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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*