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Trending topics and open questions in anaerobic ammonium oxidation

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Anaerobic ammonium-oxidizing (anammox) bacteria are major players in the biological nitrogen cycle and can be applied in wastewater treatment for the removal of nitrogen compounds. Anammox bacteria anaerobically convert the substrates ammonium and nitrite into dinitrogen gas in a specialized intracellular compartment called the anammoxosome. The anammox cell biology, physiology and biochemistry is of exceptional interest but also difficult to study because of the lack of a pure culture, standard cultivation techniques and genetic tools. Here we review the most important recent developments regarding the cell structure — anammoxosome and cell envelope — and anammox energy metabolism — nitrite reductase, hydrazine synthase and energy conversion — including the trending topics electro-anammox, extracellular polymeric substances and ladderane lipids.

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Introduction

Anaerobic ammonium-oxidizing (anammox) bacteria are intriguing microbes relevant in both nature and industry. They were first discovered a little more than twenty years ago in an anoxic bioreactor at the Gist-Brocades yeast factory in the Netherlands [1]. A few years later anammox was shown to be performed by an extraordinary group of deep-branching Planctomycete bacteria [2]. Anammox is the anaerobic conversion of the substrates ammonium and nitrite to dinitrogen gas. The process has two somewhat unusual and toxic intermediates: nitric oxide and the 'rocket fuel' hydrazine. The anammox reaction takes place in a dedicated intracellular anammoxosome compartment. Anammox bacteria have been detected in diverse habitats including marine oxygen-minimum zones, freshwater lakes, peat soil, and wastewater treatment plants [3]. Anammox bacteria are responsible for a major part of the nitrogen loss from oxygen minimum zones and since oxygen minimum zones are estimated to account for up to 50% of the nitrogen loss from the ocean, these microorganisms play a major role in the global nitrogen cycle [4,5]. Next to their relevance in nature, anammox bacteria are applied in a cost-effective and environment-friendly wastewater treatment (WWT) technology for the removal of nitrogen compounds [6].

Anammox bacteria are not available in pure culture but are grown in enrichment cultures. This is the reason why all anammox bacteria have the '*Candidatus*' status. The enrichment cultures can contain up to approximately 95% of one single anammox species. Anammox bacteria form a phylogenetic group in the Phylum *Planctomycetes* with the order *Brocadiales* and family *Brocadiaceae* [7]. To date, five different genera of anammox bacteria have been described: *Brocadia*, *Kuenenia*, *Scalindua*, *Anammoxoglobus* and *Jettenia* with *Kuenenia stuttgartiensis* as the so-called type strain. Anammox bacteria grow slowly, with typical doubling times ranging from one to several weeks. However, faster growth, 2–5 days doubling time, has also been reported [8–11]. Anammox bacteria cannot be cultivated with traditional microbiological techniques such as agar plates. Instead, they are mainly cultivated in one of two types of bioreactors: the sequencing batch reactor (ca. 75% enrichment, flocular biomass) and membrane bioreactor (95% enrichment, planktonic cells) [8,12,13]. The growth characteristics of anammox bacteria and the lack of a genetic system make studying their cell biology, physiology or biochemistry tedious and challenging. Nonetheless, huge efforts have been made to proceed in these fields and this has resulted in important and big steps forward in understanding how the anammox cell works. Here we review the most recent findings regarding anammox biochemistry, physiology and cell biology including trending topics for current and future research.

Cell biology

The cell structure of anammox bacteria has intrigued scientists ever since the description of the first anammox bacterium *Brocadia anammoxidans* and its anammoxosome compartment [2,14] (M Strous, PhD thesis, Delft University of Technology, 2000). The anammox cell consists of three compartments; the anammoxosome, cytoplasm and periplasm, and is covered by a surface protein (S-)layer. Anammox further contains unique membrane lipids called ladderanes (see Box 1) and when

Box 1 Trending topics — Ladderane lipids.

