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Anaerobic Ammonium-Oxidizing Bacteria: Unique Microorganisms with Exceptional Properties

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INTRODUCTION

From the 1940s to the 1970s, several studies indicated that a microbe was missing from nature that could anaerobically oxidize ammonium, with nitrate or nitrite, to dinitrogen gas and that the nitrogen cycle (Fig. 1) thus contained more reactions than was known at that time (8, 29, 73). This hypothesis was based on field observations that much less ammonium accumulated in anoxic water bodies than was expected from Redfield stoichiometry and thermodynamic calculations. In the early 1990s, the first experimental indications for this process were obtained when ammonium was found to be converted to dinitrogen gas at the expense of nitrate in an anoxic fluidized-bed bioreactor at the Gist-Brocades yeast factory in The Netherlands. Only a few years later, the bacteria responsible for anaerobic ammonium oxidation (anammox) were enriched and identified as a new planctomycete (86, 92). However, what no one could have predicted was that in addition to being the missing link in the nitrogen cycle, these anammox bacteria would also defy other microbiological concepts. Anammox bacteria do not conform to the typical characteristics of bacteria but instead share features with all three domains of life, *Bacteria*, *Archaea*, and *Eukarya*, making them extremely interesting from an evolutionary perspective. Furthermore, anammox bacteria were shown to be of high interest with regard to their unusual metabolism and significance in the fields of wastewater application and microbial ecology.

Planctomycetes

The *Planctomycetes* comprise a phylum of the domain *Bacteria* and are ultrastructurally distinct from other bacteria in that they have intracytoplasmic membranes that compartmentalize the cell (Fig. 2). All *Planctomycetes* available in pure culture are aerobic chemoorganoheterotrophic bacteria. Their compartmentalization is in some cases more complex than in others but always involves an intracytoplasmic membrane that defines a major cell compartment. Based on chemical analyses, electron microscopy observations, genome sequencing, and resistance to beta-lactam and other cell wall-targeting antibiotics, some *Planctomycetes* were proposed to have a proteinaceous cell wall lacking the otherwise universal bacterial cell wall polymer peptidoglycan and an outer membrane typical of Gram-negative bacteria (22, 47, 53, 84). The outermost planctomycete membrane has been defined as the cytoplasmic membrane based on the detection of RNA directly on its inner side by immunogold labeling. The other, innermost membrane has been defined as an intracytoplasmic membrane, as it is inside the cytoplasmic membrane. The outermost cytoplasmic compartment of the cell (between these two membranes) has been named the "paryphoplasm." The location of the paryphoplasm is the same as that of the periplasm of Gram-negative bacteria, but where the former is inside the essential cell boundary, the latter is not. The organization of the cell envelope of the *Planctomycetes* was therefore proposed to be fundamentally different from that of Gram-negative bacteria (56).

In the planctomycetes *Pirellula* and *Isosphaera* (Fig. 2A and B), the intracytoplasmic membrane surrounds a single interior cell compartment, the "riboplasm," which holds the cell DNA as well as the ribosomes (56). In *Isosphaera*, the intracytoplasmic membrane exhibits a large invagination into the riboplasm. In the planctomycetes *Gemmata* and anammox bacteria (Fig. 2C and D), the riboplasm itself contains a second membrane-bound compartment (56, 86). In *Gemmata*, this compartment is surrounded

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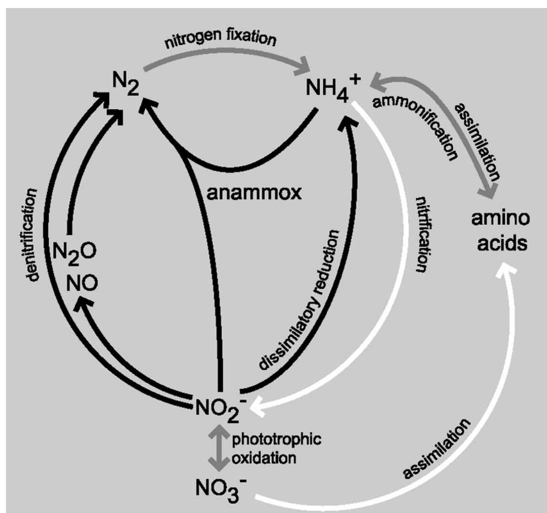

FIG 1 The biological nitrogen cycle. Black arrows, anaerobic reactions; white arrows, aerobic reactions; gray arrows, both aerobic and anaerobic reactions.

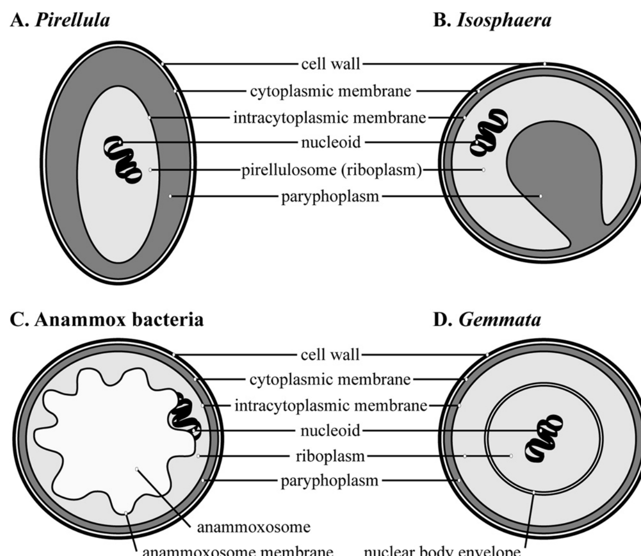

FIG 2 Cellular compartmentalization in *Planctomycetes.* (Partly based on reference 56 with kind permission from Springer Science+Business Media.)

by a double membrane and contains the cell DNA. In anammox bacteria, the compartment is bound by a single bilayer membrane and has been named the "anammoxosome." The cytoplasm in anammox bacteria was thus proposed to be divided into three cytoplasmic compartments separated by single bilayer membranes. The outermost compartment, the paryphoplasm, occurs as an outer rim, defined on its outer side by the cytoplasmic membrane and cell wall and on the inner side by the intracytoplasmic membrane. The middle compartment, the riboplasm, contains ribosomes and the nucleoid. Finally, the innermost ribosome-free compartment, the anammoxosome, occupies most of the cell volume and is bound by the anammoxosome membrane.

