Global impact and application of the anaerobic ammonium-oxidizing (anammox) bacteria

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Abstract

In the anaerobic ammonium oxidation (anammox) process, ammonia is oxidized with nitrite as primary electron acceptor under strictly anoxic conditions. The reaction is catalysed by a specialized group of planctomycete-like bacteria. These anammox bacteria use a complex reaction mechanism involving hydrazine as an intermediate. The reactions are assumed to be carried out in a unique prokaryotic organelle, the anammoxosome. This organelle is surrounded by ladderane lipids, which make the organelle nearly impermeable to hydrazine and protons. The localization of the major anammox protein, hydrazine oxidoreductase, was determined via immunogold labelling to be inside the anammoxosome. The anammox bacteria have been detected in many marine and freshwater ecosystems and were estimated to contribute up to 50% of oceanic nitrogen loss. Furthermore, the anammox process is currently implemented in water treatment for the low-cost removal of ammonia from high-strength waste streams. Recent findings suggested that the anammox bacteria may also use organic acids to convert nitrate and nitrite into dinitrogen gas when ammonia is in short supply.

Introduction

Only 6 years ago, the microbial lithotroph 'missing from nature', capable of anaerobic ammonium oxidation (anammox), was identified as *Candidatus* 'Brocadia anammoxidans' using cell purification and a molecular approach [1]. The notion that ammonium could be oxidized under anoxic conditions was already reported in the 1960s and 1970s, and came from calculations based on the Redfield ratio in marine ecosystems [2] and from thermodynamic calculations [3]. The identification of the anammox bacteria was preceded by the discovery of the process in a denitrifying pilot plant [4], and the enrichment of the responsible bacteria in a continuous reactor system with very efficient biomass retention [5–7]. The physiology of anammox bacteria has been relatively well studied using the biomass available in laboratory enrichment cultures. These bacteria grow very slowly (doubling time 11 days) due to a low substrate conversion rate [7], although in a recent study a doubling time of 1.8 days was estimated from experiments in an anaerobic biological filter inoculated with a preculture [8]. The specific activity in this reactor was 2.1 fmol/cell per day, which is half of the activity reported by Kuypers et al. [9] in the Black Sea and comparable with the activity in sequencing batch reactors [7]. Therefore the calculated doubling time is unlikely.

The yield (expressed as dry biomass per ammonium oxidized) is similar to that of aerobic nitrifiers and reflects normal thermodynamic efficiency. The K_s values of anammox bacteria for ammonium and nitrite are below the detection level (<5 μM) [10] and the bacteria are reversibly inhibited by very low levels (<1 μM) of oxygen and irreversibly inhibited by high nitrite (>10 mM) concentrations [7,10]. All anammox bacteria investigated so far produce hydrazine from hydroxylamine [11,12]. The hydrazine oxidoreductase involved in this reaction was purified from *Brocadia anammoxidans* [13]. Using immunogold and electron microscopic analysis, the enzyme was found to be present exclusively inside a membrane-bound prokaryotic organelle (the anammoxosome) [14]. This organelle was found to have a membrane nearly exclusively composed of unique ladderane lipids [15–17], with three to five linearly concatenated cyclobutane rings. The ladderane lipids are present as both ether and ester lipids in anammox bacteria. In addition, the membranes of anammox and other planctomycete bacteria contain hopanoids [18].

The phylogeny of the anammox bacteria was established using purified cells in a full molecular analysis based on the complete 16 S rRNA gene sequence and FISH (fluorescence *in situ* hybridization) with specific oligonucleotide probes [11]. Using these specific 16 S rRNA probes, anammox bacteria were detected in many freshwater and marine

Key words: anaerobic ammonium oxidation (anammox), hydrazine, nitrite, nitrogen cycle, nitrogen removal, planctomycete.

Abbreviations used: CANON, completely autotrophic nitrogen-removal over nitrite; FISH, fluorescence *in situ* hybridization; OMZ, oxygen minimum zone.

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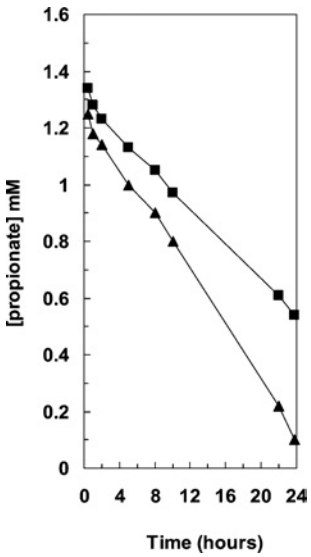
Figure 1 | Conversion of propionate in two anammox reactors that had never previously seen propionate



ecosystems [11]. Until now, the anammox bacteria belong to three genera: *Brocadia* including *B. anammoxidans* and *Brocadia fulgida*, *Kuenenia* including *Kuenenia stuttgartiensis*, and *Scalindua* including *Scalindua wagneri*, *Scalindua brodae* and *Scalindua sorokinii* [11]. Phylogenic analysis has shown that the three genera are monophyletic and branch off deep inside the planctomycete lineage of descent. All three genera share the same metabolism and have a similar ultrastructure [12], indicating that the capability for anaerobic ammonium oxidation seems to have evolved only once.

