anammox bacteria is their capability to synthesize hydrazine. According to the proposal by Strous *et al.* (2006), this synthesis would proceed by the combination of NO and ammonium together with the input of three electrons catalyzed by the putative enzyme HZS (Eqn. 5). Besides nitric oxide reductases making nitrous oxide (N_2O) from NO, HZS would be the second type of enzyme capable of forging an N–N bond. From genome analysis supported by preliminary proteomic data, the authors postulated that HZS might be encoded by one or more genes in the cluster kuste2854-2861 (Fig. 5a). Our recent work verified this hypothesis (Kartal *et al.*, 2011b). The purification of the complex demonstrated it to be a heterotrimeric protein of kuste2859-2861. Kuste2859 is entirely composed of beta propeller sheets, providing a rigid platform for the catalytic part of the enzyme. At its N-terminal part also kuste2861 is mainly structured by beta propeller sheets, while it has two *c*-type cytochromes in its C-terminal region. These might act in electron transfer as proposed in Fig. 5b. Kuste2860 also contains two *c*-type cytochromes. This protein is related to cytochrome *c* peroxidase (Atack & Kelly, 2007; Poulos, 2010) and to mauG, which is the key enzyme of tryptophan tryptophylquinone (TTQ) biosynthesis (Wilmot & Davidson, 2009). (TTQ is a protein-derived catalytic cofactor of methylamine dehydrogenase first described for *Paracoccus denitrificans*.) The homology suggests that kuste2860 is the catalytic part of the complex. Remarkably, kuste2859 and 2860 are fused in *S. profunda* (scal00025), while the kuste2861 orthologue (scal01318) is located in another part of the genome next to scal01317, which is the kustc1061 orthologue of this organism (Van de Vossenberg *et al.*, 2012).

Hydrazine synthase comprises about 20% of the protein complement of *K. stuttgartiensis*. The presence of N-terminal signal sequences in the subunits (Fig. 5) indicate that the complex is targeted toward another cell compartment, the anammoxosome, as was suggested by immunogold labeling (Karlsson *et al.*, 2009). The isolated enzyme complex shows poor activity. Appreciable activity (20 nmol h^{-1} mg^{-1} of protein) is only measured by coupling electron input and hydrazine conversion to N_2 through kustc1061, which is insensitive toward the substrate NO (Eqns 4 and 5). Still, this activity is only about 1% of the *in vivo* rate. The loss in activity already occurs during cell breaking, indicating that this is due to the disruption of a tightly coupled multicomponent system (Kartal *et al.*, 2011b). Even if fully active, HZS would be a very slow enzyme, possibly explaining the slow growth rate of anammox bacteria.

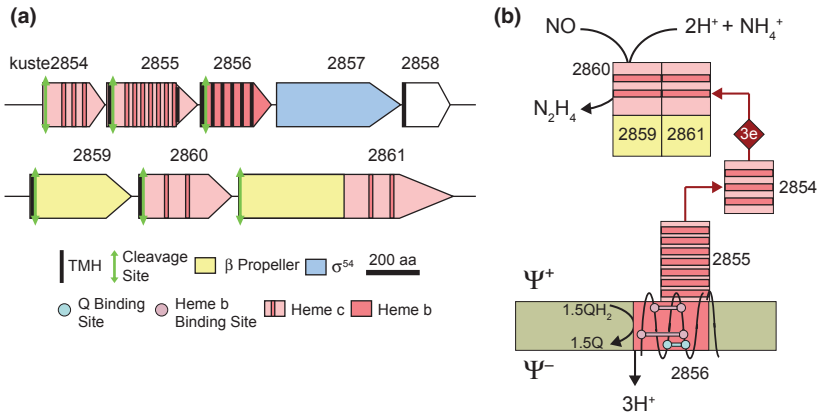

Fig. 5. Gene cluster organization of the hydrazine synthase system in the *Kuenenia stuttgartiensis* genome (a) and the proposed functional organization of its gene products (b). (a) Lengths of the gene products and the position of structural motifs are drawn to scale (aa: amino acids). (b) Putative heme *b*- and quinone (Q)-binding sites were derived from sequence comparison with respect to the highly homologous γ subunit (FdnI, cyt b_{556}) of menaquinone-dependent formate dehydrogenase (FDH-N, FdnGHI) from *Escherichia coli* (Jormakka *et al.*, 2002). Structural motifs are specified in the Figure. Numbers refer to the kuste gene numbers. Cleavage site, N-terminal cleavage site; TMH, transmembrane-spanning helix.

$$NO + NH_4^+ \rightarrow \frac{1}{4}N_2H_4 + \frac{3}{4}N_2 + H_2O + H^+ \qquad (10)$$

Hydrazine synthase also performs another reaction: It can oxidize its product, hydrazine, to N_2. The rate of the reaction is even 100-fold higher than its *in vitro* reductive, that is, physiological reaction. The net result of the forward and backward reactions is the disproportionation of NO and ammonium into hydrazine and N_2 (Eqn. 10). N_2 formation at the expense of hydrazine is not productive at all because hydrazine is the ultimate energy source. Therefore, a dedicated enzyme (kustc0694) is indeed very useful to scavenge hydrazine and to feed the electron transport chain for *pmf* generation. Major questions surround the catalytic mechanism underlying hydrazine synthesis and the role of kustc1061. One may note that the three-electron reduction of NO would yield an intermediate at the level of hydroxylamine. If so, its spillage would find the perfect cleanup service: Kustc1061 that recycles hydroxylamine back into NO and three electrons. Regardless of the answer to these questions, HZS depends on HAO-like proteins as backup systems (Kartal *et al.*, 2011b).

Another important question is what the electron donor for the HZS reaction is. Here, the genome may give another clue. Apart from the catalytic module (kuste2859-2861) and a sigma-54 transcriptional regulator (kuste2857), the gene cluster kuste2854-2861 has three additional genes (kuste2854-2856), all of which are equipped with N-terminal leaders (Fig. 5a). Kuste2854 is a triheme cytochrome *c*, which would make it a good candidate to transfer the three electrons needed for hydrazine synthesis. Kuste2855 harbors seven *c*-type hemes and contains a C-terminal TMH, potentially serving as a membrane anchor, and kuste2856 has significant homology to the γ subunit (FdnI, cyt b_{556}) of menaquinone (MQ)-dependent formate dehydrogenase (FDH-N, FdnGHI). FDH-N from *Escherichia coli* is structurally well characterized (Jormakka *et al.*, 2002). Cyt b_{556} is the MQ-binding part, and the binding occurs at the cytoplasmic side of the membrane. This subunit contains four TMHs at which four histidines coordinate two heme *b* molecules: one near the cytoplasmic (negative or *n*-side) and the other at the periplasmic face (positive site, *p*-side). All relevant amino acids related to protein structure and functionality are fully conserved in kuste2856, except that the latter contains an additional (fifth) TMH at the C-terminus. Figure 5b schematically illustrates this phenomenon. We should note that MQ ($E_0' = -0.075$ V) is the major quinone species in *K. stuttgartiensis* (N. M. de Almeida, unpublished result). It is possible to infer through analogy that reduced menaquinone (menaquinol, MQH_2) ultimately serves as the electron donor for hydrazine synthesis and release its protons at the cytoplasmic (*n*-) face. This would happen at the expense of *pmf*, yet would still provide the driving force for hydrazine synthesis ($E_0' = 0.06$ V). The disruption of this system and the use of nonphysiological (bovine heart) cyt *c* in the assays could also be an explanation for the low activity of the isolated HZS.

Nitric oxide and nitrite reduction

Strous *et al.* (2006) predicted that the intermediate NO would be produced via nitrite reduction by NirS (cyt cd_1). The genome of *K. stuttgartiensis* harbors the gene cluster kuste4136-4140 coding for the cyt cd_1 protein (kuste4136) in which all structural and functional amino acids identified in the atomic-resolution structures of NirS are conserved (Fülöp *et al.*, 1995; Baker *et al.*, 1997; Sjögren & Hajdu, 2001). Kuste4137 encodes a monoheme cyt *c* (NirC) that might act as the one-electron shuttle in nitrite reduction. Besides these, the cluster codes for proteins with strong sequence resemblance to the biosynthesis enzymes of the catalytic heme d_1. Hence, on the basis of genetic information, the cyt cd_1 could function as a nitrite reductase. However, in *K. stuttgartiensis*, NirS and NirC are only transcribed at low mRNA levels and are barely detectable in the proteome (Kartal *et al.*, 2011b). In striking contrast, NirS is one of the most abundant proteins in *S. profunda* (Van de Vossenberg *et al.*, 2012). It is possible that even low amounts of a highly active NirS in *K. stuttgartiensis* suffice as the nitrite reductase, but then, why would it be so highly expressed in *S. profunda*? To complicate things further, cd_1 nitrite reductase is fully absent in KSU-1, but this organism expresses the copper-containing protein NirK (Hira *et al.*, 2012). Perhaps, anammox bacteria contain additional nitrite reductases making NO. In this respect, HAO-like kustc0458 and kuste4574 could be possible candidates, whereas kustc1061 is available for NO formation from hydroxylamine. Thus, it seems that anammox bacteria have paved different roads to the essential intermediate NO, possibly lending the organisms metabolic flexibility in response to environmental changes.

Anammox bacteria take advantage of the oxidative power of the very reactive free-radical NO. Denitrifying bacteria that make NO as an intermediate in nitrate/nitrite respiration place NO-associated reactions out of the cell in the periplasm and have a variety of enzymes at their disposal to remove it efficiently. In these bacteria, different types of NO reductases (Nor) convert nitric oxide into the potent greenhouse gas N_2O (Tavares *et al.*, 2006; Hemp & Gennis, 2008; Watmough *et al.*, 2009; Kraft *et al.*, 2011; Martínez-Espinosa *et al.*, 2011; Stein,

2011). Other organisms that are confronted with NO attacks from the environment employ NorVW flavorubredoxin for detoxification. *Kuenenia stuttgartiensis* encodes and expresses NorVW (kuste3160) (Strous *et al.*, 2006; Kartal *et al.*, 2011a,b). Its *S. profunda* orthologue (scal000274) is also expressed, and this marine species contains an additional quinol-dependent qNor (scal02135) and a partial NorB (scal00292) as candidates for NO reduction (Van de Vossenberg *et al.*, 2012). Anammox bacteria, at least the freshwater representatives, are remarkably resistant toward nitric oxide and easily cope with 5000 p.p.m. NO, even using it for their metabolism (Kartal *et al.*, 2010b). Still, the organisms make no or only very little N_2O themselves, which is one of the advantages in their application (Kampschreur *et al.*, 2008, 2009; Kartal *et al.*, 2010b). In fact, most of what is being released from these systems is the result of denitrifying activity of aerobic ammonium oxidizers.

Energy conservation of anammox bacteria

bc$_1$ Complexes of anammox bacteria

In the proposed model for the anammox energy metabolism, the bc_1 complex has a central role (Fig. 3). As it is crucial for many other organisms, mitochondria and chloroplasts (cyt b_6f), the complex has been well investigated over the years, and the crystal structures were resolved for several species (see for reviews: Crofts, 2004; Osyczka *et al.*, 2005; Crofts *et al.*, 2006; Cooley, 2010; Mulkidjanian, 2010). Anammox bacteria have invented some interesting variations on the common theme. These are encoded in the *K. stuttgartiensis* genome by three gene clusters: kuste3096-3097, kustd1480-1485 and kuste4570-4574 (Fig. 6a). mRNA deep sequencing and proteomic analyses revealed that all three complexes are expressed at the transcriptional and protein levels, albeit in different amounts, kuste4570-4574 being the major species (Strous *et al.*, 2006; de Almeida *et al.*, 2011; Kartal *et al.*, 2011a, b).