Anammox Bacteria

Since their discovery in the 1990s (48), anammox bacteria have been found in many different environments, such as wastewater treatment plants (35), lakes (81), marine suboxic zones (52), and coastal sediments (79). Anammox bacteria are key players in the nitrogen cycle (Fig. 1), where they were discovered to be a major source of dinitrogen gas on a global scale (14, 21, 50, 88). The contribution of anammox bacteria has been investigated in all major oxygen minimum zones (OMZ) in the ocean (Black Sea, Chilean and Peruvian OMZ, Namibian OMZ, and Arabian Sea) that contribute significantly to the loss of fixed nitrogen from the ocean (32, 49, 50, 52). In all those studies, the anammox process was the major pathway for the loss of fixed nitrogen, as was documented by stable isotope measurements, ladderane lipids, fluorescence *in situ* hybridization (FISH), and quantitative PCR (qPCR) of functional genes (32, 51, 52). So far, the capability of anammox is limited to a very specific group of the *Brocadiales* (see below), while denitrification occurs in bacteria, archaea, and even eukaryotes (33). In general, ecosystems with surplus organic electron donors will favor the processes of denitrification and dissimilatory nitrate reduction to ammonia (33), while oligotrophic systems with low oxygen concentrations but with an ample supply of ammonium from anaerobic mineralization will most likely stimulate the anammox process. Anammox bacteria have also been applied in wastewater treatment for the removal of ammonium (38, 65, 93). Since the startup of the first anammox wastewater treatment plant in 2002 in Rotterdam, Netherlands, anammox is emerging as an attractive alternative to conventional nitrogen removal from wastewater all over the world.

So far, five anammox "*Candidatus*" genera (34) have been described, with 16S rRNA gene sequence identities of the species ranging between 87 and 99% (79). "*Candidatus* Kuenenia" (76), "*Candidatus* Brocadia" (43, 48, 86), "*Candidatus* Anammoxoglobus" (41), and "*Candidatus* Jettenia" (67a) have all been enriched from activated sludge. The fifth genus, "*Candidatus* Scalindua" (50, 77), has been enriched from natural habitats, especially from marine sediments and oxygen minimum zones (44, 45, 46, 51, 64, 66, 79, 96, 105). Despite the relatively large phylogenetic distance, all anammox organisms belong to the same order, *Brocadiales* (34), which forms a monophyletic group, or clade, deeply branching inside the phylum *Planctomycetes* (78, 79, 86).

Anammox bacteria are coccoid bacteria with an average diameter ranging between 800 and 1,100 nm (97). They are anaerobic chemolithoautotrophs and thus are physiologically distinct from the other known *Planctomycetes*. Anammox bacteria use nitrite as the electron acceptor to form dinitrogen gas as the final product (91). The highly toxic "rocket fuel" hydrazine (N_2H_4) and nitric oxide (NO) are the two intermediates of this process (40, 74, 75, 87, 91). Carbon fixation proceeds through the acetyl coenzyme A (CoA) pathway (80, 89). The catabolic anammox reaction is carried out 15 times to fix 1 molecule of carbon dioxide with nitrite as the electron donor, leading to the anaerobic production of nitrate in anabolism (15, 87).

Next to ammonium, organic and inorganic compounds can be used as alternative electron donors, e.g., propionate, acetate, and formate by "*Candidatus* Kuenenia stuttgartiensis," "*Candidatus* Anammoxoglobus propionicus," "*Candidatus* Scalindua" spp., and "*Candidatus* Brocadia" spp.; methylamines by "*Candidatus* Brocadia fulgida"; as well as ferrous iron by "*Candidatus* Kuenenia stuttgartiensis" (41, 42, 89, 96). At least for "*Candidatus* Kuenenia stuttgartiensis" and "*Candidatus* Scalindua" spp., it has been shown that, besides nitrite, iron and manganese oxides can also be

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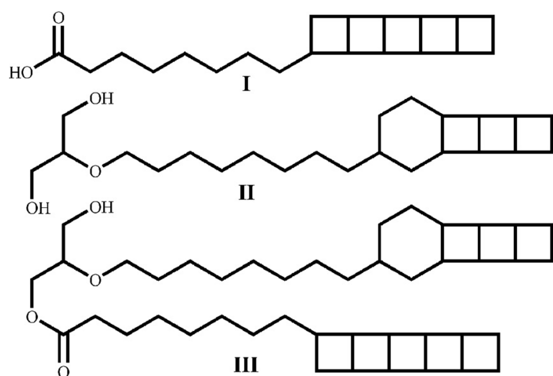

FIG 3 Structures of three characteristic ladderane lipids. I, ladderane fatty acid containing ring system Y; II, ladderane monoalkyl glycerol ether containing ring system X; III, ladderane glycerol ether/ester containing both ring systems X and Y. Lipids containing ladderane moieties X and Y are abundant membrane lipids in anammox bacteria. (Adapted from reference 35 with kind permission from Springer Science+Business Media.)

used as electron acceptors (89, 96). Additionally, a shortage of ammonium can be overcome by the dissimilatory reduction of nitrate to ammonium (DNRA) by "*Candidatus* Kuenenia stuttgartiensis" and "*Candidatus* Scalindua" spp. using formate as the electron donor (39, 96).

Anammox enrichment cultures contain about 80 to 95% anammox bacteria and are grown either as aggregates or as single cells in bioreactors, with very effective biomass retention (37, 87, 95). Their extremely long generation time is one of the reasons why they cannot be grown with standard microbial cultivation methods: they divide only once every 1 week (single cells) or 2 weeks (aggregated cells) under optimal conditions. So far, it has not been possible to grow anammox bacteria in pure culture. They can, however, be physically purified from an enrichment culture by using density gradient centrifugation (86) and subsequently used in further experiments. For electron microscopic studies, such isolation is not necessary because these bacteria are easily recognized by their unique structures by electron microscopy.

In addition to the cell plan, the anammox membrane lipids are also atypical. Anammox lipids contain a combination of ester-linked (typical of the *Bacteria* and *Eukarya*) and ether-linked (typical of the *Archaea*) fatty acids. Lipids are taxonomic markers that determine the membrane structure, and lipid membranes are essential to enable the existence of concentration gradients of ions and metabolites. Anammox bacteria contain unique membrane lipids named ladderanes (50, 77, 82, 83). Ladderanes contain one or both of two different ring systems, ring systems X and Y (Fig. 3). Ring system X contains three cyclobutane moieties and one cyclohexane moiety substituted with an octyl chain, which is ether bound at its ultimate carbon atom to the glycerol unit. Ring system Y contains five linearly concatenated cyclobutane rings substituted with a heptyl chain, which contains a methyl ester moiety at its ultimate carbon atom. All rings in ring systems X and Y are fused by *cis*-ring junctions, resulting in a staircase-like arrangement of the fused rings, defined as ladderane. Lipids containing ladderane moieties X and Y represent 34% of the total lipids in "*Candidatus* Brocadia anammoxidans" (83). The structure of the ladderane membrane lipids is unique in nature and has so far been found only in anammox bacteria. These ladderane lipids are major membrane lipids of anammox membranes and are hypothesized to make these membranes highly impermeable (82, 83) to prevent the excess loss of ions and metabolites and to provide structural integrity to the cell. This was also verified *in situ* with mixtures containing purified ladderane phospholipids that constituted both monolayers and bilayers (10). These lipid systems were shown to have a high lipid-packing density and a relatively rigid nature but also conveyed fluid-like behavior.