In the present paper, the oxidation of organic acids, the recent estimates of the role of anammox in the global nitrogen cycle and the implementation of anammox bacteria in water treatment are discussed.

Use of organic acids by anammox bacteria

Anammox bacteria have been described as obligate chemolithoautotrophs [1] and CO_2 incorporation into biomass was shown by microautoradiography [11]. However, many chemolithoautotrophs can use organic compounds as a supplementary carbon source. Therefore the effect of organic compounds on anammox bacteria was recently investigated [19]. It was shown that alcohols inhibited anammox bacteria, while short-chain fatty acids were converted by them. Methanol was the most potent inhibitor, leading to complete and irreversible loss of activity at concentrations as low as 0.5 mM. Propionate and acetate were consumed at a rate of approx. 48 μmol/h per g of protein by Percoll-purified anammox cells. Other compounds tested like glucose, alanine or starch had little effect on the anammox bacteria. Propionate was oxidized immediately after the addition to anammox reactors that had never seen propionate before (Figure 1). Propionate was recovered mainly as CO_2, with nitrate or nitrite as the electron acceptor. The anammox bacteria carried out propionate oxidation simultaneously with anaerobic ammonium oxidation. In an anammox enrichment culture fed with propionate for 150 days, the relative amounts of anammox cells and denitrifiers did not change significantly over time, indicating that anammox bacteria could compete successfully with heterotrophic denitrifying bacteria for propionate in the presence of excess ammonium. Labelling experiments with ^{15}N clearly showed that nitrite was the intermediate of nitrate reduction. Thus anammox bacteria were shown to be capable of reducing nitrate to nitrite. The biochemical mechanism of nitrite reduction remains unclear. In principle, anammox bacteria could make use of the regular denitrification pathway to produce dinitrogen gas, but previous experiments showed that nitrous oxide had no effect on the anammox reaction [20]. Alternatively, anammox bacteria could first reduce nitrite to ammonium and subsequently oxidize ammonium in the anammox reaction. This route would be consistent with the observation that anammox cell extracts do contain high activities (500 nmol/min per mg of protein) of calcium-dependent cytochrome *c* nitrite reductase [21]. Taken together, these results show that anammox bacteria have a more versatile metabolism than previously assumed.

Global distribution and contribution of anammox bacteria

Anammox bacteria are not restricted to wastewater treatment systems. Different studies have detected the presence and activity of anammox bacteria in more than 30 natural freshwater and marine ecosystems all over the world (Table 1). The detection and quantification of the anammox bacteria in these systems was based on different combinations of microbiological and biogeochemical techniques: enrichment cultures, nutrient profiles, ^{15}N-labelling incubations with sediments or water samples, ladderane and membrane lipid analysis, FISH microscopy, and/or 16 S rRNA gene sequencing [11]. The ecosystems where anammox was found first were anoxic sediments and anoxic water columns [22–24]. In the sediments with low organic carbon content, anammox accounted for 20–79% of total N_2 production [22–26]. Recently, Trimmer et al. [27] observed that the activity of the anammox bacteria in these sediments is probably regulated by the availability of NO_3^- and NO_2^- and the relative size of the anammox population. Engström et al. [25] found that the relative importance of anammox to the production of dinitrogen gas was inversely correlated to the remineralized solute production, benthic oxygen consumption and amount of chlorophyll a in the surface sediment [24,25]. These correlations suggest a strong competition between different reductants for pore water nitrite. The studies in the anoxic water columns of the Black Sea and the Golfe Dulce showed that anammox was responsible for 20–50% of the total N_2 production [9,28]. Recently, it was also discovered that the anammox bacteria are mainly responsible for nitrogen loss in the OMZ (oxygen minimum zone) of one of the most productive regions of the world's oceans, the Benguela upwelling system [29]. The upwelling of nutrient-rich South Atlantic mid-waters in the Benguela current system along the

Table 1 | **Distribution of anammox bacteria in natural and man-made ecosystems around the world**