Anammox bacteria contain membrane lipids that are unique for anammox bacteria and are used as lipid biomarkers [43–46]. These membrane lipids are called ladderanes and consist of (three or five) linearly concatenated cyclobutane rings. These hydrocarbon tails are bound to a polar head group by ester or ether bonds. All membranes of the anammox cell contain ladderane lipids [21]. Molecular modeling indicated that their role might be in rendering the anammox membranes less permeable and more rigid [43]. It was hypothesized that the slow growing anammox bacteria need a less permeable membrane to limit passive diffusion of valuable intermediates, protons and/or toxic intermediates such as hydrazine. Although hypotheses have been put forward and studies have been conducted it is not yet clear exactly how ladderane lipids are synthesized by anammox bacteria [47,48•]. In addition, isolating individual ladderane lipids from anammox cells for biophysical characterization is difficult due to the complex mixture of lipids. However, recently the total chemical synthesis of two natural and one unnatural ladderane lipid was developed [49•,50••]. Biophysical characterization showed that the densely packed ladderane membranes indeed have low proton permeability but normal hydrazine permeability compared to a conventional membrane [50••]. This strongly indicates that the biological function of ladderane lipids is to prevent the breakdown of the proton motive force during the relatively slow anammox energy metabolism. Next, it would be very interesting to perform biophysical characterization of ladderane membranes that include the other anammox membrane lipids such as straight chain and branched fatty acids and hopanoids [51,52].

Box 2 Trending topics — Extracellular polymeric substances.

Anammox prefers to grow in flocs. Because of this characteristic, the first highly enriched anammox culture was grown in a sequencing batch reactor [12] which selects for aggregates. It was only after the application of a membrane filter bioreactor that anammox was coerced to grow as planktonic cells [8]. The large propensity of anammox to grow in aggregates is achieved by the excretion of extracellular polymeric substances (EPS). EPS consists of polysaccharides, (glyco)proteins, nucleic acids and lipids (reviewed by Flemming [53]). For bacteria, EPS has a huge variety of functions, but is of industrial interest mainly due to the ability to aid in sedimentation and to serve as coating material [53–55]. Planktonic cells of anammox are stimulated to form aggregates by adverse environmental conditions (low temperature, low pH, oxygen, high nitrogen load), but can also be stimulated by variations in nutrient availability [56,57•,58•]. These conditions have in common that they cause an increase in the intracellular concentration of c-di-GMP in anammox bacteria [57•,58•]. The genome of *Jettenia caeni* contains no less than thirteen genes encoding putative enzymes involved in c-di-GMP turnover [58•]. In particular, JcaA was identified as an enzyme capable of both synthesis and degradation of c-di-GMP in *J. caeni*. In other bacteria aggregation and biofilm formation is dependent on the concentration of c-di-GMP [59,60]. Perhaps then unsurprisingly, the increase of c-di-GMP in anammox increases the production of precursors of the EPS layer itself and of known EPS compounds like alginate and exopolysaccharide poly-*N*-acetylglucosamine [56, 57•,58•,61]. The anammox EPS contains relatively many hydrophobic groups and this hydrophobicity increases floc formation ability [62]. The composition and properties of EPS differ slightly between the different anammox species [63] but in general anammox EPS contains more protein and is more hydrophobic compared to the EPS of other bacteria and through these properties has higher aggregation ability. Fully formed flocs are not uniform in composition, but are instead stratified [56,64] and contain gas pockets [65]. The reported stratification is different, depending on the enrichment and culturing methods [64]. Similarities include an outer layer that can consist of mainly nucleic acids [64] and either α- or β-polysaccharides with an even distribution of protein throughout [56,64]. It has also been postulated that anammox EPS can be used as carbon and energy source during starvation [61] and extracellular enzymes have been detected in activated sludge and biofilms that can potentially degrade EPS components. The anammox EPS is currently extensively investigated due to its relevance in the industrial application of anammox.

grown in aggregates, excrete large amounts of extracellular polymeric substances (EPS, see Box 2).