The ladderane phospholipid and core lipid contents of four genera of anammox bacteria were studied: "*Candidatus* Anammoxoglobus propionicus," "*Candidatus* Brocadia fulgida," "*Candidatus* Scalindua" spp., and "*Candidatus* Kuenenia stuttgartiensis" (9, 70). Each species of anammox bacteria contained C_{18} and C_{20} ladderane fatty acids with either three or five linearly condensed cyclobutane rings and a ladderane monoether containing a C_{20} alkyl moiety with three cyclobutane rings. Additionally, two new C_{22} ladderane fatty acid lipids were identified in "*Candidatus* Anammoxoglobus propionicus." In contrast to the ladderane core lipids, large variations in the distribution of ladderane phospholipids were observed, i.e., different combinations of hydrophobic tail types attached to the glycerol backbone sn-1 position, in combination with different types of polar headgroups (phosphocholine, phosphoethanolamine, or phosphoglycerol) attached to the sn-3 position (9, 70). Also, all four investigated species contained a C_{27} hopanoid ketone and bacteriohopanetetrol, indicating that hopanoids are synthesized by anammox bacteria (70). Bacteriohopanoids were later suggested to play a role in maintaining the optimal equilibrium between membrane fluidity and rigidity in anammox cells (11).

How the anammox ladderane lipids are synthesized is largely unknown. Although part of the ladderane fatty acids could be produced through the known pathway of type II fatty acid biosynthesis, it is unlikely that the cyclobutane rings and the cyclohexane ring are formed via this pathway (68). It was previously hypothesized that ladderane lipids are formed from the cyclization of polyunsaturated fatty acids (82). Later, a comparative genomic study (69) indicated two putative pathways for ladderane biosynthesis. In the first one, the ladderane lipids would indeed be produced by using polyunsaturated fatty acids as precursors, and subsequently, the oxidative cyclization or radical cascading (by *S*-adenosylmethionine [SAM] radical/B_{12} enzyme and SAM methyl transferase) of polyunsaturated fatty acids would produce ladderane lipids. For the alternative pathway, it was proposed that the extended lipid biosynthesis gene clusters detected in the anammox bacterium "*Candidatus* Kuenenia stuttgartiensis" may encode a presently unknown pathway for ladderane biosynthesis that feeds the ladderane moieties into fatty acid biosynthesis.

As we have seen already, the anammox bacteria deviate on a number of points from the textbook description of *Bacteria*. Here we review what is known about the compartmentalized cell plan of anammox bacteria: the anammoxosome, riboplasm, paryphoplasm, and anammox cell wall. Furthermore, we briefly discuss what is known about anammox cell division so far. Overall, the focus is on the anammoxosome and anammox cell wall. Theories and experimental evidence concerning the functional significance of compartmentalization in these anammox bacteria suggest that the anammoxosome is a compartment dedicated to energy metabolism by generating a proton motive force for the synthesis of ATP. Although there is some debate about the organization of the anammox cell envelope, it does not seem to resemble that of either Gram-negative or Gram-positive bacteria, being proposed to be

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proteinaceous and to lack both an outer membrane and peptidoglycan. However, the "*Candidatus* Kuenenia stuttgartiensis" genome (89) revealed several characteristics that do suggest the presence of a Gram-negative-like cell wall with an outer membrane and periplasmic space.

THE ANAMMOXOSOME

Functional Significance

Invagination of the anammoxosome membrane. The anammoxosome membrane is present in anammox bacteria in a curved configuration, in some cases with deep tubular protrusions of the membrane into the interior of the anammoxosome (99). There are two main advantages of a curved membrane (61). Proteins can bind selectively on the curvature and thus create a microenvironment on the membrane, leading to the preferential localization of ion channels in protrusions. As well as creating a microenvironment, a curved membrane increases the membrane surface and subsequently maximizes the amount of membrane available for use by membrane-bound metabolic processes. The latter point is especially true for the mitochondrial cristae (for a review, see reference 58) and is also extremely interesting in the case of the anammoxosome. The anammoxosome is hypothesized to be the site where all catabolic processes of anammox metabolism take place, most likely inside the membrane (see below). The anammoxosome would thus have a function similar to that of the mitochondrion. In this hypothesis, the anammoxosome membrane is energized by the translocation of protons to the anammoxosome, and a proton motive force is created, which drives ATP synthesis. Therefore, it is highly possible that the anammox bacteria actively enlarge the area of the anammoxosome membrane, by a curvature of the membrane, to enhance their metabolic activity (i.e., rate). How the anammoxosome membrane is folded is unclear. If an active folding mechanism is absent, the anammoxosome must be hypotonic, with the osmotic pressure folding its membrane inward to reach osmotic equilibrium. However, there are different ways to actively bend a membrane (61): changes in lipid composition, the influence of integral membrane proteins and cytoskeletal proteins, and microtubule motor activity. Filamentous tubule-like structures that might perform motor activity were observed (see Fig. 6A), but a relationship with membrane curvature was not apparent.

Energy metabolism. The function of the anammoxosome has been hypothesized to be the production of energy, analogous to the function of mitochondria in eukaryotes (56, 101). This hypothesis was initially based upon the immunogold localization of a hydroxylamine oxidoreductase (HAO)-like enzyme to the anammoxosomes of both "*Candidatus* Brocadia anammoxidans" and "*Candidatus* Kuenenia stuttgartiensis" (see Fig. 5A) (36, 56), indicating that anammox catabolism takes place there. The genome of "*Candidatus* Kuenenia stuttgartiensis" encodes 10 HAO-like octaheme proteins, 6 of which are highly expressed in the proteome and transcriptome (40). A biochemical model (Fig. 4) has been proposed (89) and recently validated (40), where the anaerobic oxidation of ammonium is catalyzed by several cytochrome *c* proteins. By using both complete transcriptome and proteome data, it was shown that "*Candidatus* Kuenenia stuttgartiensis" expressed a cd1 NirS nitrite reductase with the ability to reduce nitrite to NO. The production and functional role of NO were investigated by inhibitor studies, which indicated that NO is an

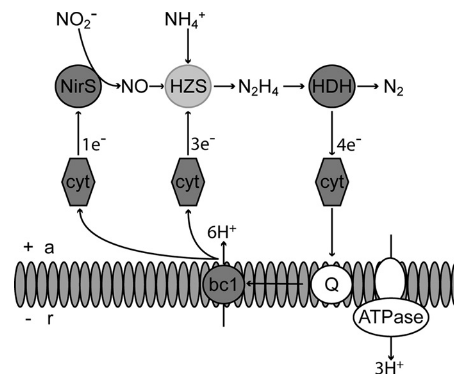

FIG 4 Postulated anaerobic ammonium oxidation coupled to the anammoxosome membrane in anammox bacteria resulting in a proton motive force and subsequent ATP synthesis via membrane-bound ATPases. Heme-containing enzymes are shown in gray. bc1, cytochrome bc_1 complex; cyt, cytochrome; HDH, hydrazine dehydrogenase; HZS, hydrazine synthase; NirS, nitrite reductase; Q, coenzyme Q; a, anammoxosome compartment; r, riboplasm compartment. (Adapted from reference 100 with permission of the publisher. Copyright 2010 Blackwell Publishing Ltd.)