The canonical bc_1 complex is a dimer of three core components: the Rieske 2Fe-2S iron-sulfur protein, a monoheme cyt *c*, and membrane-bound quinone-binding cytochrome b_6 (Crofts, 2004; Osyczka *et al.*, 2005; Cooley, 2010) (Fig. 6b). Cyt *c* and the Rieske iron-sulfur protein reside at the (periplasmic) *p*-side. After translation, the latter protein is transported by the twin-arginine (*tat*) translocon. Cyt b_6 traverses the membrane eight times (TMH-A–H), at which four highly conserved histidines at the entrances and exits of TMH-B and TMH-D coordinate two heme *b* molecules, facing the cytoplasmic (b_H) and periplasmic (b_L) sides, respectively (Fig. 6b). The architecture resembles FdnI (cyt b_{556}) described above with the major difference that cyt b_6 can bind two quinones: one near the cytoplasm (Q_i) and the other

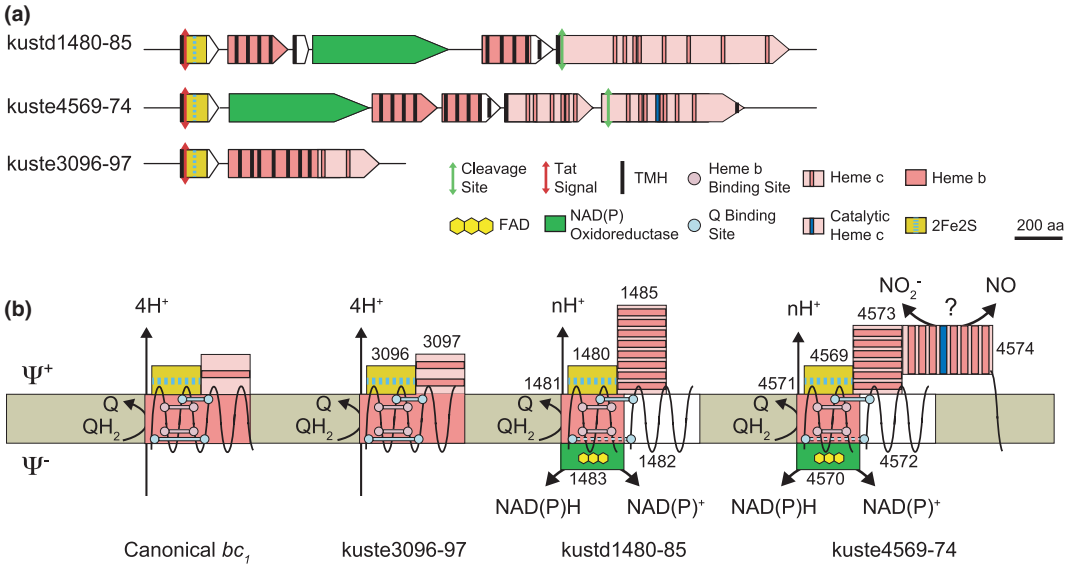

Fig. 6. Gene cluster organization of the three bc_1 complexes in the *Kuenenia stuttgartiensis* genome (a) and the proposed functional organization of their gene products (b). (a) Lengths of the gene products and the position of structural motifs are drawn to scale (aa: amino acids). (b) Putative heme *b*- and quinone (Q)-binding sites were derived from sequence comparison with respect to the canonical bacterial bc_1 complex (left-hand figure), which is represented as its monomeric three-subunit complex for simplicity (see also text). Numbers refer to the kust gene numbers as in (a). Structural motifs are specified in the Figure. 2Fe2S, Rieske 2Fe-2S iron-sulfur cluster; cleavage site, N-terminal cleavage site; *tat* signal, twin-arginine translocation signal; TMH, transmembrane-spanning helix. The catalytic heme *c* is as specified in Fig. 4.

positioned at the periplasm (Q_o). Binding of Q_i is achieved by amino acids at the TMH-A membrane entrance and the loop in between TMH-D and TMH-E (Fig. 6b). Q_o is sandwiched by amino acid stretches at the end of TMH-C and TMH-E. The catalytic function of the bc_1 complex is coupling the oxidation of the two-electron carrier quinol with the reduction of two cyt *c*-type cytochromes. Hereby, two protons from Q_oH_2 are released at the *p*-side. By an ingenious mechanism ('Q-cycle'), proposed by Peter Mitchell (1975a,b), an oxidized Q_i gets reduced and takes two protons from the cytoplasm, altogether giving an apparent net proton translocation stoichiometry of $4H^+/2e$. The mechanism, also known as 'oxidant-induced reduction' or 'electron bifurcation', exploits the large difference in midpoint redox potentials of the quinone-semiquinone (i.e. the one-electron reduced species) and the semiquinone-quinol couples.

Now, what is different about bc_1 complexes in anammox bacteria? Nothing special regarding the Rieske iron-sulfur proteins in the three bc_1 complexes; according to alignments, these are conserved with regard to other known species. The N-terminal signal sequences (*tat* signals) indicate that the encoded proteins are exported (Fig. 6). By use of the *tat* signal, fully assembled proteins – usually equipped with iron-sulfur clusters – are carried across the membrane (Coulthurst & Palmer, 2008; Yuan *et al.*, 2010; Robinson *et al.*, 2011). Kuste3097 appears to be a fusion protein between cyt b_6 and a diheme cyt *c* at the C-terminal part. The N-terminal amino acid sequence is fully conserved with respect to known cyt b_6 proteins. Apart from their Rieske factors, the arrangements of kustd1480-1485 and kuste4570-4574 are more complex (Fig. 6b). Rather than mono- or diheme cyt *c*, the kustd1480-1485 complex harbors an octaheme *c*-type protein (kustd1485). The presence of an N-terminal cleavage site suggests that it is exported after translation. In the kuste4570-4574 complex, even two multiheme proteins are present: a hexaheme *c*-type protein (kuste4573) and the HAO-like octaheme protein kuste4574 referred to previously. Their N-terminal leaders are indicative of protein export. Remarkably, in both complexes kustd1480-85 and kuste4569-74, cyt b_6 is split into two genes with their division at similar positions. Kustd1481 and kuste4571 contain four TMHs each and both show a high degree of sequence identity (69%) to each other and to TMHs A-D in common cyt b_6. The conserved regions include histidines involved in the coordination of the two *b*-type hemes and the first halves of the amino acid stretches related to Q_o and Q_i binding. Kustd1484 and kustd4572 are 47% identical, and both have five TMHs that are homologous to the C-terminal part of cyt b_6. Sequence identities include the second halves of Q_o-binding and – to a lesser extent – the Q_i-binding motifs. It remains to be established whether the complexes will bind one or two quinones. The presence of genes coding for FAD-containing NAD(P) oxidoreductase in both complexes is surprising (Fig. 6a and b). Both gene products lack N-terminal cleavage sites indicating their residence in the cytoplasm. The question then is what these particular bc_1 complexes are doing. Thinking of the bifurcation principle, which also applies to flavines and their semiquinones (Buckel & Thauer, 2012), it is possible to speculate that they couple the oxidation of (mena)quinol to the reduction of an electron acceptor of higher redox potential and one of low redox potential: NAD(P) ($E_0' = -0.32$ V). This would solve the serious problem of NAD(P)H synthesis in an elegant way. In the most abundant complex, which harbors HAO-like kuste4574, the high-redox-potential electron acceptor might be nitrite with NO as the reduced product ($E_0' = +0.38$ V). Again, this is what the reading of the genome and comparative literature analyses suggest. Ultimate proof will come from the isolation and characterization of these complexes.

ATP synthesis

Like in all respiratory systems, the redox reactions and proton movements related to the central catabolic reactions discussed above should result in a net proton translocation across a semi-permeable membrane system, thus creating *pmf*. The proton-motive force can then be utilized to drive ATP synthesis by the membrane-bound ATP synthase complex. As already mentioned, four gene clusters are found in the *K. stuttgartiensis* genome that code for such complexes: One typical proton-translocating F_1F_o ATPase (ATPase-1; kuste3787-3796), two closely related F-ATPases that typically lack the delta subunit (ATPase-2, kuste4592-4600; ATPase-3, kustc0572-0579), and a prokaryotic V-type ATPase (V-ATPase-4, kuste3864-3871) (Strous *et al.*, 2006; Van Niftrik *et al.*, 2010) (Fig. 7). ATPase-1 is the most abundant species. Immunogold labeling with antibodies raised against its catalytic subunits showed that ATPase-1 is mostly localized at the anammoxosome and to a lesser degree near the outermost (paryphoplasm) membrane (Van Niftrik *et al.*, 2010). ATPase-2 and ATPase-3 belong to a divergent sodium-dependent type, which has been suggested to extrude sodium ions (Dibrova *et al.*, 2010). The latter could provide the means to build a sodium-motive force, to drive sodium-dependent processes such as import of substrates. This still remains to be verified. Moreover, the localization of these ATPases and the answer to the question whether or under which conditions both atypical ATPases are expressed remain elusive.

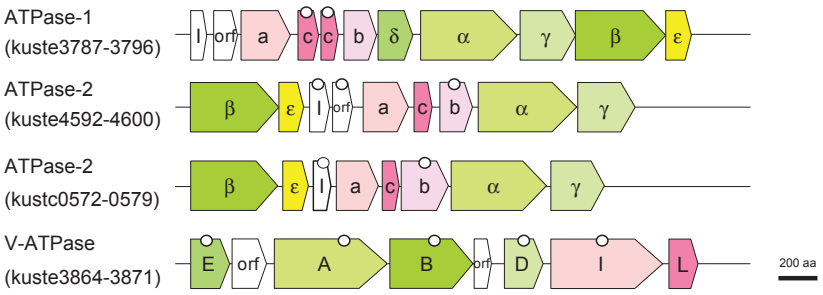

Fig. 7. Four ATPases in the *Kuenenia stuttgartiensis* genome. Gene products are labeled according to the homologous subunits in related ATPases. Genes that have been annotated in the *K. stuttgartiensis* genome as unknown proteins or that were erroneously annotated are indicated with white circles on top (Van Niftrik *et al.*, 2010). Lengths of the polypeptides are drawn to scale (aa: amino acids).

The anammoxosome and energy metabolism

The anammoxosome is hypothesized to constitute the power station of the anammox cell. As already pointed out, there is compelling evidence for such a role: Major part of cytochrome *c* proteins is present in close proximity to the inner rim of the anammoxosome membrane, the HAO-like kustc1061 and HZS are specifically present inside the organelle, and most of ATPase-1 can be detected at its membrane (see above and Neumann *et al.*, 2011). Still, conclusive evidence will only come from the isolation of the anammoxosomes and the demonstration that the conversion of ammonium and nitrite in these organelles results in the generation of *pmf*. This will be a formidable task, requiring dedicated methods to peel off the cell wall and outer membrane layers one by one.