Anammoxosome

The anammoxosome compartment is the location of the energy metabolism and is devoid of ribosomes and DNA. It does contain two conspicuous structures: iron-rich nanoparticles with an unknown function [15,16•] and tubule-like structures containing, or associated with, the nitrite oxidoreductase (NXR, see 'Physiology & biochemistry') enzyme [17]. In addition, the anammoxosome membrane is highly folded and contains ATPases [18,19] — all fitting to its role in energy conversion. Key metabolic enzymes hydrazine synthase (HZS; kuste2859-61), hydrazine dehydrogenase (HDH; kustc0694), hydroxylamine oxidase (HOX; kustc1061) and one of the putative nitrite reductases [20] the HAO-like protein kustc0458 with its redox partner kustc0457 were localized to the anammoxosome [17]. The anammoxosome can be isolated from the cell [21] and isolated anammoxosomes were shown to be able to perform the anammox reaction. However, the N_2 production rate of isolated anammoxosomes was only comparable to that of intact cells in the presence of hydrazine. Isolated anammoxosomes probably need hydrazine as an external electron source due to the absence of the surrounding cytoplasm compartment. Without the cytoplasm, the electron flow is most likely impaired as a result of the absence of electron acceptors such as NAD^+ and ferredoxins and the carbon fixation pathway (see also 'Physiology & Biochemistry'). The hypothesis is that the anammox reaction is coupled to an electron transport chain in the anammoxosome membrane giving rise to a proton motive force and subsequent ATP synthesis (inside the cytoplasm) by the anammoxosome membrane-bound ATPases. Even though the presence of the anammox reaction and metabolic proteins inside the anammoxosome has been firmly established, the link to the anammoxosome membrane with its proposed electron transport chain, proton motive force and ATP synthesis still needs experimental validation.

Periplasm and peptidoglycan

Recently, the outermost cell compartment of anammox bacteria [22•] and other Planctomycetes [23] was redefined as a periplasmic space typical of Gram-negative bacteria. Complementary techniques showed the presence of peptidoglycan in the cell envelope of *Kuenenia*

stuttgartiensis — a structure of which the Planctomycetes were previously proposed to be devoid of. Cryotransmission electron microscopy of vitreous sections revealed an additional layer (4.5–6 nm thick) between the two outermost membranes. In addition, boiling in SDS resulted in sacculi that could be disintegrated by using lysozyme — an enzyme which cleaves the β-1,4 bonds of the peptidoglycan sugar backbone. The presence of peptidoglycan was also confirmed by growing cells in the presence of D-amino acid dipeptide probes that are incorporated in the peptide stem of growing peptidoglycan. The composition of the *K. stuttgartiensis* peptidoglycan was determined through muropeptide analysis which suggested that the composition (i.e. abundance of certain muropeptides) differs from that of the model organism *Escherichia coli*. This fits to other data showing that the anammox cell is more fragile and more prone to osmotic effects and that the observed peptidoglycan layer is thinner and not visible with standard electron microscopy methods. It would be very interesting to investigate whether these differences in peptidoglycan have an effect on the properties and function of the cell wall and bacterium.

Surface protein layer

The anammox bacterium *K. stuttgartiensis* has an S-layer as outermost layer of the cell [24,25•]. The S-layer has a hexagonal symmetry with unit cells consisting of six protein subunits. The S-layer is formed by the 160 kDa glycoprotein kustd1514. The kustd1514 protein is heavily (*O*-)glycosylated; with a modified mass of 250 kDa. In addition, the individual glycans contain an extensive degree of methylation. S-layers are common in both Archaea and Bacteria and can have multiple functions [26,27] such as protection, adhesion and cell shape and integrity. Laboratory strains of bacteria often lose their S-layer over time indicating that the S-layer function in bacteria is more important in natural environments. However, even though *K. stuttgartiensis* has been maintained in the lab over 10 years, it still contains the S-layer. This could indicate that the *K. stuttgartiensis* S-layer might serve an important function such as cell integrity by functioning as an exoskeleton. Considering the cellular fragility of the cells and the atypical peptidoglycan this can very well be the case. However, until genetic tools are available, the functional assessment of such structures remains challenging.

Outer membrane

As for peptidoglycan, it was also proposed that anammox bacteria did not contain an outer membrane. This hypothesis was refuted by the characterization of an outer membrane protein (OMP) in the outer membrane of *K. stuttgartiensis* [28•] which was previously also suggested by genomic analysis [29]. Outer membranes can be distinguished from cytoplasmic membranes by the presence of lipopolysaccharides and (general or specific) channel-forming β-barrel OMPs. Lipid bilayer essays have shown the presence of at least two channel-forming OMPs in membrane fractions of *K. stuttgartiensis*. One of these is the highly abundant kustd1878 protein which is hypothesized to form a specific pore that is moderately cation-selective. Strikingly, kustd1878 was also observed to be present after S-layer enrichment [24]. This indicates that the S-layer (see 'Surface protein layer') is strongly linked to the outer membrane or maybe even to the kustd1878 OMP itself. The latter option fuels speculations about a function for kustd1878 in S-layer export — a function which has been shown before for OMPs [26]. This putative function for the kustd1878 OMP should be tested in lipid bilayer assays with the S-layer protein kustd1514 as substrate. In addition, the presence of LPS still needs verification.