essential intermediate in anammox metabolism (40). The second step in the proposed model was the combination of NO with ammonium to form the highly reactive and volatile hydrazine intermediate. In a set of dedicated stable isotope experiments, Kartal et al. (40) were able to show that hydrazine is indeed turned over *in vivo*. Although no enzyme complex is known to convert NO and ammonium into hydrazine, there was a highly transcribed and expressed gene cluster (kuste2859 to kuste2861) identified in "*Candidatus* Kuenenia stuttgartiensis." Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and two-dimensional gel electrophoresis showed that this complex was visible as three very dominant spots. This protein complex was then purified to homogeneity as a multimer of about 240 kDa. In a coupled assay together with a very active hydrazine dehydrogenase (HDH), the production of $^{29}N_2$ could be established by using ^{15}N-ammonium and ^{14}NO as substrates. The protein complex encoded by the gene cluster kuste2859 to kuste2861 was tentatively named hydrazine synthase. This N_2H_4 synthase and the NO reductase of canonical denitrifying microbes are so far the only two enzymes known to be able to forge the bonding of two nitrogen atoms. It is very intriguing that most, if not all, of the nitrogen gas in Earth's atmosphere is made by the oxidizing power of nitric oxide. This may reflect the hypothesis that NO was one of the first deep redox sinks on our planet (19, 25). In anammox metabolism, hydrazine is finally oxidized to dinitrogen gas by a set of HDHs (i.e., kustc0694, one of the HAO-like proteins). The four electrons derived from this oxidation are transferred to soluble cytochrome *c* electron carriers (12, 30), ubiquinone, the cytochrome bc_1 complex (complex III), soluble cytochrome *c* electron carriers, and, finally, nitrite reductase and hydrazine synthase. As the hydrazine dehydrogenase is inhibited by an accumulation of hydroxylamine (and NO), one of the other HAO-like proteins (kustc1061) has been proposed to function as a safety valve, converting any hydroxylamine into NO, which can be fed directly into the hydrazine synthase.

In the model shown in Fig. 4, the anammox reaction establishes a proton gradient by the translocation of protons from the riboplasm to the anammoxosome. This results in an electrochemical proton gradient directed from the anammoxosome to the

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riboplasm, with the riboplasm being alkaline and negatively charged compared to the anammoxosome. This proton motive force has a drawing force on the protons from inside to outside the anammoxosome, which can be used to drive the synthesis of ATP catalyzed by membrane-bound ATPases located in the anammoxosome membrane. Protons would flow passively back into the riboplasm (with the electrochemical proton gradient, downhill) through proton pores formed by the ATPases. The anammoxosome membrane-bound ATPases would be located with their hydrophilic ATP-synthesizing domain in the riboplasm and their hydrophobic proton-translocating domain in the anammoxosome membrane. The synthesized ATP would then be released into the riboplasm.

There is further experimental evidence that supports the proposed model and function of the anammoxosome. The presence of ATPases on the anammoxosome membrane was verified by immunogold localization (100). The genome of "*Candidatus* Kuenenia stuttgartiensis" encodes four putative ATPase gene clusters: one typical F-ATPase (F-ATPase-1), two atypical F-ATPases (F-ATPase-2 and -3) lacking the delta subunit, and a prokaryotic V-ATPase (V-ATPase-4). Transcriptomic, proteomic, and immunoblot analyses with antisera directed at the catalytic subunits indicated that F-ATPase-1 was the most significant membrane-bound ATPase under the growth conditions investigated. Immunogold localization showed that this F-ATPase was present predominantly on the innermost (anammoxosome) membrane and outermost membrane of the anammox cell (Fig. 5C and D). This is consistent with preliminary results using ^{31}P nuclear magnetic resonance (NMR) that indicated the presence of two pH peaks and thus, most likely, two energized membranes (94). However, genes encoding a putative nucleotide transporter could not be found in the genome of "*Candidatus* Kuenenia stuttgartiensis."

Experimental evidence that further supports the proposed model that the anammoxosome compartment is dedicated to the generation of energy is the demonstration that all, or almost all, cytochrome *c* proteins are located in the anammoxosome of "*Candidatus* Kuenenia stuttgartiensis," as determined by cytochrome peroxidase staining (97). Staining was observed only inside the anammoxosome and was most intense in a 150-nm rim along the inside of the anammoxosome membrane and in membrane curvatures (Fig. 5B). This suggests that the anammox enzymes are indeed either attached to or associated with the anammoxosome membrane and that they reside on the anammoxosome side of this membrane, as proposed. The specific locations of enzymes in places where the membrane was curved further strengthen the idea of an energy-generating organelle, where the membrane is folded to enhance catabolic activity. In bacteria, cytochromes with heme *c* (such as HAO and nitrite reductase) have so far been found only in the periplasmic space (for a review, see references 3 and 4). The absence of peroxidase staining in the paryphoplasm compartment is consistent with the idea that this compartment is a cytoplasmic, and not a periplasmic, compartment.

Limitation of diffusion. Anammox bacteria depend on an electrochemical ion gradient across a membrane for sufficient ATP synthesis. Because anammox catabolism is slow, only a few protons are translocated per unit of time, whereas the dissipation of the resulting electrochemical gradient by passive diffusion is independent of the growth rate and proceeds at a normal speed. Therefore, the passive diffusion of protons across a biological membrane is relatively more important and leads to a higher energy loss in the case of anammox. For comparison, in mitochondria, the energy loss due to passive diffusion of protons is already 10% (28). Therefore, it appears that a special, less permeable membrane could be essential for anammox cells. Furthermore, anammox intermediates such as hydrazine readily diffuse through biomembranes. For this reason, the limitation of the diffusion of both anammox intermediates and protons is extremely important for these bacteria. Since anammox catabolism takes place inside the anammoxosome, the anammoxosome membrane might be dedicated to the limitation of diffusion by means of the dense and rigid ladderane lipids (which have a lower degree of rotational freedom) as a specific adaptation to their unusual metabolism. The higher density of the anammoxosome membrane has been confirmed by permeability tests with fluorophores and the molecular modeling of a lipid bilayer composed of one type of ladderane lipid (83). The density of the ladderane part of this model membrane was calculated to be significantly higher (up to 1.5 kg/liter) than that of a conventional membrane (at most 1.0 kg/liter). The packing of the model membrane is probably still suboptimal compared to that of an *in vivo* ladderane membrane, which is much more complex, with many different lipids.