If the anammoxosome is the power plant, the straightforward question is: For what purpose do these microorganisms place their bioenergetic machinery inside this organelle? With the current state of our knowledge, an answer can only be speculative. Denitrifying bacteria reduce nitrate to N_2 via nitrite, NO, and N_2O. Except for nitrate reduction by the nitrate reductase (Nar) system, all reactions reside at the periplasm, which represents the *p*-side of the chemiosmotic system (Richardson, 2000; Simon, 2002; Simon *et al.*, 2008; Kraft *et al.*, 2011). Nitrite, NO, and N_2O reduction by themselves do not contribute to *pmf* generation, except when electron transfer in these reactions proceeds via the bc_1 complex. Nevertheless, the high catalytic activities of these reductases enable the organisms to metabolize at high rate, which results in rapid growth. In contrast, anammox bacteria have to deal with the very sluggish HZS, and they have to express this enzyme at high levels to achieve appreciable metabolic activity. The periplasmic space presumably would not be sufficient to harbor the required amount of enzyme. Next, when localized at the periplasm, the enzymes are exposed to a large surface where intermediates can diffuse out. Indeed, it is well known that denitrifying bacteria release significant amounts of NO and N_2O, especially during metabolic shifts as a result of environmental changes (pH, aerobic–anaerobic transitions) (Betlach & Tiedje, 1981; Baumann *et al.*, 1996, 1997; Otte *et al.*, 1996; Saleh-Lakha *et al.*, 2009). These microorganisms may cope with these losses because they metabolize very rapidly and NO and N_2O conversions contribute relatively little to energy conservation. However, for anammox bacteria, such a loss of intermediates would be detrimental. The 'simple' solution is the containment of the catabolism within a special organelle. Curvature of the membrane system provides extra space for respiratory enzymes, whereas NO and hydrazine that escape from the anammoxosome can partly diffuse back into the organelle. Membranes are the barriers for the passage of charged compounds even as small as protons, but with a flaw. Protons passively diffuse through the membrane at a certain rate, independent of the metabolic activity of the cell, thus dissipating the *pmf*. In mitochondria that operate at a high rate, leakage accounts for an estimated 10% energy loss (Haines, 2001). Again, this would also be detrimental for the slowly metabolizing anammox bacteria. Obviously, densely packed ladderanes might raise a better barrier to proton, NO, or hydrazine leakage than common lipids. Nevertheless, the finding that those intermediates can be detected outside the cell indicates that ladderanes are not perfect. Moreover, cell aggregation could also be beneficial, allowing anammox bacteria to share residual losses with their companions in the biofilm.

Substrate uptake and substrate trafficking

Anammox bacteria have to acquire their substrates, ammonium and nitrite in particular, from environments where the concentrations are generally low. CO_2/bicarbonate as the sole carbon source is sufficiently present in most anaerobic systems, but it still has to be taken up.

Nitrate is subjected to export or import depending on its role in the metabolism. The anammox cell is layered by three membrane systems. Consequently, substrates will pass two or three membrane layers to be used in anabolic (cytoplasm) or catabolic (anammoxosome) reactions, respectively. Considering that transcription and translation occur in the cytoplasm ('riboplasm'), inherent problems are the proper sorting toward the anammoxosome or the other membrane systems, and the correct topological orientation of membrane-bound transport systems. Anammox bacteria possess the common bacterial set of protein export systems: the *sec*-translocon for the translocation of proteins that typically have a cleavable N-terminal TMH and the *tat*-translocon (Medema *et al.*, 2010). Cleavage of the N-terminal leader is performed by type I signal peptidase that serves both the *sec* and *tat* translocons (Auclair *et al.*, 2012). The encoding gene and all other components of the *sec* and *tat* systems are found as single copies in the *K. stuttgartiensis* genome. Next, genes encoding type II and type IV signal peptidases are detectable in the genome, also as single copies. Their presence suggests additional transport systems for subsets of proteins including those that are located extracellularly (Paetzel *et al.*, 2002). The peptidases recognize specific amino acids in the N- or sometimes C-terminal region for cleavage. Despite detailed analyses, Medema *et al.* (2010) were unable to detect any features that could be related to targeting toward the anammoxosome or cytoplasmic membranes.

In the outer membrane of Gram-negative bacteria, numerous proteins function as porins and transporters (see for a recent review: Fairman *et al.*, 2011). These outer membrane proteins (OMPs) are structured by 8-24 β-barrel strands forming a channel through which components can pass the membrane. Passage can be aspecific for a range of compounds, but many OMPs act as very specific molecular sieves. The specific ones are equipped with an ingenious ratchet mechanism, preventing substrate backflow out of the cell. In this way, compounds can be accumulated. Substrate-specific sieves can be expressed in high copy numbers. The analysis of the *K. stuttgartiensis* genome by the HHomp toolkit (http://toolkit.tuebingen.mpg.de/hhomp) reveals the presence of at least 25 different OMP-like proteins in the organism, belonging to different families, but most of these are still annotated as 'unknown' or 'hypothetical' (Speth *et al.*, 2012b). Such an annotation also concerns kuste1878, which is in fact one of the most abundant proteins encountered during protein fractionation (N. M. de Almeida and W. J. Maalcke, unpublished result). Hence, the outer membrane of *K. stuttgartiensis* seems to be gated. This would leave anammox substrates to cross one or two more membrane barriers.

From bioenergetic and topological points of view, substrate trafficking in anammox bacteria represents an interesting case. Experimentally, these matters are still *terra incognita*, and the following discussion is solely based on genome analysis. Assuming that the outermost membrane is not fully closed, the compartment surrounding the cytoplasm/riboplasm represents a periplasmic space (*p*-side), while the cytoplasm itself is the negative (*n*-) side, which is alkaline in common bacteria. The anammoxosome constitutes a second *p*-side, presumably of acidic pH (Van der Star *et al.*, 2010). Consequently, negatively charged molecules to be directed to the anammoxosome first have to be taken up against the *pmf* and subsequently benefit from it during export from the cytoplasm. The opposite holds for positively charged compounds. The net result should be an increase in concentration to serve the need of metabolic enzymes. As outlined next, anammox bacteria employ general sets of channel proteins. Thus, a similar protein should support both import (into the cytoplasm) and export (into the anammoxosome) of its substrate. Anammox bacteria rely on members of the major facilitator superfamily (MFS) for the transport of their key substrates (Fig. 8a). Strictly speaking, these are not transporters that derive energy from ATP hydrolysis or the *pmf* to drive processes, but they facilitate the channeling through a membrane (see for a review: Law *et al.*, 2008). Importantly, MFS proteins work bidirectionally: They mediate both substrate import and substrate export. The resolution of the crystal structures of a number of key members of MFS proteins allowed a detailed insight into the molecular mechanism of substrate translocation. As a common principle, the membrane-spanning helices surround a pore with a narrow slit permitting passage of only the dedicated substrates. These substrates are scavenged in a vestibule at the entrance side. The channels occur in open or closed conformations to control transport.

Inspection of the *K. stuttgartiensis* genome reveals that the organism uses members of the AmtB/Rh family for ammonium uptake. In fact, five distinct genes coding for such proteins were annotated, and these are all expressed (Fig. 8a) (Strous *et al.*, 2006; Kartal *et al.*, 2011b). Kustc1009, kustc1012, and kustc1015 are located in the same gene cluster, the latter two of which are preceded by P_{II} proteins (kustc1010 and kustc1014). The cytoplasmic P_{II} protein is the master controller of ammonium metabolism, a covalent linkage with UMP directing its activity (Arcondéguy *et al.*, 2001; Leigh & Dodsworth, 2007; Forchhammer, 2008). Among others, the proteins in the non-UMP-bound state are able to dock to the AmtB exit, in this way plugging the transport channel (Andrade *et al.*, 2005). The cytoplasmic C-terminal amino acids of AmtB are essential in this interaction. The homology to

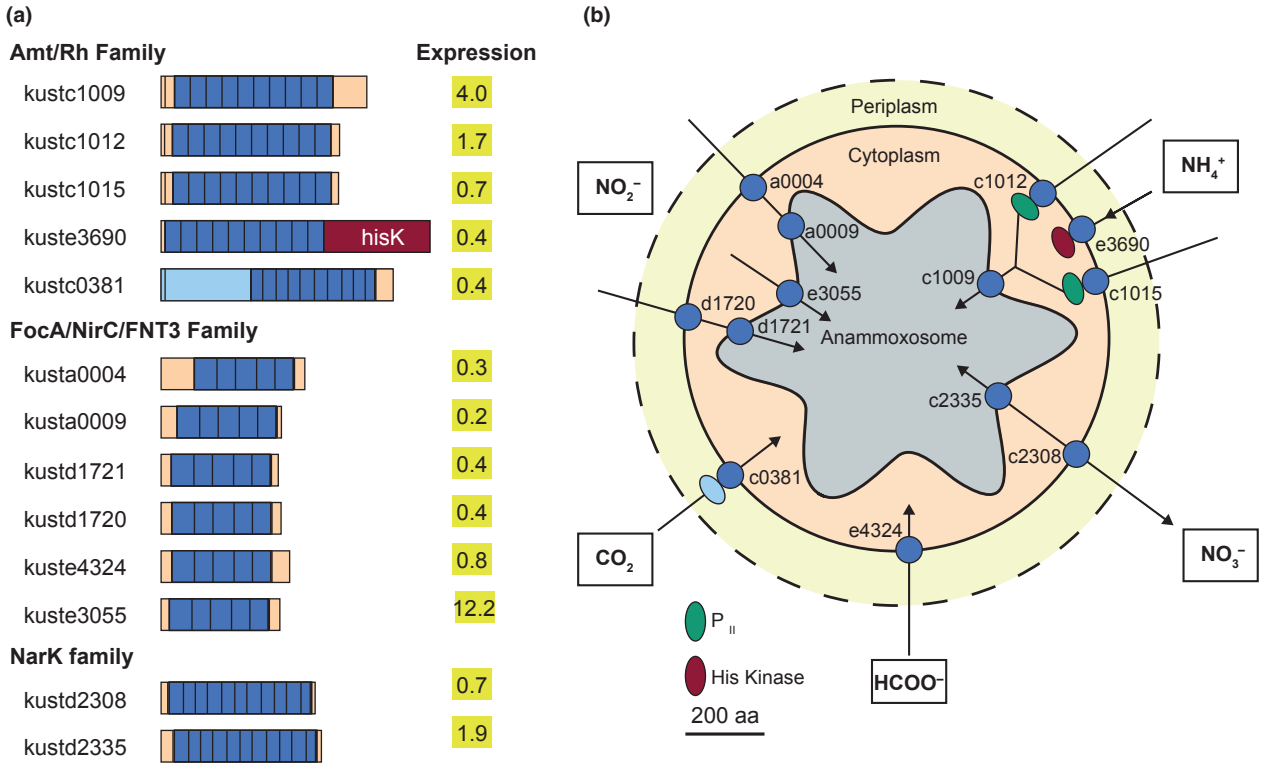

Fig. 8. Genes coding for different members of the major facilitator superfamily (MFS) in the *Kuenenia stuttgartiensis* genome (a) and the putative localization of their gene products in the anammox cell (b). In (a) the parts of the gene products showing high sequence identity with known MFS members, including the positions of transmembrane-spanning helices (vertical black lines), are highlighted in blue. N- or C-terminal segments localized in the cytoplasm or at its opposite side are colored orange and light blue, respectively. Lengths of the polypeptides are drawn to scale (aa: amino acids). Expression values are expressed as n-fold coverage of Solexa deep RNA sequencing of the *K. stuttgartiensis* transcriptome (Strous *et al.*, 2006; Kartal *et al.*, 2011b). Codes in (b) refer to the kust codes specified in (a). P_{II}: P_{II} protein involved in the regulation of ammonium metabolism, hisK: His Kinase, histidine kinase domain.