Physiology & biochemistry

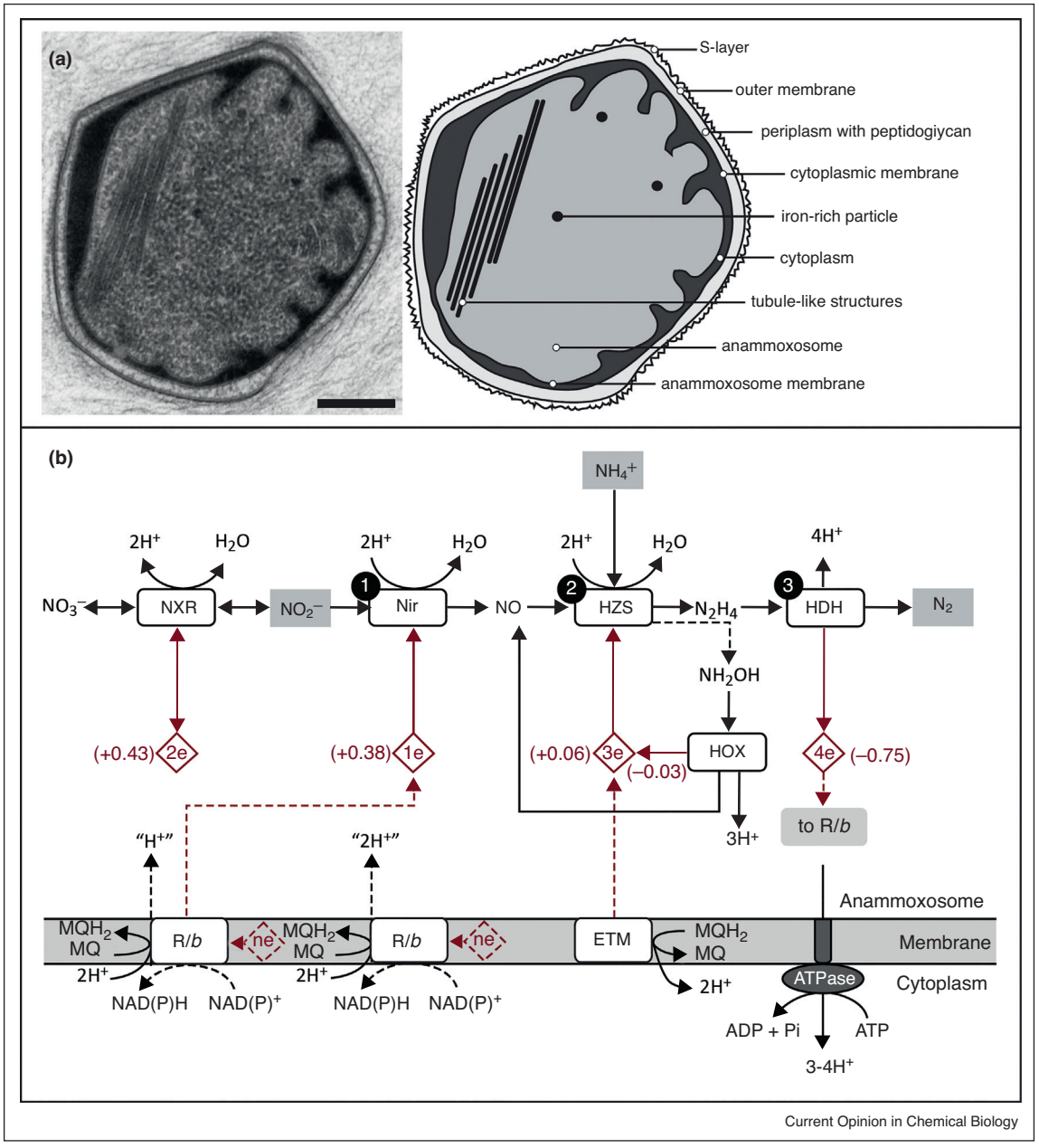
As mentioned before, anammox bacteria obtain their energy for growth by the anaerobic oxidation of ammonium coupled to the reduction of nitrite leading to dinitrogen gas as end product (Figure 1, reviewed by Kartal *et al.* [20,30••]). First, nitrite is reduced to nitric oxide by a nitrite reductase (Nir) using one electron. Next, nitric oxide is combined with ammonium to form hydrazine by hydrazine synthase (HZS) using three electrons. Finally, hydrazine is oxidized to dinitrogen gas by hydrazine dehydrogenase (HDH) releasing the four electrons to power the previous two redox reactions. It has been proposed that some of the electrons produced by the oxidation of hydrazine are lost to carbon fixation and that these are replenished by the oxidation of nitrite to nitrate by NXR. In addition, the highly abundant hydroxylamine oxidase (HOX) protein is postulated to convert any hydroxylamine that might be lost from the HZS enzyme (see 'Hydrazine synthase') back to nitric oxide and thus back to HZS.