The presence of ladderane lipids in the anammoxosome membrane has been demonstrated by the enrichment of intact anammoxosomes from "*Candidatus* Brocadia anammoxidans" cells (83). Lipid analysis showed a strong enrichment in ladderane lipids in this enriched anammoxosome fraction: 53% of total lipids (compared to 34% in the intact cell fraction). With such a dense membrane, the anammoxosome would need specific transporters to regulate the transport of ammonium and nitrite. The "*Candidatus* Kuenenia stuttgartiensis" genome (89) encodes four ammonium transporters (Amt), four formate/nitrite transporters (FocA), and two nitrate/nitrite transporters (NarK), whose locations are the subjects of further research.

Iron-containing particles and tubule-like structures. Next to the curved membrane, the anammoxosome was observed to contain two conspicuous structures: electron-dense particles and tubule-like structures. The electron-dense particles had diameters of 16 to 25 nm and varied in number from 1 to as many as 20 per anammoxosome (Fig. 6B and C) (99). Transmission electron microscopy (TEM)–energy-dispersive X-ray (EDX) analysis showed that the electron-dense particles contained iron and might thus represent bacterioferritins. The genome of "*Candidatus* Kuenenia stuttgartiensis" (89) was found to contain two genes, kuste3640 and kuste4480, with 32 to 48% sequence identity to the *Escherichia coli* bacterioferritin protein Bfr. The heme-containing bacterioferritins, like the heme-free ferritins, are iron storage proteins. They can solubilize and store iron(III) in the form of an inorganic mineral (1). However, the physiological role of bacterioferritin is not entirely clear (2). In aerobic organisms, bacterioferritins might detoxify as well as store iron at the same time, which is especially important for these organisms. In the presence of oxygen, iron(II) is both scarce and toxic; the predominant form of iron is the insoluble iron(III), while iron(II) reacts with oxygen, generating toxic free radicals in the "Fenton" reaction. In anaerobes, detoxification is presumably less important, and the function of bacterioferritin is less well understood. Whether the anammox iron particles are indeed bacterioferritins remains to be investigated. Whatever their identity, several putative functions exist: they could function in iron respiration (89), be a response to iron scar-

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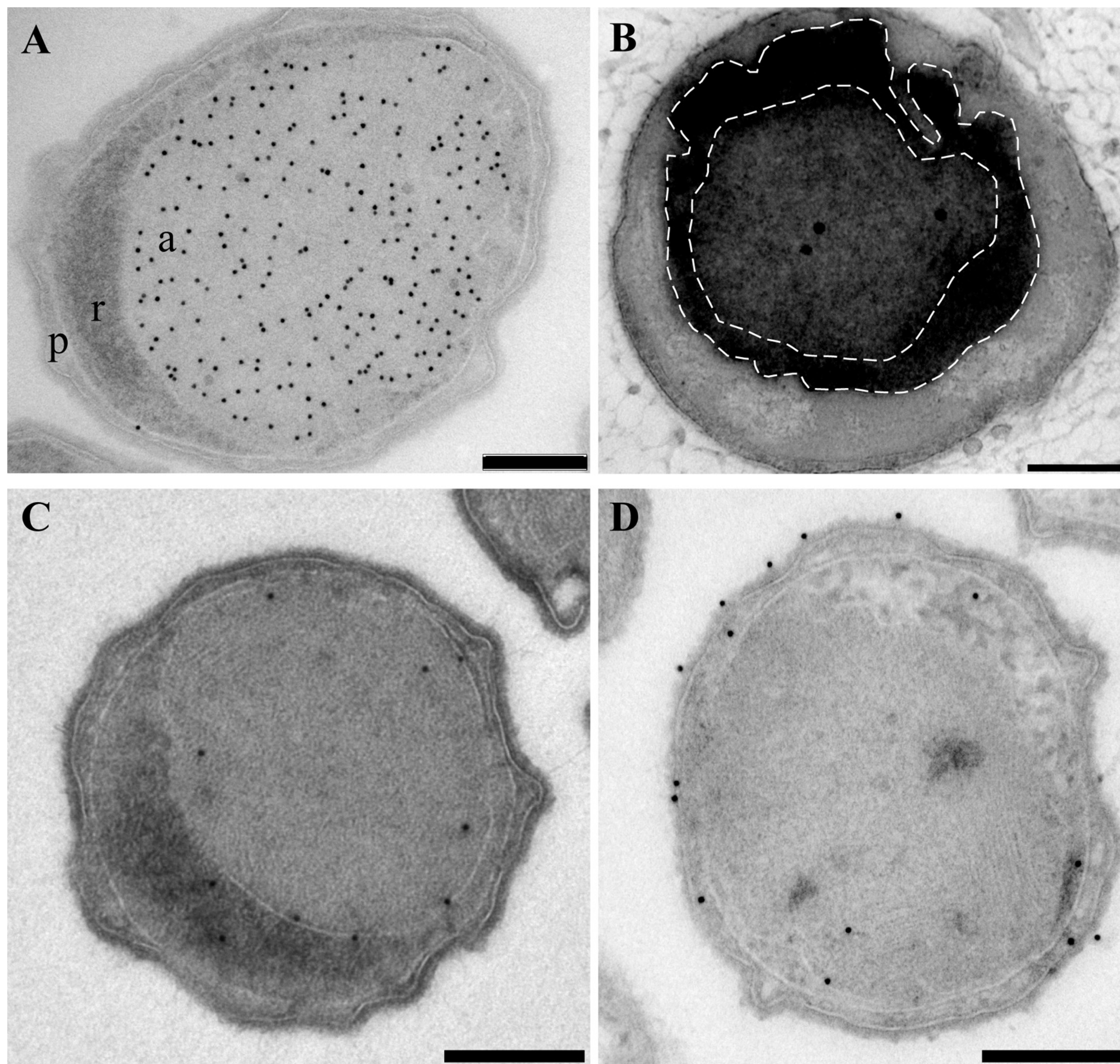

FIG 5 Experimental evidence for the anammoxosome compartment of anammox bacteria being a dedicated energy generator. (A) Immunogold localization of an anammox hydroxylamine oxidoreductase-like protein shows its location in the anammoxosome compartment in rehydrated cryosections of "*Candidatus* Kuenenia stuttgartiensis." a, anammoxosome; r, riboplasm; p, paryphoplasm. Scale bar, 200 nm. (B) Cytochrome peroxidase staining localizes cytochrome *c* proteins to the anammoxosome in chemically fixed and Epon-embedded thin sections of "*Candidatus* Kuenenia stuttgartiensis." Intense staining occurs in close proximity to the anammoxosome membrane, as outlined by the dashed lines. Scale bar, 200 nm. (Adapted from reference 97.) (C and D) Immunogold localization of the catalytic beta subunit of the F-ATPase-1 gene cluster localizes this ATPase to the outermost membrane and the anammoxosome membrane in rehydrated cryosections of "*Candidatus* Kuenenia stuttgartiensis." Scale bar, 250 nm. (Adapted from reference 100 with permission of the publisher. Copyright 2010 Blackwell Publishing Ltd.)