known AmtBs and the presence of P_{II} proteins in the kustc1009-1015 gene cluster suggest that they are involved in ammonium transport, kustc1012, and kustc1015 in direct connection with their cognate P_{II} proteins. Atomic structures of AmtB proteins from three different microbial species have been resolved (Khademi *et al.*, 2004; Zheng *et al.*, 2004; Andrade *et al.*, 2005). All three share identical homotrimeric architecture of protomers, each having 11 TMHs. The comparison of the kustc1009, kustc1012, and kustc1015 amino acid sequences with those of structurally well-studied AmtBs demonstrates the conservation of essential structural and functional features. However, anammox proteins contain an additional N-terminal TMH, which might be cleaved during maturation. A second difference is that kustc1009 contains a much longer C-terminal amino acid stretch than the others (Fig. 8a), which possibly prevents P_{II} binding. (one may note that kustc1009 lacks a cognate P_{II}). By analogy with known systems, one might infer that kustc1012 and kustc1015 mediate ammonium import from the periplasm into the cytoplasm (Fig. 8b). This would leave kustc1009 as the ammonium channel into the anammoxosome. The *K. stuttgartiensis* genome encodes a fourth AmtB homologue, kuste3690. Its characteristic feature is a histidine kinase domain near the C-terminus that is predicted to be localized in the cytoplasm. The presence of the kinase domain suggests a signaling function for kuste3690. The fifth candidate, kustc0381, belongs to the Rh subfamily and shares all essential sequence motifs with the *Nitrosomonas europaea* Rh protein, which was identified as a CO_2 transporter (Li *et al.*, 2007). Like other MFS proteins, translocation seems to be regulated by open–closed state conformers. The molecular trigger behind its opening or closing is not understood, but Li *et al.* (2007) suggested that a currently unknown (metabolic) protein could play a role in its control. Intriguingly, kustc0381 has an N-terminal extension of ~230 amino acids. This extension follows a noncleavable TMH right after the translation start, and the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM-2.0/) predicts its localization

outside the cytoplasm. The polypeptide could have a role in substrate (CO_2) accumulation, channel closing, or both. However, the lack of homology to a known protein (s) leaves its function elusive. Considering that CO_2 fixation takes place in the cytoplasm (see next section), kustc0381 should be bound to the cytoplasmic membrane (Fig. 8b).

Transport of nitrite is facilitated by the FocA/NirC proteins, which are also members of the MFS. FocA proteins mediate formate import and export, while NirC proteins catalyze reversible nitrite transfer across the membrane at high rates (Moir & Wood, 2001; Falke *et al.*, 2010). Unlike NirC, FocA proteins are well defined by crystal structures from three bacterial species (Wang *et al.*, 2009; Waight *et al.*, 2010; Lü *et al.*, 2011). At the moment, a third branch (FNT3) has been added to the family represented by the hydrosulfide ion channel (HSC), the crystal structure of which was recently resolved from *Clostridium difficile* (Czyzewski & Wang, 2012). In this organism, HSC efficiently expels HS^- from the cell. Like other anion channels, the protein is not very specific and it is capable of formate and nitrite translocation as well. FocA and HSC share many structural properties. Both form symmetric pentamers with each protomer comprising six TMHs. In addition, both proteins have many conserved amino acids in common that are related to substrate accumulation and transport. As many of these features are shared with NirCs, on the basis of amino acid sequences alone, it is rather difficult to predict what the physiological function of a particular protein is. However, their localization in the genome may give a hint: The channel proteins tend to be present in close proximity to genes coding for enzymes that deal with the metabolism of the molecules they translocate.

The genome of *K. stuttgartiensis* has five genes that code for FocA/NirC-like proteins, four of these occurring in tandem (kusta0004 and kusta0009; kustd1721 and kustd1720). Kusta0004 shares the highest sequence identity (67%) with kustd1721, while kusta0009 is most related to kustd1720 (66% sequence identity). A particular property of kusta0009, and to a lesser extent of kustd1720, is the presence of a relatively long N-terminal sequence predicted to be in the cytoplasm. In known FocA proteins, this part of the protein was demonstrated to undergo a pH-dependent structural change, thereby opening or closing the transport channel (Lü *et al.*, 2011). Multiple sequence alignment places these four anammox proteins inbetween FocA and NirC proteins. The kustd1720-1721 tandem is localized immediately upstream of nitrate:nitrite oxidoreductase reductase (*Nxr*) locus (kustd1713-1699; see below), indicating that all four could have a role in nitrite transport with one partner localized on the anammoxosome membrane and the counterpart on the cytoplasmic membrane (Fig. 8b). The fifth gene coding for a FocA/NirC protein (kuste4324) is an orphan. Kuste4324 shows higher sequence identity to FocA than to NirC proteins. If localized on the cytoplasmic membrane, kuste4324 could be a good candidate to serve in formate uptake. Re-evaluation of the *K. stuttgartiensis* genome resulted in the identification of one more member of the FocA/NirC/FNT3 family, kuste3055, which is annotated as a conserved hypothetical protein. This protein is 38% identical to HSC, and all structural and functional amino acids are conserved. Remarkably, kuste3055 is by far the most highly expressed transporter (Fig. 8a). Considering (1) the aspecific substrate use of HSC, (2) its proficient properties in substrate export from the cytoplasm, and (3) the absence of a clear role for hydrogen sulfide in anammox metabolism, kuste3055 could be an additional shuttle to supply the anammoxosome with nitrite.

Nitrate plays a dual role in anammox metabolism. It is the product of nitrite oxidation, generating reducing equivalents for CO_2 fixation, and it is the terminal electron acceptor for organic electron donor oxidation. Both nitrite oxidation and nitrate reduction are catalyzed by Nxr, which is most likely localized in the anammoxosome (see below). Such localization would need at least two transporters to take nitrate across the anammoxosome and cytoplasmic membranes. Indeed, genes coding for two of these are found in the genome of *K. stuttgartiensis* (kuste2308 and kuste2335) (Fig. 8a). The gene products of these are 66% identical to each other and belong to the NarK MFS of nitrate channel proteins. The family splits into two branches: NarK1 members act as high-affinity nitrate/H^+ symporters, and NarK2 proteins are low-affinity nitrate/nitrite antiporters (Moir & Wood, 2001; Goddard *et al.*, 2008). Multiple sequence alignments assign both kuste2308 and kuste2335 to the NirK1 subfamily. Unfortunately, the amino acid sequences do not give an indication about their specific localization, and the assignment made in Fig. 8b is provisional.

This ends our discussion on the wide-open status of our knowledge on the transfer of key substrates; the summarizing scheme presented in Fig. 8b is tentative at best. The ultimate picture on transport systems will be much more complicated due to the need of common inorganic compounds (phosphate, calcium, magnesium, etc.), the role of trace metals (Fe, Ni, Cu, Mn, Mo) in cellular metabolism, and the fact that the *K. stuttgartiensis* genome possesses a variety of putative uptake systems for organic compounds, including ABC transporters. Besides these, the genome of *S. profunda* contains many genes involved in oligopeptide transport systems, which suggests these bacteria are capable of oxidizing decaying organic matter (Van de Vossenberg *et al.*, 2012). Again, the presence of transporters for organic compounds in the

genomes of anammox bacteria indicates that these microorganisms are not just chemolithoautotrophic specialists, but are metabolically much more versatile.

Anammox anabolism

CO_2 fixation

The genome of *K. stuttgartiensis* contains all relevant genes of the acetyl-CoA (Wood–Ljungdahl) pathway for CO_2 fixation, and these are all expressed (Strous *et al.*, 2006; Kartal *et al.*, 2011a,b). In agreement, the activity of its key enzyme, CO-dehydrogenase/acetyl-CoA synthase (ACS) was also demonstrated (Strous *et al.*, 2006). Intermediary metabolism starting from acetyl-CoA is accounted for by the repertoire of gluconeogenesis and citric acid (TCA) cycle enzymes. However, *K. stuttgartiensis* lacks the gene(s) coding for citrate synthase/citrate lyase, which would imply that the TCA cycle is operating in the reductive direction. On the other hand, the *S. profunda* genome contains two putative citrate synthase genes (scal03477 and scal01583) (Van de Vossenberg *et al.*, 2012). As the *K. stuttgartiensis* genome is still not fully closed, it is possible that the missing gene(s) could be in those gaps.

At first glance, the acetyl-CoA pathway is energetically efficient. For instance, compared with the Calvin–Benson–Bassham cycle (Eqn. 12), the Wood–Ljunghdahl pathway (Eqn. 11) is less ATP-demanding per hexose-6-phosphate (hexose-6-P) made from CO_2.

$$6CO_2 + 24[H] + 6ATP \rightarrow \text{hexose-P} + 6ADP + 5Pi \quad (11)$$

$$6CO_2 + 12NADPH + 18ATP \rightarrow \text{hexose-P} + 12NADP^+ + 18ADP + 17Pi \quad (12)$$

Acetyl-CoA synthesis includes a number of low-redox-potential reactions that require strong reductants ([H]): CO_2 reduction to formate ($E_0' = -0.42$ V), reduction of CO_2 to CO ($E_0' = -0.52$ V) in acetyl-CoA synthesis, and the reductive carboxylation of the latter to make pyruvate ($E_0' = -0.47$ V). Besides these, NADPH ($E_0' = -0.32$ V) is required as the electron donor in a variety of other anabolic reactions. Considering the extremely low redox potential of hydrazine oxidation to N_2 ($E_0' = -0.75$ V), these reductions should not be thermodynamic obstacles. However, there could be two concerns: (1) In organisms that use the acetyl-CoA pathway, electron transfer in the CO_2 reduction steps is mediated by low-redox-potential ferredoxins. Ferredoxins are usually encoded together with the catalytic enzymes, but in the anammox gene clusters that code for these enzyme systems, ferredoxin genes are consistently absent (at least in *K. stuttgartiensis*). When lacking ferredoxins, electrons for CO_2 fixation could be derived from the oxidation of (mena)quinol that is produced during hydrazine oxidation (Fig. 9). With menaquinol as electron donor ($E_0' = -0.075$V), all reduction reactions mentioned would become endergonic. (2) The electrons that are withdrawn from the Q-pool for CO_2 fixation have to be replenished. Under autotrophic conditions, this is only achieved by the oxidation of nitrite to nitrate (Eqn. 3), but nitrite is a poor reductant ($E_0' = +0.42$ V, see next section).