Nitrite reductase

The nitrite reductase (typically *nirS* or *nirK*) is a curiosity in anammox bacteria. *K. stuttgartiensis* harbours the genes required for a canonical *nirS*, but these are barely expressed. Interestingly, *nirS* is expressed by *Scalindua brodae* [31], while the (meta)genome of *Jettenia caeni* strain KSU-1 lacks *nirS* [32], but encodes for a *nirK* instead. The *Brocadia* species do not encode for any nitrite reductase genes in their genomes whatsoever [33,34]. There seems to be no common theme (i.e. enzyme) for nitrite reduction among the various anammox species, which could imply that there is a missing (unknown) nitrite reductase. This missing nitrite reductase is postulated to be one of the hydroxylamine oxidoreductase (HAO)-like proteins kustc0457-0458 or kuste4574 based on protein sequence analysis [20] but this still awaits experimental verification.

The anammox metabolic pathway might be different for individual anammox species. N-tracer experiments indicated that, unlike *K. stuttgartiensis*, anammox species *Brocadia sinica* reduces nitrite to hydroxylamine instead

Figure 1



Anammox cell structure and function.
(a) Transmission electron micrograph and cartoon of a section through an anammox cell. Scale bar; 200 nm. Micrograph used with permission from van Niftrik *et al.* [15]. **(b)** Schematic overview of the anammox energy metabolism. The main reactions are indicated by numbers: **1.** nitrite reduction to nitric oxide by Nir, **2.** combining nitric oxide with ammonium to form hydrazine by HZS, and **3.** the oxidation of hydrazine to dinitrogen gas by HDH. NXR; nitrite oxidoreductase, Nir; nitrite reductase, HZS; hydrazine synthase, HDH; hydrazine dehydrogenas, HOX; hydroxylamine oxidase, R/b; Rieske-heme *b* complexes (*bc*1 complexes), ETM; electron transfer module. Modified with permission from Kartal and Keltjens [30••].

of nitric oxide [35•]. This trait may be shared by other anammox species that lack known *nir* genes for NO production. An interesting question is therefore how much metabolic versatility there is among anammox species, especially concerning the anammox intermediates (see also Box 3).

Hydrazine synthase

The enzyme responsible for the synthesis of hydrazine is unique to anammox, and is therefore used as a biomarker [36]. *K. stuttgartiensis* hydrazine synthase (HZS) consists of three subunits (α, β and γ). In *Scalindua brodae* and *Scalindua profunda* the β-subunits and γ-subunits are

Box 3 Trending topics — electro-anammox.

The direct transfer of electrons among bacteria and between bacteria and electrodes is currently a hot topic. Over the last few years indications have been found suggesting that anammox might also be a player in this field. For example; anammox can be coerced to grow as a biofilm on electrodes and consume nitrogenous compounds faster when exposed to an electric field [66,67]. Direct electron transfer would allow anammox bacteria to live as part of an electric system, coupling the biological anammox process to man-made electrical systems. This tightly coupled bioelectrochemical system may solve some issues in the application of anammox bacteria in WWT. The first issue is that the anammox biomass has to be retained for effective functioning of a WWT plant. This can be achieved by use of a mechanical plate settler [68], or by growing the biomass as a biofilm [69]. As anammox can grow as a biofilm on the electrode, the WWT plant would be more robust against the dilution of the active biomass. The second issue is the difficulty regulating and modulating the activity of anammox bacteria in a WWT plant. The application of an electric field would allow for the modulation of the anammox reaction [66,67], by increasing or decreasing the intensity of the field. Direct experimental evidence for direct electron transfer performed by anammox does not yet exist, but offers up chances for exiting new research.