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city (although the anammox bacteria are not grown under iron-limiting conditions), function as a storage facility for the numerous heme-containing enzymes that are involved in the electron transport chain, or be iron-rich proteins themselves.

In addition to the electron-dense particles, tubule-like structures inside the anammoxosome have been observed (56, 101) (Fig. 6A). These structures seem to be hexagonal in shape when transsectioned and constructed of three identical units. Each separate unit, i.e., the electron-dense parts of the hexagonal-shaped structures, has a width of 9.4 nm on average, and together, these units form long tubule-like structures, which are at times arranged in packed arrays that can stretch the full length of the anammoxosome. It has been hypothesized that these tubule-like structures might have a cytoskeletal function (in anammoxosome division or membrane curvature) or be a highly ordered protein themselves. The many trimeric heme complexes may be putative candidates for these structures.

THE RIBOPLASM

The riboplasm of anammox bacteria mostly resembles the standard cytoplasmic compartment of other bacteria. The riboplasm contains the ribosomes and the nucleoid; it is the site where the transcription and translation processes are assumed to take place. However, how anammox bacteria are capable of specifically transporting proteins from the riboplasm to either the paryphoplasm or anammoxosome remains unclear. No specific signal peptides

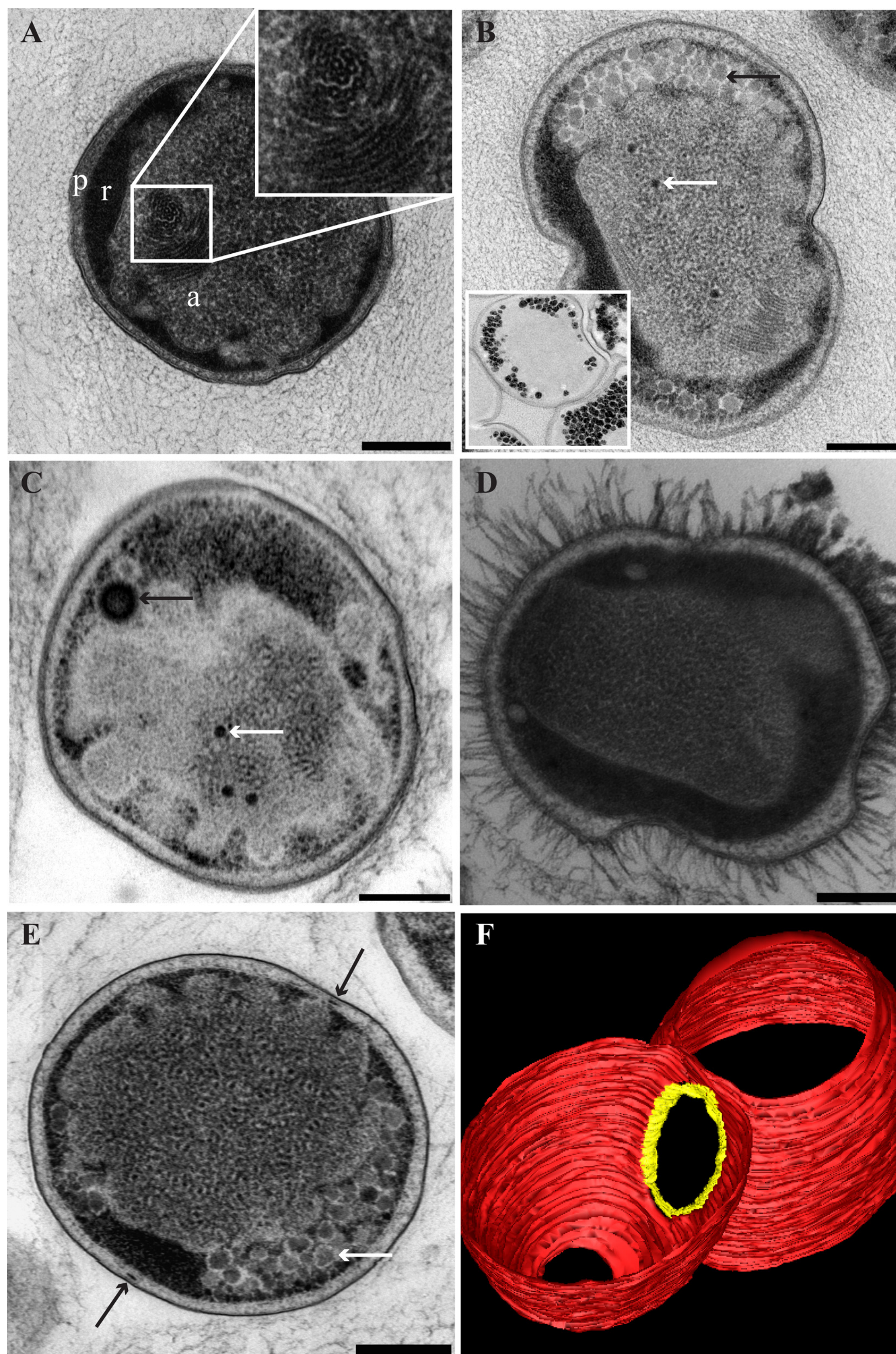

FIG 6 Transmission electron micrographs and electron tomography model of different high-pressure-frozen, freeze-substituted, and Epon-embedded anammox bacteria. (A) "*Candidatus* Kuenenia stuttgartiensis" cell showing tubule-like structures (inset) inside the anammoxosome. a, anammoxosome; r, riboplasm; p, paryphoplasm. (Adapted from reference 98.) (B) "*Candidatus* Anammoxoglobus propionicus" cell showing glycogen storage (black arrow) and iron-containing anammoxosome particles (white arrow). The inset shows a glycogen-stained "*Candidatus* Kuenenia stuttgartiensis" cell. (Adapted from reference 97.) (C) "*Candidatus* Brocadia fulgida" cell showing riboplasmic particles (black arrow) and iron-containing anammoxosome particles (white arrow). (Adapted from reference 97.) (D) "*Candidatus* Scalindua" sp. cell showing pilus-like cell appendages. (E) "*Candidatus* Kuenenia stuttgartiensis" cell showing the onset of cell division and the appearance of the cell division ring in the paryphoplasm (black arrows). The white arrow shows glycogen storage particles. (Adapted from reference 98.) (F) Snapshot of an electron tomography model showing the cell wall (in red) and the cell division ring (in yellow) of a "*Candidatus* Brocadia fulgida" cell. Scale bars, 200 nm. (Adapted from reference 98.)