Above, we proposed a bifurcation mechanism for NAD(P)H generation in concert with menaquinol oxidation catalyzed by bc_1 complexes in anammox bacteria. Analysis of the genome shows that *K. stuttgartiensis* contains two other, more conventional means to make NAD(P)H (Strous *et al.*, 2006): A sodium-dependent NADH:quinone oxidoreductase (Na^+-NQR; NqrA-E, kuste3325-3329); and a H^+-translocating NADH:quinone oxidoreductase (NADH dehydrogenase, complex I; NuoA-N, kuste2660-2672). NqrA-E in *K. stuttgartiensis* are fully conserved with known Na^+-NQRs (Verkhovsky & Bogachev, 2010), except that in the anammox bacterium, NqrE is a fusion product of the NqrE and NqrF subunits found in other organisms. The exergonic NADH oxidation to quinone reduction ($\Delta G^{\circ\prime} = -47$ kJ per reaction with menaquinone as electron acceptor) is utilized by Na^+-NQR to translocate sodium ions, thus creating a sodium-motive force (Verkhovsky & Bogachev, 2010). Consequently, in anammox bacteria, a sodium-motive force might be employed to drive the opposite unfavorable reaction, namely NAD^+ reduction with quinol. To this end, the alternative Na^+-translocating ATPases described above might act in creating such a sodium-motive force. If so, the next question is where the particular ATPases and NQR complex are localized, on the cytoplasmic, anammoxosome membranes, or both.

Complex I is ubiquitously present in respiratory systems. The enzyme system has been the topic of extensive research for many years (see for recent reviews: Sazanov, 2007; Hirst, 2009; Efremov & Sazanov, 2011a,b, 2012). In prokaryotes, the core complex is composed of 14 subunits. The resolution of the atomic structure of the *Thermus thermophilus* complex I (Sazanov & Hinchliffe, 2006; Efremov *et al.*, 2010; Efremov & Sazanov, 2011a) led to the understanding of the way its machinery works. Complex I has an unusual L-shaped architecture comprising hydrophilic and membrane-bound branches (Fig. 10a). In the hydrophilic part (localized in the cytoplasm), a series of 2Fe-2S and 4Fe-4S clusters wire the electrons derived from NADH oxidation via FMN in the catalytic subunit (NuoF) toward quinone at the membrane interface. In

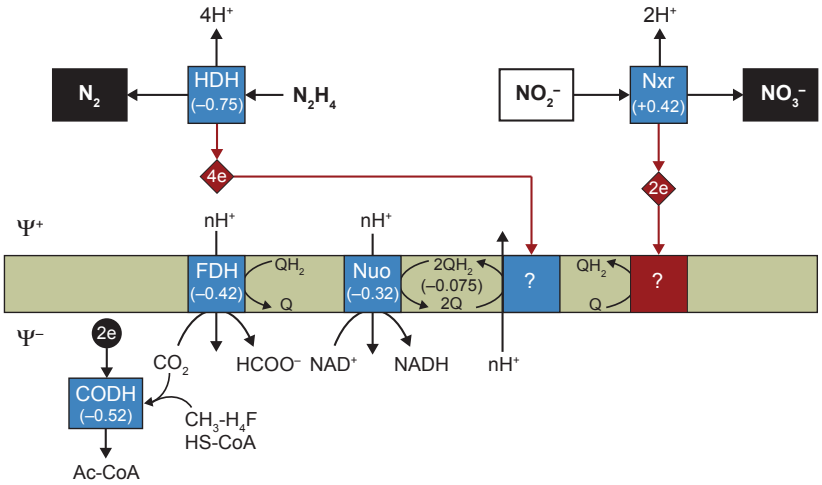

Fig. 9. Connection between the central catabolic and anabolic reactions in anammox metabolism. Standard redox potentials (V) of the different redox processes are given in parentheses. CODH: CO dehydrogenase/acetyl-CoA (Ac-CoA) synthetase, FDH: formate dehydrogenase, HDH: hydrazine dehydrogenase, Nuo: NADH:quinone (Q) oxidoreductase (complex I), Nxr: nitrite:nitrate oxidoreductase, CH_3-H_4F: methyl-tetrahydrofolate. The question marks depict the unknown systems/mechanisms that feed electrons from hydrazine and nitrite oxidation into the quinone pool.

this transport, the 2Fe-2S cluster in NuoE and one 4Fe-4S cluster in NuoG do not have a clear role. The energy released in the redox process sets membrane-bound H^+ transport channels in move, such that most likely four protons are driven across the membrane per NADH oxidized, in agreement with the common understanding of a $4H^+/2e$ reaction stoichiometry. The prokaryotic 14-subunit machinery seems to be fully conserved in NuoA-N from *K. stuttgartiensis* (Fig. 10a). Importantly, for over 50 years, it has been known that complex I acts reversibly: It is able to perform *pmf*-driven NAD^+ reduction with quinol as electron donor (Chance & Hollunger, 1961). The latter mechanism could account for the reverse electron-transport-driven NADH generation in anammox. In brief, these organisms have three putative systems to perform the energetically unfavorable reduction of NAD^+ from quinol: (1) bifurcation in the bc_1 complex, using (2) H^+- and (3) Na^+-driven NADH:quinone oxidoreductases.

Energetically, CO_2 reduction to formate with (mena) quinol is a greater challenge ($\Delta G^{\circ\prime}$ = +67 kJ per reaction). In the *K. stuttgartiensis* genome, a large gene cluster (kustc0821-0842) encodes a remarkable enzyme system (Strous *et al.*, 2006). It comprises the nearly full complement of the H^+-dependent complex I (Fig. 10b) with modifications that are reminiscent of the formate:hydrogen lyase (Hyf) system from *E. coli* (Andrews *et al.*, 1991, 1997). The comparison of the amino acid sequences of the particular *K. stuttgartiensis* subunits with those of *T. thermophilus* complex I shows that they are conserved, including sites related to binding NAD(H), FMN, the iron-sulfur clusters, and the putative quinone-binding site. However, in the *K. stuttgartiensis* complex NuoC/D is split into two separate proteins (NuoC and NuoD, kustc0824 and kustc0825, respectively). Such a split is not uncommon. Moreover, the essential NuoG subunit (with its two 2Fe-2S and two 4Fe-4S clusters) is absent at first sight. However, this subunit is found in the N-terminal part of the molybdo-*bis*-pyranopterin guanine dinucleotide (Mo-*bis*PGD) formate dehydrogenase catalytic subunit (FdhA, kustc0828). The latter shows an almost-complete conservation in its central and C-terminal parts of all relevant amino acids that in the crystal structure of formate dehydrogenase H (FDH-H) from *E. coli* (Boyington *et al.*, 1997) have been implicated with binding of the substrate and of the cofactors. This conservation includes four cysteines that coordinate a 4Fe-4S cluster. On the other hand, kustc0828 lacks a selenocysteine that is typical for FDH-H and several other formate dehydrogenases. One more difference is the presence of a set of cysteines in the C-terminal part of *K. stuttgartiensis* NuoF (kustc0827), which might give room to two extra 4Fe-4S clusters. A schematic representation of these findings is presented in Fig. 10b. Still, the question is what the function of this complex is. Considering the presence of catalytic and binding sites for formate, NADH, and quinone, it is conceivable that the enzyme couples the oxidation of NADH to reduction of CO_2 to formate and of menaquinone to menaquinol (Eqn. 13), again by electron bifurcation through their flavin (FMN) prosthetic

groups. This reaction is exergonic, perhaps permitting the translocation of one or two protons across the membrane for energy conservation.

$$2\text{NADH} + \text{CO}_2 + \text{MQ} + \text{H}^+ \rightarrow 2\text{NAD}^+ + \text{HCOO}^- + \text{MQH}_2\ (\Delta G^{\circ\prime} = -28\ \text{kJ/reaction}) \quad (13)$$

$$\text{CO}_2 + \text{methyl-H}_4\text{folate} + \text{HS-CoA} + 2\text{e} \rightarrow \text{acetyl-CoA} + \text{H}_4\text{folate} \quad (14)$$

On top of the thermodynamic mountain, we find acetyl-CoA synthesis involving CO_2 reduction and the subsequent condensation of intermediary CO with the methyl group of methyl-H_4folate and coenzyme A (Eqn. 14). This reaction is catalyzed by a soluble enzymatic machinery composed of a two-subunit B_{12}-containing methyltransferase that takes the methyl group from H_4folate and hands it over to the CO-dehydrogenase/acetyl-CoA synthetase (ACS) (Fig. 10c) (see for a review: Ragsdale & Pierce, 2008). Among others, this reaction includes CO_2 reduction to its carbonyl stage, which is characterized by a very low redox potential. Until recently (Huang *et al.*, 2012), it was not understood where the low-redox-potential electrons come from to drive the reduction. Strous *et al.* (2006) identified a gene cluster in the *K. stuttgartiensis* genome (kustd1547-1538) that encodes all components related to acetyl-CoA synthesis, including the conserved set of accessory proteins for the assembly and maturation of the unique NiFeS catalytic clusters of ACS (Fig. 10c). Still, one conspicuous candidate, ferredoxin, is absent. Intriguingly, immediately downstream of this cluster, there are three genes (kustd1552-1550) coding for (1) the catalytic subunit of formate dehydrogenase, including the additional 2Fe-2S and 4Fe-4S present in NuoG (kustd1550), (2) a NuoF- (kustd1551), and (3) a NuoE orthologue (kustd1552): the FMN-binding catalytic subunit of complex I. All three are predicted to be soluble enzymes. Based on analogy, it is possible to speculate that this enzyme system could couple the oxidation of two molecules of formate ($E_0' = -0.42$ V) to NAD^+ reduction ($E_0' = -0.32$ V), thereby generating two low-redox-potential electrons for CO_2 reduction ($E_0' = -0.52$ V). One may note that the net Gibbs free energy change of the coupled reactions is $\Delta G^{\circ\prime} = 0$ kJ per