fused into a single gene (scabro01046 and sca00025, respectively) [31,37]. The crystal structure of the HZS multi-enzyme complex from *K. stuttgartiensis* was recently determined [38••]. HZS is a dimer of heterotrimers (α2β2γ2; kuste2861, kuste2859, kuste2860 respectively). The alpha and gamma subunit each contain an active site connected by a major tunnel. The tunnel connects two heme groups present in subunit alpha (αI) and gamma (γI) and branches off towards the surface of the protein at the halfway point between the two sites. The tunnel allows for diffusion of nitric oxide from the surface to the active site of subunit gamma, where the nitrogen is proposed to be reduced from oxidation number +II to −I by electrons obtained from tetraheme cytochrome *c* protein kuste2854, resulting in the putative production of hydroxylamine. Hydroxylamine is then proposed to diffuse through the major tunnel to the active site of subunit α, where it could bind in a tight hydrophobic pocket of the catalase-like heme αI. The N–O bond would then be polarized, enabling ammonium, which enters through a second (minor) tunnel, to perform a nucleophilic attack on the nitrogen of hydroxylamine, producing hydrazine through comproportionation. Hydrazine would leave the enzyme from the branch of the major tunnel to the surface. The β-subunit is proposed to play a role in modulating transport through the tunnel. Although purification of HZS indicated that the enzyme can catalyze the proposed reaction [39,40], the specific activity is very low.

Hydrazine dehydrogenase

The last step of the anammox process is the oxidation of hydrazine by an HAO-like enzyme hydrazine dehydrogenase (HDH). There are a number of HDH paralogues in each anammox genome. By direct purification, the *K. stuttgartiensis* HDH was identified to be kustc0694 [40,41•], which catalyses the four electron oxidation of hydrazine. This reaction is strongly inhibited by hydroxylamine and NO.

Energy conversion

An open question in the anammox metabolism is how the energy from the oxidation of hydrazine is conserved into an electrochemical gradient [20]. A complexome analysis [42••] found evidence for an unusual Rieske-heme *b* complex (*bc*1 complex), which would result in the conversion of energy trough NAD(P)H, reduced quinone and possibly a proton motive force. Evidence for this respiratory complex generating a proton motive force is lacking, but the major ATPase in anammox, the proton-pumping F_1F_O-ATPase, was localized to the anammoxosome membrane [18,19]. This suggests that a proton motive force is being generated over the anammoxosome membrane and used for ATP production. A surprising finding is that anammox bacterium *K. stuttgartiensis* seems to express and assemble a set of respiratory complexes that utilize sodium ions instead of protons. Anammox bacteria may therefore have the potential to also use a sodium motive force [42••]. The sodium gradient could be established by a membrane bound NADH:quinone oxidoreductase found in the complexome analysis. The sodium gradient may then be utilized for the reduction of ferredoxin for use in autotrophic carbon fixation [42••].

Conclusions

The enigmatic anammox bacteria are of major interest to the scientific community for varying reasons. A major part of current anammox research focuses on the application; with EPS (Box 2) and electro-anammox (Box 3) as trending topics. From the cell biological perspective, important questions are; how the anammox reaction is coupled to energy conversion, whether anammox peptidoglycan is different from typical peptidoglycan and why, what the function of the anammox S-layer is and how it relates to the OMP kustd1878, whether the outer membrane of anammox bacteria contains LPS, and how ladderane lipids (Box 1) are synthesized. From the physiological and biochemical perspective, important topics are which enzyme performs nitrite reduction to nitric oxide, how can we get HZS to be active upon purification, and what is the role of hydroxylamine in the anammox reaction of different anammox species. Without a pure culture, standard cultivation methods and a genetic system, these topics for current and future research are a major experimental challenge but of outstanding scientific interest.

Conflict of interest statement

Nothing declared.

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