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could be detected for different compartments, and it has been hypothesized that protein sorting might be achieved through both the secretory (Sec) pathway (both the paryphoplasm and anammoxosome) and the twin-arginine translocation (Tat) system (anammoxosome) with additional chaperones to achieve specificity and facilitate separate translocation routes (62).

In addition, anammox bacteria were found to store glycogen in their riboplasmic compartment (Fig. 6B and E) (97). The exact role of glycogen in bacteria is not entirely clear. There are indications that glycogen functions as an energy and carbon storage compound, providing energy and carbon for cell survival under conditions of stress or starvation (31); that glycogen storage is linked to excess carbon and/or the lack of a required nutrient (especially nitrogen) in the medium; or that it is used for the formation of a biofilm (6).

Two anammox bacteria, "*Candidatus* Brocadia fulgida" and "*Candidatus* Anammoxoglobus propionicus," were also shown to contain additional, larger, particles in the riboplasm (Fig. 6C) that most resembled polyhydroxyalkanoates (PHAs) or polyphosphate storage (97). However, the identity and function of these larger particles remain to be investigated.

THE CELL WALL AND PARYPHOPLASM

There are many questions remaining concerning the anammox cell plan, especially whether the outermost anammox compartment (the paryphoplasm) is a cytoplasmic compartment or a periplasmic space. In analogy to the other *Planctomycetes*, the anammox cell envelope has been defined as having a proteinaceous cell wall without peptidoglycan and an outer membrane. In the *Planctomycetes*, the cytoplasmic membrane has been defined as such based on the finding of RNA in the outermost, paryphoplasm compartment (55, 56). However, no biochemical analysis has been performed on the anammox cell envelope, and although RNase-gold labeling indicated the presence of RNA in the paryphoplasm compartment, no conclusions can be drawn from this, considering the narrow anammox paryphoplasm region and the length of the antibody complex (R. Webb, personal communication). However, electron microscopic observations suggested that the anammox cell envelope organization is the same as that of the other *Planctomycetes*.

To explore potential similarities to a Gram-negative cell plan, the genome of "*Candidatus* Kuenenia stuttgartiensis" (89) was examined by comparative genomic analysis, which indicated that "*Candidatus* Kuenenia stuttgartiensis" may be genetically capable of the biogenesis of a periplasm and outer membrane. First, a number of open reading frames (ORFs) were homologous to outer membrane porins. These porin homologues were absent in the genome of the planctomycete *Rhodopirellula baltica*. Second, the "*Candidatus* Kuenenia stuttgartiensis" genome encodes the complete TonB system, a protein complex that relays energy from the cytoplasmic membrane to the outer membrane to drive a number of outer membrane receptors, five of which were also encoded in the genome. Third, "*Candidatus* Kuenenia stuttgartiensis" encoded a number of typical three-component Gram-negative multidrug exporters, which consist of a cytoplasmic membrane, a periplasmic subunit, and an outer membrane subunit ("gated porins"). Fourth, a partial peptidoglycan biogenesis pathway was encoded, including a number of penicillin-binding proteins. The only step not present in the peptidoglycan pathway of this anammox bacterium was the ability to cross-link the glycan backbone. With respect to all these four points, *R. baltica*, another planctomycete with a publicly available genome, contains hardly any genetic potential for a Gram-negative cell wall structure or peptidoglycan synthesis. This may indicate that the paryphoplasm in "*Candidatus* Kuenenia stuttgartiensis" may actually be more similar to a "regular" periplasm.

In contrast to the genomic evidence that could support the paryphoplasm being a periplasm-like space, there is experimental evidence that supports the paryphoplasm being a cytoplasmic compartment with the cytoplasmic membrane on its outer side and the absence of a typical bacterial cell wall. First, neither peptidoglycan nor a typical outer membrane can be observed on transmission electron micrographs of all known species of anammox bacteria when examined after cryofixation and freeze-substitution or via classical chemical fixation (97, 99). Second, anammox bacteria and other non-anammox *Planctomycetes* are prone to osmotic collapse under both hypotonic and hypertonic conditions (see references 54, 56, and 90), an indication that their structural integrity, normally derived from the presence of a cell wall, is not optimal. Third, the cell division ring of anammox bacteria is situated in the paryphoplasm compartment (98). In general, the bacterial cell division ring is inside, and closely opposed to, the cytoplasmic membrane, indicating that the membrane outside the paryphoplasm is the cytoplasmic membrane. Fourth, the apparent absence of cytochrome *c* proteins in the paryphoplasm, as indicated by cytochrome peroxidase staining (97), supports the notion that this cannot be a typical periplasmic space analogous to that of Gram-negative bacteria. Fifth, immunogold localization showed the F-ATPase to be present on both the anammoxosome and the outermost membrane of the anammox cell. This indicates that the outermost anammox membrane is an energized, cytoplasmic membrane, as was initially proposed (56), and not a relatively permeable outer membrane typical of Gram-negative bacteria.

The genetic potential found in the "*Candidatus* Kuenenia stuttgartiensis" genome for producing a Gram-negative-like cell wall could be a cryptic result of lateral gene transfer or a remainder of the evolutionary ancestor of anammox bacteria, which would then be a Gram-negative bacterium. Anammox bacteria certainly do not live in osmotically protected areas, like the pathogenic cell wall-less *Mycoplasma* species, and therefore are in need of some form of structural integrity. Other *Planctomycetes* possess proteinaceous cell walls, and their cells show considerable structural integrity and in some cases are even able to withstand treatment with 10% SDS at 100°C (47, 53), conditions under which Gram-negative cell walls and especially outer membranes would be expected to disintegrate. Perhaps, in the case of anammox, the ladderane lipids provide the structural integrity that most other bacteria derive from their cell wall. These lipids have been found predominantly in the anammoxosome membrane but also occur in one or both of the other two anammox cell membranes (83). Alternatively, we may in fact lack some structural information, and there may be yet another layer to the anammox cell. There is indeed some evidence of a regular protein surface layer (S-layer) lattice in "*Candidatus* Kuenenia stuttgartiensis" from freeze-fracture replicas (24). In archaea, which often do not contain other cell wall components besides S-layers, S-layers have been proposed to maintain cell shape and can be viewed as exoskeletons that contribute to mechanical and osmotic cell stabilization (20). Perhaps, in the anammox bacteria where, as in the *Archaea*, no other cell

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wall components have been found, structural integrity is also derived from an S-layer lattice. We are currently investigating the composition of the anammox cell wall and the presence of an outer membrane, an exoskeleton (S-layer), or an endoskeleton (cytoskeleton).

Finally, some "*Candidatus* Scalindua" species were observed to contain pilus-like appendages (Fig. 6D). Whether the other anammox genera can also produce pilus-like cell appendages is unknown, but cellular appendages were never observed.