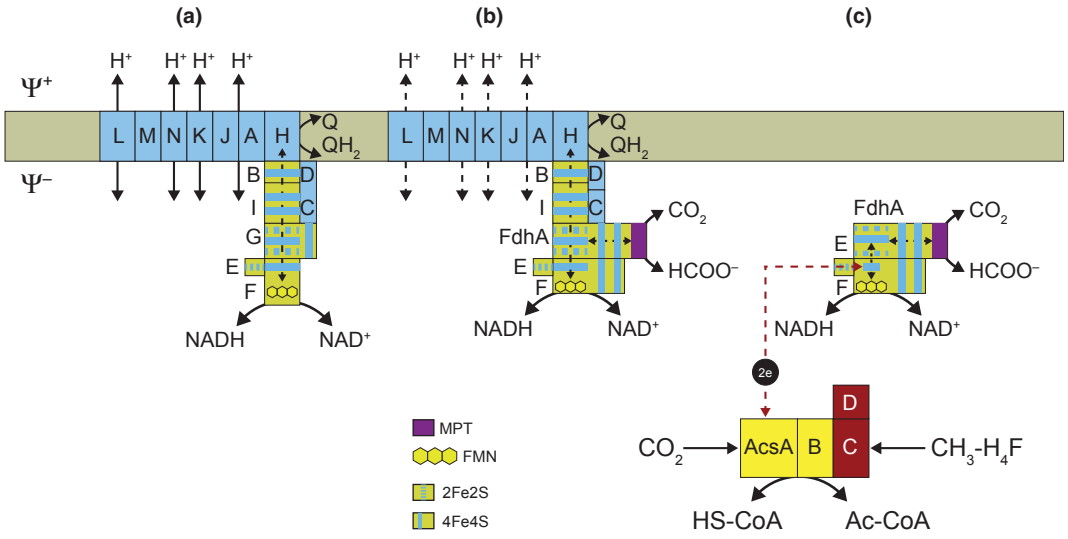

Fig. 10. Proposed functional organization of (a) the NADH:quinone (Q) oxidoreductase (Nuo, complex I), (b) formate dehydrogenase, and (c) acetyl-CoA synthetase systems in *Kuenenia stuttgartiensis*. Letters refer to the subunits in the *Escherichia coli* complex I (Nuo). In (a), the *K. stuttgartiensis* subunits are the following: A: kuste2660, B: kuste2661, CD: kuste2662, E: kuste2663, F: kuste2664, G: kuste2665, H: kuste2666, I: kuste2667, J: kuste2668, K: kuste2669, L: kuste2670, M: kuste2671, N: kuste2672. (b) The *K. stuttgartiensis* formate dehydrogenase system is suggested to be composed of the following components (again, letters refer to the *E. coli* Nuo complex subunits): A: kustc0822, B: kustc0823, C: kustc0824, D: kustc0825, E: kustc0826, F: kustc0827, H: kustc0834, I: kustc0835, J: kustc0836, K: kustc0837, L: kustc0838, M: kustc0840, N: kustc0842, and formate dehydrogenase subunit A (FdhA): kustc0828. (c) The *K. stuttgartiensis* acetyl-CoA (Acs) system comprises AcsA (kustd1546), AcsB (kustd1545), and the corronoid-containing part (red) involved in methyl group transfer: AcsC (kustd1542) and AcsD (kustd1539). Electrons for CO_2 reduction are suggested to be generated by a formate:NADH oxidoreductase complex (see text) localized in the genome in close vicinity to the Acs system and composed of NuoE (kustd1552), NuoF (kustd1551), and FdhA (kustd1550) subunits. 4Fe4S (full bar): 4Fe-4S cubane cluster, 2Fe2S (dashed bar): 2Fe-2S cluster, FMN: FMN prosthetic group, MPT: Mo-*bis*PGD molydopterin prosthetic group, Ac-CoA: acetyl-CoA, CH_3-H_4F: methyl-tetrahydrofolate.

reaction. Besides this, oxidant-driven reductions might play a role in two more reductive carboxylations: the synthesis of pyruvate ($E_0' = -0.47$ V) and 2-ketoglutarate ($E_0' = -0.49$ V). Genome analysis suggests that pyruvate synthesis in *K. stuttgartiensis* is catalyzed by the gene products of kuste2338-2339 and two different enzyme complexes (kuste2317-2318 and kustc0356-0358) might function in 2-ketoglutarate formation. Again, gene clusters coding for these complexes lack the genes encoding the ferredoxin (delta) subunits that are usually associated with these enzymes.

Anammox bacteria are not unique in the above respects. BLAST searches with the *K. stuttgartiensis* formate dehydrogenases and associated Nuo subunits as queries reveal a multitude of anaerobic microorganisms with similar properties, including homoacetogens, which perhaps points to a general electron bifurcation principle. Moreover, in *Moorella thermoacetica* and in *Carboxythermus hydrogenoformans*, the gene cluster coding for the acetyl-CoA syntheses system is immediately followed by a set of genes encoding a heterodisulfide reductase system (Pierce *et al.*, 2008). In hydrogenotrofic methanogenic archaea, the heterodisulfide reductase system couples the terminal methane-forming reaction to the endergonic first step, CO_2 reduction by electron bifurcation (Thauer *et al.*, 2008; Kaster *et al.*, 2011; Buckel & Thauer, 2012).

Nitrite oxidation

Anammox energy metabolism relies on a cyclic electron flow of hydrazine oxidation coupled to nitrite reduction and hydrazine synthesis (Fig. 3). Reduction reactions associated with CO_2 fixation (Fig. 9) and loss of intermediates (NO, hydrazine) represent a drain from the pool of electrons. Consequently, the electron pool has to be replenished. The observation that growth of anammox is associated with nitrate formation (Eqns 1–3) strongly suggests that the necessary electrons are derived from the oxidation of nitrite, which requires a dedicated nitrite: nitrate oxidoreductase (Nxr) system. Canonical nitrate reductase is a heterotrimeric protein (NarGHI), of which the atomic structure is resolved (Bertero *et al.*, 2003; Jormakka *et al.*, 2004). The enzyme catalyzes the menaquinol-dependent reduction of nitrate to nitrite (Eqn. 15).

$$MQH_2 + NO_3^- \rightarrow MQ + NO_2^- + H_2O(\Delta G^{\circ\prime} = -95\ \text{kJ/reaction}) \quad (15)$$

Nitrate reduction is a key step in the energy metabolism of nitrate-respiring species (see for reviews: Richardson, 2000; Simon, 2002; Rothery *et al.*, 2008; Simon *et al.*, 2008; Kraft *et al.*, 2011). In these organisms, the two-electron reduction of nitrate with the concommitant uptake of 2 H^+ takes place in the cytoplasm. NarG is the catalytic part that has a Mo-*bis*PGD cofactor similar to formate dehydrogenases. MQH_2 oxidation together with the release of 2 H^+ resides at the periplasm and is catalyzed by the membrane-bound NarI subunit. The net result is the apparent translocation of $2H^+/2e$ out of the cell, thus contributing to the build-up of *pmf*. Electron transfer to Mo-*bis*PGD in NarG proceeds via a 4Fe-4S cluster in this subunit, four 4Fe-4S clusters in NarH, and two heme *b*'s in NarI.

The genome of *K. stuttgartiensis* codes for a highly complex gene cluster (kustd1713-1699) that includes nitrate reductase components and a variety of other proteins (Fig. 11a) (Strous *et al.*, 2006; de Almeida *et al.*, 2011). The *K. stuttgartiensis* Nxr system clearly contains NarG (kustd1700) and NarH (kustd1703) homologues, but NarI is absent (Fig. 11a). Instead, this subunit is substituted by kustd1704 that has 24% sequence identity to the gamma subunit (EbdC) of ethylbenzene dehydrogenase (EbdABC), which is another member of Mo-*bis*PGD family (Johnson *et al.*, 2001; Kloer *et al.*, 2006). In the atomic structure of this nonmembrane-bound enzyme, EbdC binds two heme *b* molecules (Kloer *et al.*, 2006). The amino acids involved in the binding of the hemes are conserved in kustd1704. The kustd1713-1699 gene cluster also harbors kustd1709, which is a paralogue of kustd1704. Kustd1709 is remarkable because it contains an additional C-terminal TMH and it is fused with a diheme *c* that has a high sequence identity (41%) with kustc0457, the redox partner of HAO-like kustc0458. The analogy is even more intriguing as kustd1699 and kustd1713 code for proteins that closely resemble the putative blue copper cupredoxin kustc0456 that is associated with kustc0457-0458. Kustd1699 and kust1713 might serve as mobile electron carriers, a function that may also be hypothesized for kustd1705, kustd1708, and kustd1711, which are mono-, tetra-, and diheme *c* proteins, respectively (Fig. 11b). Besides the components mentioned, kustd1713-1699 comprise (1) two potential chaperones (kustd1702 and kustd1706), (2) kustd1701, which is characterized by four cysteines in the C-terminal part with spacings that are typical for adrenodoxin/putidaredoxin-like 2Fe-2S proteins, and (3) several membrane-anchored proteins (kustd1707, kustd1709, kustd1710, kustd1712) (Fig. 11a,b). At its N-terminal part, kustd1712, which is predicted to contain 16 TMHs, displays a remarkable sequence identity to cydA of cyt *bd* terminal oxidase (CydAB). CydA (nine TMHs) was suggested to bind one heme *b* and one quinol, both at the periplasmic side (Borisov *et al.*, 2011). In kust1712, the amino acids that are suggested to bind the heme b and quinol, as well as their localization near the periplasmic face are conserved with respect to CydA.

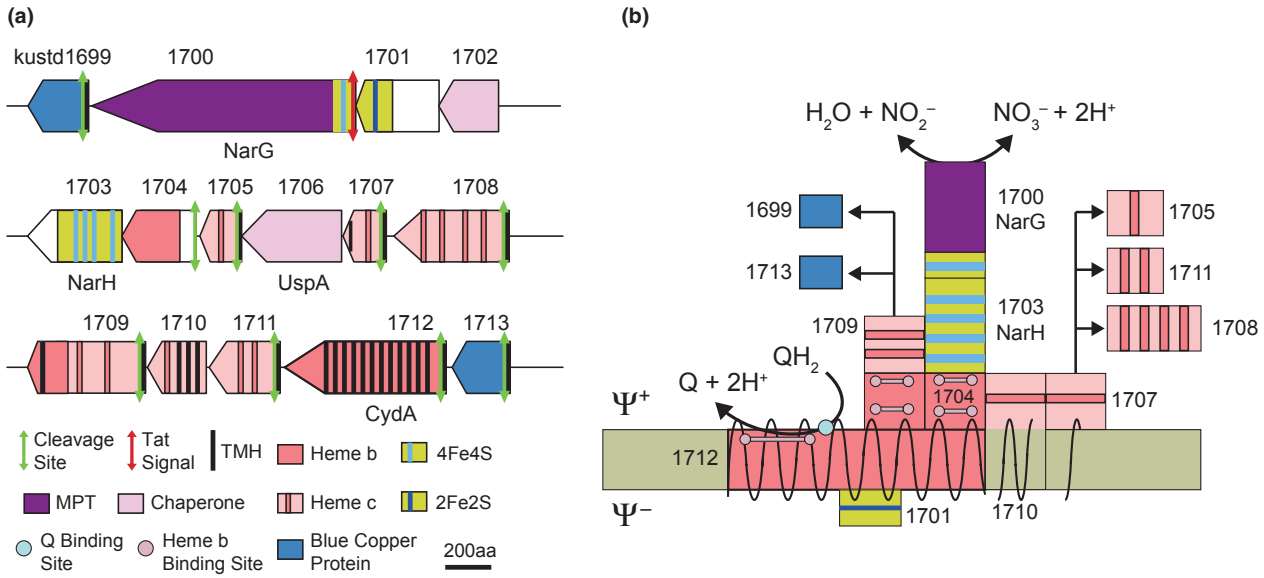

Fig. 11. Gene organization of the nitrite:nitrate oxidoreductase cluster in the *Kuenenia stuttgartiensis* genome (a) and the proposed functional organization of its gene products (b). (a) Lengths of the gene products and the position of structural motifs are drawn to scale (aa: amino acids). CydA (kustd1712), subunit showing significant sequence identity to subunit 1 of cytochrome bd quinol oxidase (CydAB). (b) Putative heme *b*- and quinone (Q)-binding sites in kustd1712 were inferred from sequence comparison with respect to the CydA (Borisov *et al.*, 2011). Numbers refer to the kust gene numbers as in (a). Structural motifs are specified in the Figure. Cleavage site: N-terminal cleavage site, *tat* signal: twin-arginine translocation signal, TMH: transmembrane-spanning helix, 4Fe4S: 4Fe-4S cubane cluster, 2Fe2S: putative novel adrenodoxin/putidaredoxn-like 2Fe-2S protein, MPT: Mo-*bis*PGD molydopterin prosthetic group.