Place	Method of detection	Reference
Man-made systems		
Delft/Nijmegen, The Netherlands	^{15}N, enrichment, FISH, lipids, clone library	[1,4,11,15,36]
Stuttgart, Germany	Enrichment, FISH, clone library	[37]
Dubendorf, Switzerland	Enrichment, FISH, clone library	[38–40]
Hannover, Germany	Enrichment, FISH	[41]
Kumamoto, Japan	Enrichment, FISH	[42,43]
Sydney, Australia	Enrichment	[44,45]
Athens, GA, U.S.A.	Enrichment	[46]
Kirinya, Jinja, Uganda	FISH	[47]
Ghent, Belgium	Enrichment, FISH, clone library	[48]
Pitsea, U.K.	Enrichment, FISH, lipids, clone library	[49]
Hangzhou, China	Enrichment	[50,51]
Kyungsan, Korea	Enrichment	[52]
Kanagawa, Japan	Enrichment, FISH	[53]
Lyngby, Denmark	Enrichment	[54]
Mechernich, Germany	Enrichment	[55]
Santiago de Compostela, Spain	Enrichment, FISH	[56,57]
Yongin, Korea	Enrichment, FISH	[58]
Beijing, China	Enrichment	[59,60]
Chiba, Japan	Enrichment, FISH	[8]
Guangzhou, China	Enrichment, clone library	[61]
Perth, Australia	Enrichment, FISH	[62]
Natural systems		
Skagerak (North Sea)	^{15}N, nutrient profiles	[22]
Black Sea	^{15}N, nutrient profiles, FISH, lipids, clone library	[9]
Golfo Dulce, Costa Rica	^{15}N, nutrient profiles	[63]
Thames Estuary, U.K.	^{15}N	[27,64]
Arctic Sea (East Greenland)	^{15}N, nutrient profiles	[65]
Arctic Sea (NW Greenland)	^{15}N, nutrient profiles	[65]
Mertz Sea, Antarctica	Clone library	[66]
Randers Fjord, Denmark	^{15}N, nutrient profiles, FISH, clone library	[67]
Benguela OMZ, Namibia	^{15}N, nutrient profiles, FISH, lipids, clone library	[29]
Chesapeake Bay, U.S.A.	^{15}N, FISH, clone library	[68]
Gullmarsfjorden, Sweden	^{15}N, nutrient profiles	[25]
Long Island, U.S.A.	^{15}N, nutrient profiles	[25]

southwest African continental margin sustains some of the highest primary production rates in the ocean. Although the upwelling water is generally well oxygenated, bottom waters become severely oxygen-depleted over large areas of the southwest African shelf due to aerobic mineralization of sinking algal biomass [29]. The strong N deficit in the OMZ was until now attributed to denitrification, but the study of Kuypers et al. [29] showed unequivocally that the anammox bacteria are responsible for most of the nitrogen loss in the Benguela OMZ waters. Based on their observations, it is likely that anammox also plays an important role in other OMZ waters of the ocean.

Application of the anammox process

In recent years, there has been an enormous increase in research efforts dedicated to the more applied aspects of the anammox process (Table 1). This is not surprising considering the 90% reduction of operational costs that the implementation of the anammox process would achieve [30]. In wastewater treatment, anammox would have the potential to replace the conventional denitrification step, if preceded by partial nitrification to nitrite. In this way the nitrification aeration costs would also be reduced by 50%. The current price estimate for a partial nitrification anammox process is approx. 0.75 Euro per kg of N removed [30]. Previous feasibility studies with various types of wastewater have shown that the activity of the anammox bacteria is not negatively affected by the chemical composition of the applied waters [30]. It has now been independently established in several different laboratories and semi-technical plants that anammox bacteria can be enriched from various types of wastewater sludge [30], indicating that

anammox bacteria are indigenous in many treatment plants throughout the world (Table 1). However, the enrichment time varies between 100 and 600 days [30,31]. The highest nitrogen removal by anammox bacteria of approx. 9 kg of N/m^3 per reactor day was achieved in gas lift reactors [31].

Alternatively, the anammox process can be combined with partial nitrification in one reactor [the CANON (completely autotrophic nitrogen-removal over nitrite) process] [32]. In this process, growth of nitrite oxidizers has to be prevented. Long run experimental studies have shown that this is possible if dual competition occurs for both nitrite and oxygen [33]. This requires that nitrogen load and aeration have to be well balanced in the operation of CANON reactors [34]. Two-dimensional dynamic models show that in CANON biofilms complex nitrite profiles can be expected, which vary not only over the biofilm depth but also across the surface [35] (see http://www.biofilms.bt.tudelft.nl; dynamic CANON-simulations).

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