CELL DIVISION

Anammox bacteria divide by constrictive binary fission (98), as opposed to the other known *Planctomycetes*, which reproduce by budding (22). The doubling time of anammox bacteria is on the order of weeks (87) rather than the minutes for model organisms such as *Escherichia coli*. The first sign of cell division is the appearance of a division ring in the outermost compartment (Fig. 6E and F), the paryphoplasm, followed by a slight invagination of the cell wall. The cell then doubles in size by the elongation of the two poles, during which the anammoxosome also becomes elongated and slightly invaginated. After elongation, the constriction continues until the cells are almost entirely pinched off. In this way, the anammoxosome is divided equally among the daughter cells.

In the search for possible candidates for genes encoding division ring proteins, the "*Candidatus* Kuenenia stuttgartiensis" genome (89) was investigated for the 10 known essential divisome genes. The divisome is a multiprotein complex with FtsZ (Fts, filamentous temperature sensitive) as the key player (13). Driven by GTP hydrolysis, FtsZ assembles into a ringlike structure at the midcell (5, 16, 57, 63, 71), recruits at least 9 other essential proteins (26, 103), and constricts to separate the two daughter cells. Interestingly, members of the *Planctomycetes* and *Chlamydiae* are the only phyla among bacteria with no obvious homologue for the otherwise ubiquitous cell division gene *ftsZ* (59, 104).

In the "*Candidatus* Kuenenia stuttgartiensis" genome, the so-called FtsA-independent divisomal complex (27, 102) (*ftsK*, *ftsQ*, *ftsB*, *ftsL*, *ftsW*, and *ftsI*) was complete. The *ftsL*, *ftsI*, *ftsW*, and *ftsQ* genes were part of a putative division cell wall (*dcw*) operon (see also reference 67), which in *Escherichia coli* and most other bacteria harbors genes involved in cell division and peptidoglycan precursor biosynthesis. The FtsA-independent divisomal complex is believed to assemble independently from the Z-ring complex and to be recruited to the midcell once the Z-ring complex is established (27, 102). Clear homologues of genes encoding the Z-ring complex (*ftsZ*, *ftsA*, and *zipA*) and *ftsN* were not found in the "*Candidatus* Kuenenia stuttgartiensis" genome. Despite the absence of *ftsZ* in the "*Candidatus* Kuenenia stuttgartiensis" genome, a division ring is present during anammox cell division. The genome was therefore searched, and based on the presence of an ATP/GTP-binding site (P loop) and associated synergy loops (also called T7 loops, involved in GTPase activity), a possible novel cell division ring gene that was unrelated to *ftsZ* was identified. This gene (kustd1438) codes for a 3,690-amino-acid (aa)-long protein that contains a 22-aa-long signal peptide, which is consistent with the location of the division ring in the paryphoplasm. However, on the level of primary structure, kustd1438 and FtsZ are not homologous. Immunogold localization using an antibody raised against the encoded kustd1438 protein showed that it was indeed part of the division ring and, on the basis of sequence analysis, might actively contribute to ring constriction or assembly via GTP hydrolysis. Genomic analyses of other *Planctomycetes* and *Chlamydiae* revealed no putative functional homologues of the newly identified gene, suggesting that it is specific to anammox bacteria.

CONCLUSIONS AND OUTLOOK

There is still much to learn about the metabolism and cell biology of anammox bacteria. Future studies will continue to focus on the function of the anammoxosome, on the identification and location of metabolic proteins, and on the generation of a proton motive force and subsequent ATP synthesis. The key lies in the isolation of large quantities of free anammoxosomes from the cells. Furthermore, the nature and function of the paryphoplasm also require further investigation. Finally, the anammox cell wall needs extensive (biochemical) analysis to decipher whether its composition resembles most that of Gram-negative bacteria or *Archaea*.

The compartmentalization in anammox bacteria is linked to metabolism and has a specific cellular function: catabolism. The hypothesis as to why this compartmentalization was accomplished is that the division of membrane tasks over two different types of membranes gives more freedom to the organism in the optimization of either membrane. The anammoxosome membrane can be used to generate and maintain a proton motive force for ATP synthesis and to keep the valuable intermediates of the anammox process inside the anammoxosome as much as possible. Thus, this membrane has to be relatively impermeable, as is accomplished by the presence of the rigid ladderane lipids. The cytoplasmic membrane can be used for homeostasis, such as the control of intracellular ion concentrations and transport processes, and thus has to be relatively flexible and permeable. By dividing these tasks, the cell can overcome the problem of requiring a single membrane to be both impermeable and permeable, and the use of an intracytoplasmic compartment for ATP synthesis via this proton motive force would result in the total control of the physical chemistry of the proton motive force and thus lead to more efficient energy transduction.

From an evolutionary perspective, the implication of bacterial organelles with specific cellular functions is food for thought. The exact place of the *Planctomycetes* in the tree of life has been a somewhat heated debate among scientists over the last few years. Using rRNA phylogeny, it was suggested that the *Planctomycetes* are an ancient lineage situated at the root of the bacterial tree (7). On the other hand, others have argued that the number of nucleotide positions used in this phylogenetic analysis is too low to support this conclusion and that the bacterial ancestor is a hyperthermophile (18). Here the *Planctomycetes* are not placed at the root but at the third branch of divergence in the domain *Bacteria*. From the sequencing of the complete genomes of some of the *Planctomycetes*, it appears that the evolutionary relationship of this phylum is indeed not straightforward. Although the genome of the planctomycete *Gemmata obscuriglobus* revealed eukaryotic signature proteins (85), the genome of "*Candidatus* Kuenenia stuttgartiensis" indicated that anammox bacteria are more related to the *Chlamydiae*, obligate intracellular parasites, than to eukaryotes and might have evolved from a Gram-negative bacterium (89). So the question remains, Are the *Planctomycetes* a relatively new phylum that evolved their compartmentalization separately from the eukaryotes (convergent evolution), or is the last universal common ancestor (LUCA) complex and/or eukaryote-like,

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and are the *Planctomycetes* the last examples to survive (23) before evolution proceeded to the less complex but perhaps more efficient prokaryotic cell types? Recently, it was argued that the unusual features of the *Planctomycetes*, *Verrucomicrobia*, and *Chlamydiae* (PVC) superphylum (104) could indeed imply the existence of continuity and intermediate steps between the domains *Bacteria*, *Archaea*, and *Eukarya* and suggest that the LUCA was complex (17, 72). This hypothesis was disputed by others who argued that comparative genome analyses have never revealed a link between the *Eukarya* and *Planctomycetes* and that the features of the PVC superphylum either are a result of convergent evolution or were acquired through lateral gene transfer (60).

Whether a relatively old or new addition to the tree of life, anammox bacteria have turned out to be of high (micro)biological interest with regard to their unusual metabolism and cell biology. Also, the significance of anammox bacteria in nature and their increasing application in wastewater treatment make these unique prokaryotes with their exceptional properties an important subject of future studies.

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