Besides the complexity of the Nxr system, another unusual aspect might be its localization. All proteins, except the two chaperones and the adrenodoxin-like kust1701, contain an N-terminal cleavage site, which implies their transport across a membrane after translation. Furthermore, heme *c*-containing hydrophilic regions of the membrane-bound components are most likely localized outside the cytoplasm. Translocation applies to NarG (kust1700), which has a clear *tat* signal, possibly taking NarH (kustd103) as its passenger during transport. Such localization would be contrary to the one of canonical NarGH, where NarG is protected from N-terminal cleavage (Ize *et al.*, 2009). The straightforward guess for the localization of the Nxr system of anammox bacteria would be the anammoxosome; nevertheless, this remains to be established experimentally.

From the above overview, it is possible to infer that NarG kustd1700, NarH kustd1703, and heme *b*-containing kustd1704 – and/or kustd1709 – represent the catalytic part of the nitrate:nitrite oxidoreductase complex. Although nitrate reduction is of relevance during growth with alternative organic and inorganic electron donors (see next) and anammox bacteria are capable of nitrate reduction *in vivo* and *in vitro* (Van de Graaf *et al.*, 1997; Schalk *et al.*, 2000; Güven *et al.*, 2005; Kartal *et al.*, 2007b), the primary function of the nitrate reductase system would be nitrite oxidation, particularly under autotrophic conditions. Indeed, kustd1700, kustd1703, and kustd1704 show highest sequence identity to the Nxr components of nitrite-oxidizing species, most notably to '*Candidatus* Nitrospira defluvii' (Lücker *et al.*, 2010). The genome of *N. defluvii* encodes several other proteins that closely resemble other kustd1713-1699 components, indicating that *N. defluvii* has an Nxr system of comparable complexity. Interestingly, *N. defluvii* contains four kustd1712 homologues.

The presence of the CydA homologue kustd1712 might suggest that nitrite oxidation is coupled to (mena) quinone reduction (reaction 15 reversed), thus feeding the Q-pool (Fig. 9). However, this reversed reaction meets a gigantic bioenergetic barrier ($\Delta G^{\circ\prime}$ = +95 kJ per reaction). The only means to cross such a barrier would be membrane-bound reversed electron transport driven by the *pmf* (Strous *et al.*, 2006; de Almeida *et al.*, 2011). Considering that both proton release during nitrite oxidation and proton uptake for quinone reduction take place in the same compartment (Fig. 11) – rendering it non-electrogenic – the driving force should be provided by *pmf* only. To cross a $\Delta G^{\circ\prime}$ = +95 kJ barrier, an estimated $3H^+/e$ are required. At present, no biological system is known with such capacity (although anammox bacteria

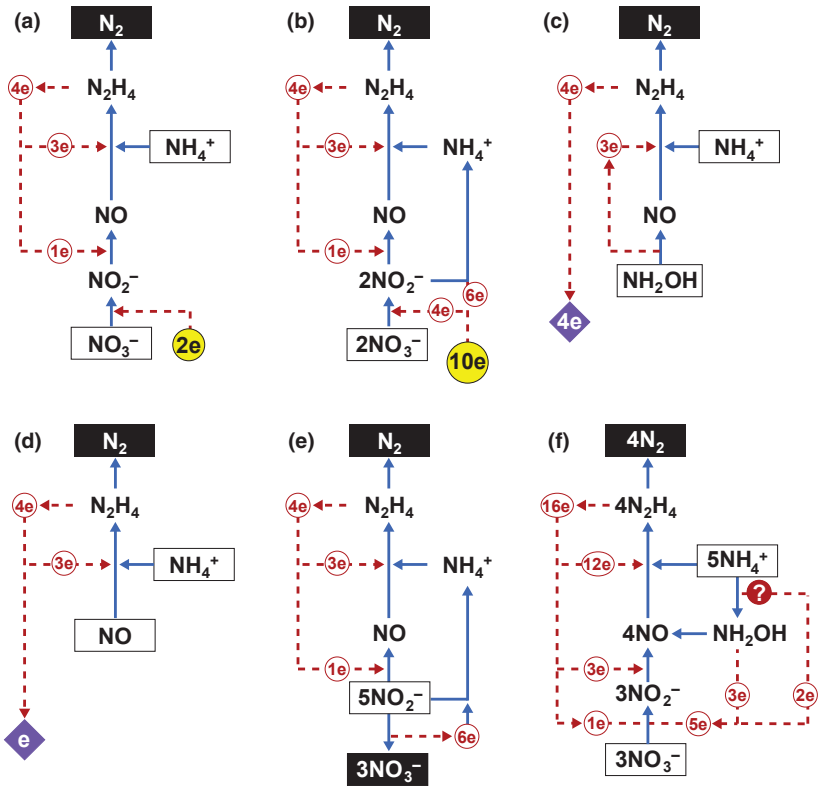

Fig. 12. Schematic representation of the metabolic versatility of anammox bacteria. (a) Ammonium and nitrate metabolism supported by an external organic or inorganic electron donor. (b) 'Disguised denitrification' by the reduction of nitrate to N_2 supported by an external (in)organic electron donor. 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 blue and dashed red lines, respectively. Substrates are boxed in white, products in black; electrons derived from external sources are marked by yellow circles and surplus electrons by purple diamonds. The latter can be used for reductive reactions related to supplementation of the anammox cycle intermediates or cell carbon fixation.

are surprising organisms, and one can never rule out another surprise). Nevertheless, there is a more attractive solution: Coupling oxidation of nitrite to nitrate ($E_0' = +0.42$ V) to the reduction of nitrite to NO ($E_0' = +0.36$ V) is energetically easier to overcome ($\Delta G^{\circ\prime} = +11.5$ kJ per reaction). In this way, the electrons from nitrite oxidation are stored in NO. When NO is subsequently fed into the anammox process, those electrons just end up in hydrazine. This hypothesis is backed up by the presence of various soluble electron carriers in the kustd1713-1699 gene cluster, which might plug their electrons from nitrite oxidation into cognate nitrite reductase(s) and other redox processes of favorable midpoint reduction potential (>0.3–0.4 V). Those electrons perhaps might even serve in hydrazine synthesis. Otherwise, quinone-binding kustd1712 could be beneficial during nitrate reduction (see next). Kustd1712 contains one or more putative proton channels, perhaps permitting proton pumping to contribute to *pmf*.

Alternative lifestyles

Anammox bacteria as facultative chemo-organotrophs

As discussed above, anammox bacteria face the problem of preventing electron drainage from the cyclic electron flow during catabolism. Loss of electrons as a result of anabolic reduction reactions or leakage of intermediates needs to be replenished at the expense of nitrite oxidation. In this respect, alternative external organic or inorganic electron donors could be of use to replenish these electrons. Indeed, it has been established that these organisms are able to convert organic and inorganic compounds to sustain their metabolism, most notably formate, acetate, and propionate (Strous *et al.*, 2006; Kartal *et al.*, 2007a,b, 2008). Rather than being incorporated into cell biomass, organic compounds are fully oxidized to CO_2. Apparently, anammox bacteria stick to their

autotrophic lifestyle. For formate and acetate consumption, the organisms can rely on the already available formate dehydrogenase and CO-DH/acetyl-CoA systems, while the presence of genes coding for the key enzymes of propionate metabolism [methylmalonyl-CoA decarboxylase (kustd2060-2061) and methylmalonyl-CoA epimerase (kuste4266)] in the *K. stuttgartiensis* genome complies with the ability of this organism to utilize propionate. A puzzling aspect is that the enzymes involved have to operate backwards (substrate oxidation) and forwards (CO_2 fixation) at the same time. In reversible systems operating at low rate under thermodynamic equilibrium – conditions that apparently apply to slow-growing bacteria – this is feasible. Next, anammox bacteria can use mono- and dimethylamine (Kartal *et al.*, 2008) and methanol (B. Kartal and B. de Wild, unpublished results) as electron donors, but the enzymes involved in their metabolism are still unknown.

$$NH_4^+ + NO_3^- + HCOO^- + H^+ \rightarrow N_2 + CO_2 + 3H_2O \ (\Delta G^{\circ\prime} = -524.4 \text{ kJ mol}^{-1}N_2) \quad (16)$$

The utilization of organic electron donors is also attractive from several other perspectives: (1) More energy is derived from this process compared with ammonium and nitrite conversion (e.g. Eqn. 2 vs. Eqn. 16 with formate as an additional electron donor). (2) In the presence of an (in)organic electron donor, nitrate may serve as the electron acceptor. Nitrate is more abundant in nature than nitrite. The product of its reduction, nitrite, can be directly fed into the catabolic cycle (Fig. 12a). (3) The specialized consumption of a certain organic substrate may result in a competitive advantage. This is nicely illustrated by the enrichment of *B. fulgida* and *A. propionicus* (Kartal *et al.*, 2007a, 2008). Both species were obtained from the same seed sludge and enriched under exactly the same conditions, yet with a subtle difference: The *B. fulgida* enrichment medium was supplemented with a low concentration of acetate, whereas a similarly low concentration of propionate was present in the *A. propionicus* enrichment medium. Although all anammox species tested were able to oxidize formate, acetate and propionate instantaneously – that is, without an induction phase – the specific rate of acetate oxidation by *B. fulgida* was 1.2-fold to threefold higher than performed by the others, while *A. propionicus* displayed a two- to fivefold higher propionate oxidation rate. Still, specific oxidation rates were only 4–6% of ammonium oxidation rate. Apparently, ammonium was the preferred electron donor, whereas the organic compounds would be cometabolized, a type of metabolism that might be classified as 'facultative chemoorganotrophy'. Nevertheless, the presence of acetate and propionate enabled *B. fulgida* and *A. propionicus* to outcompete other species including denitrifiers.

The oxidation of organic (or inorganic) electron donors allows anammox bacteria to metabolize in the absence of ammonium, when they adopt a 'disguised' denitrifying lifestyle (Kartal *et al.*, 2007b) (Fig. 12b). In this process, nitrate is first converted to nitrite, half of which is reduced to ammonium. Hereafter, ammonium and nitrite are combined to yield N_2 by the anammox pathway. The six-electron reduction of nitrite to ammonium resembles the dissimilatory nitrite reduction to ammonium (DNRA) mechanism (see above, Eqn. 8). 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 & Simon, 2009). Other microorganisms have developed different octaheme *c*-type proteins for this purpose (Atkinson *et al.*, 2007; Polyakov *et al.*, 2009). *Brocadia anammoxidans* cell extracts show significant Ca^{2+}-dependent DNRA activity, and the protein responsible for this activity could even be enriched to some degree (Kartal *et al.*, 2007b). However, the enzyme remains elusive and the *K. stuttgartiensis* genome shows no clear orthologue to known NrfAs or ammonium-forming octaheme *c*-type proteins, indicating that these anammox species must have evolved yet another variation on the theme. In contrast, a pentaheme *c* nitrite reductase (KSU1_B0055) has been annotated in anammox strain KSU-1. Despite high sequence identity with known NirfA's, KSU1_B0055 lacks one essential feature: A lysine serving as the proximal ligand to the catalytic heme 1, but it cannot be ruled out that the particular lysine stems from another part in the amino acid sequence. The identification of the anammox-specific nitrite reductase(s) making ammonium is relevant not only from the biochemical perspective but most certainly also from an ecological point of view for its application as a biomarker. While acting as 'disguised' denitrifiers, both nitrogen atoms in N_2 derive from nitrate, unlike in the standard anammox process. This will make it hard to assign N_2 production through nitrate reduction to anammox or 'true' denitrifiers. This ambiguity may lead to the underestimation of the contribution of anammox bacteria to N_2 production in nature and explain contradictory results, for instance, from the Arabian Sea (Ward *et al.*, 2009; Jensen *et al.*, 2011).

Alternative inorganic electron donors and acceptors

Also inorganic substances can serve as electron donors for *K. stuttgartiensis*, in particular Fe^{2+} (Strous *et al.*, 2006). The presence of the complete set of genes in its genome

coding for Ni-Fe hydrogenase and accessory enzymes (kustd1773-1779) implies that hydrogen is a potential electron source. On the other hand, Fe^{3+}, insoluble FeOOH, and MnO_2 act as electron acceptors (Strous *et al.*, 2006). It is not yet clear whether the reduction of these compounds represents a physiological reaction: The opposite, oxidative direction would be more beneficial to an organism in need of electrons. Consequently, 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; Richter *et al.*, 2012). Indeed, various multiheme proteins capable of such a role are present in the genome of *K. stuttgartiensis*. The conversion of inorganic and organic compounds is often mediated by members of the molybdopterin family (Rothery *et al.*, 2008). Apart from formate dehydrogenase and nitrate reductase that were already discussed, *K. stuttgartiensis* contains three other candidates (kustc0484, kustc0546, and kuste4466) with high sequence similarity to Mo-*bis*PGD-containing enzymes. Unfortunately, neither the particular amino acid sequences nor the gene contexts permit the prediction of a specific function. However, kuste2658 encodes a heme *b*-containing membrane-bound molybdopterin protein, closely resembling a novel type of bacterial sulfite oxidase (Loschi *et al.*, 2004). This observation could suggest the use of sulfur-containing electron donors, but this remains to be explored experimentally. The presence of a proton-translocating cbb_3-type terminal oxidase gene cluster (kustc0425-0430), of which all genes are expressed to a certain extent, is surprising for an obligate anaerobe. The question is whether this oxidase is active and contributes to the energy metabolism or merely acts in oxygen detoxification.

Other opportunities?

Besides the organic and inorganic compounds already mentioned, genomes of anammox bacteria have the blueprint for a metabolic repertoire for the uptake and conversion of a range of compounds derived from decaying organic matter. As for acetate and propionate, it is conceivable that specialized consumption lends species their specific niches, whereas other more versatile organisms may benefit from a diverse diet to address substrate changes in their environment. Future research is necessary to establish how substrate use and species and niche differentiation interrelate.

Surveying the metabolic potentials and pathways, a number of alternative lithotrophic lifestyles can be envisaged. These alternatives deal with exergonic disproportionation reactions of NO (Eqn. 17), hydroxylamine (Eqn. 18), and nitrite (Eqn. 19).

$$10NO + 2H_2O \rightarrow 3N_2 + 4NO_3^- + 4H^+ (\Delta G^{\circ\prime} = -332.0 \text{ kJ mol}^{-1}N_2) \quad (17)$$

$$3NH_2OH + H^+ \rightarrow N_2 + NH_4^+ + 3H_2O (\Delta G^{\circ\prime} = -680.7 \text{ kJ mol}^{-1}N_2) \quad (18)$$

$$5NO_2^- + 2H^+ \rightarrow N_2 + 3NO_3^- + H_2O (\Delta G^{\circ\prime} = -305.5 \text{ kJ mol}^{-1}N_2) \quad (19)$$

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+ (\Delta G^{\circ\prime} = -451.9 \text{ kJ mol}^{-1}N_2) \quad (20)$$

The reaction stoichiometries may look awkward, but the conversions are compatible with the known anammox pathway, and NO and hydroxylamine stimulate anammox activity as mentioned before. In fact, hydroxylamine disproportionation (Eqn. 18) may have been transiently observed in hydrazine production experiments (Van der Star *et al.*, 2008b). One may note that these compounds are found in natural habitats as free intermediates during imbalanced growth of both aerobic ammonium and nitrite-oxidizing bacteria (Schmidt *et al.*, 2004a,b; Kampschreur *et al.*, 2008, 2009; Schmidt, 2008; Yu & Chandran, 2010) and anaerobic denitrifiers (Betlach & Tiedje, 1981; Baumann *et al.*, 1996, 1997; Otte *et al.*, 1996; Saleh-Lakha *et al.*, 2009). Nitrifiers and denitrifiers are both partners and competitors of anammox bacteria in natural systems. Although they are unlikely to be primary substrates, NO and hydroxylamine might supplement the ammonium and nitrite diet. When fed into the pathway, extra electrons are harvested via hydrazine oxidation to compensate electron losses related, for instance, with CO_2 fixation (Fig. 12c and d). The disproportionation of nitrite into nitrate and N_2 (Eqn. 19) is another intriguing possibility, representing a novel type of chemolithotrophy. On good bioenergetic grounds, this possibility has been ruled out for denitrifying organisms (Strohm *et al.*, 2007). Still, nitrite disproportionation complies with the known anammox route (Fig. 12e), provided that the electrons from the oxidation of three nitrite molecules ($E_0' = +0.42$ V) can be utilized for the reduction of another nitrite into ammonium ($E_0' = +0.35$ V). A last possibility does not concur with anammox metabolism as far as we know it: The anaerobic oxidation of ammonium coupled to the reduction of nitrate to make N_2 (Eqn. 20), which is one of the two reactions predicted by Broda (1977). The problem is that it requires the activation of ammonium into an oxygen-containing species (hydroxylamine) at some point (Fig. 12f). Such activation seems to be an exclusive property of aerobic ammonium oxidizers. By the same argumentation, anaerobic ammonium oxida-

tion to N_2 ($E_0' = -0.28$ V) coupled with the reduction of inorganic electron acceptors of more positive redox potential is not very likely, unless a reactive species is generated for ammonium activation. If existent, organisms that perform these types of alternative ammonium oxidation by themselves will not be anammox bacteria as described here.

Summary and perspectives

Thirty-five years after their prediction by Broda (1977), the long-time-overlooked 'impossible' anammox bacteria are now given a central place in the nitrogen cycle. Obviously, these microorganisms owe this position to their unique ability to oxidize ammonium under anoxic conditions. They do so by a specific catabolic pathway with nitric oxide and hydrazine as intermediates (Fig. 3). Another unique feature is the catabolic machinery that is hypothesized to be contained within a special organelle: The anammoxosome. Recent research has established the nature of the catabolic pathway and identified the key enzymes involved in it. Still, this resulted in many questions to be addressed in the future.

Our analyses suggest a central role for the Q-pool in the catabolic and anabolic systems. Quinol, or rather menaquinol, is proposed to serve as the electron donor for hydrazine synthesis and the different bc_1 complexes, for the reduction of NAD^+ as well as of CO_2, in formate and acetyl-CoA syntheses. The latter three reduction reactions perhaps could take advantage of delicate electron bifurcation mechanisms (Figs 3, 9 and 10). The primary question is how the Q-pool is fed. Undoubtedly, the electrons derive from hydrazine oxidation, but how they end up in quinol and how this process is related with proton translocation remains to be elucidated (Fig. 3). The use of quinol for biosynthetic reduction reactions and the loss of diffusible intermediates (NO, N_2H_4) from the cell imply drainage of electrons from the Q-pool. These electrons have to be replenished. Under autotrophic conditions, this is achieved by the oxidation of nitrite catalyzed by an intricate nitrite:nitrate oxidoreductase (Nxr) system (Fig. 11). Nitrite oxidation coupled with quinone reduction represents a huge thermodynamic gap to bridge. A more accessible way to do so would be coupling the oxidation of nitrite to nitrate with the reduction of nitrite to NO.

The study by Kartal *et al.* (2011b) showed NO to be the intermediate in anammox metabolism. However, it is still unknown which enzyme(s) actually produce(s) NO from nitrite. Our overview indicates that anammox bacteria might take different paths: Enzymatic reactions known from other microorganisms (NirS, NirK) as well as anammox-specific ones featuring one or more of the HAO-like octaheme proteins (Fig. 4). At least one of these (kustc0694) has the specific role of HDH, the enzyme that makes the end product N_2. Another HAO-like protein (kustc1061) produces NO from hydroxylamine. What the other HAOs do and how each of these is tuned to a specific reaction (most likely by subtle changes in their catalytic sites and electron wiring circuits) remains elusive. Such mechanistic questions particularly apply to HZS, the enzyme system that forges the N–N bond from NO and ammonium to form hydrazine. Circumstantial evidence, mostly obtained from EM protein localization studies, is compelling that the above processes occur in the anammoxosome and its membrane. Conclusive evidence will only come from experiments with purified anammoxosomes. These experiments have to show that N_2 formation from ammonium and nitrite is indeed associated with anammoxosomes and that the process is coupled to the formation of *pmf* and ATP synthesis. As pointed out before, the isolation of intact anammoxosomes will be a true tour de force, considering the complicated cell plan with its multilayered membrane systems and cell wall.

The anammox bacteria have to acquire their central substrates, ammonium and nitrite, in direct cooperation and competition with other N-cycle microorganisms. Aerobic ammonium oxidizers produce nitrite, but consume ammonium. The difference in lifestyles with respect to oxygen would seem to exclude an interaction, but the contrary is the case. Both guilds interact in a delicate way. The ammonium oxidizers convert part of the available ammonium into nitrite using oxygen, thus creating the conditions for nitrogen formation by anammox bacteria. This concerted action is in fact the biological basis of a new wastewater treatment technology (Kartal *et al.*, 2010a). On the other hand, nitrite is the electron donor for aerobic nitrifiers and an electron acceptor for anaerobic denitrifying species. At first glance, these guilds would be just competitors to anammox bacteria. The actual situation is likely to be more complex. Moreover, it has become clear that anammox bacteria are not just autotrophic specialists. They use a broad range of organic and inorganic compounds as supplementary electron donors. It is very well conceivable that certain species have developed a specialization in the effective conversion of a restricted number of alternative electron donors providing them with their specific niche. Moreover, a broad substrate spectrum provides more generalist species the opportunity to respond to varying nutritional conditions. How species differentiation and metabolism relate to each other and which regulatory mechanisms underlie adaptation is a largely unknown field in anammox research. Regardless of the answer to these questions, the alternative substrates have to be gained in competition with many other microbial species. The slow-growing anammox

bacteria can survive only if they are able to scavenge their substrates down to extremely low concentrations. This requires dedicated, high-affinity uptake systems. Several of these were identified via analysis of the genomes of anammox bacteria (Fig. 8). The biochemical properties of the uptake systems and their specific localization in the anammox cell are other topics that require attention. Bearing in mind the metabolic pathways and metabolic possibilities the anammox bacteria have, we also postulated additional lithotrophic opportunities using NO, hydroxylamine, or only nitrite as substrates. These are intermediates in nitrifying and denitrifying processes. Their consumption by anammox bacteria may allow much more complicated metabolic interactions between nitrogen cycle microorganisms than considered so far.

Acknowledgements

The authors like to thank the many coworkers and funding agencies (ALW, STW, EU, ERC, STOWA, Spinoza prize, DARWIN, Paques BV, Agentschap NL) who made this work possible. B.K., N.M.dA. and W.J.M. are supported by the Netherlands Organization for Scientific Research grants (VENI, ALW, and Darwin grant numbers 863.11.003, 818.02.015, and 142.16.1201, respectively) and MSMJ by the European Research Council (advanced grant number 232937).

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