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AN INSIGHT TO ANAMMOX BACTERIAL COMMUNITIES AND THEIR DETECTION STRATEGIES- MINI REVIEW

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ABSTRACT

Anammox bacteria are the members *Planctomycete s*having unique structural and growth characteristics. The researches around the globe have acknowledged their significant role in the global and particularly in marine nitrogen cycle. However, due to slow doubling time, nutrients selectivity and sensitivity, anammox bacteria could not be grow on pure culture yet. Therefore, various methods are being applied to detect anammox bacteria and process including, enrichment culturing in bioreactors, isotopic pairing techniques, lipid analysis, 16S rRNA specific universal primers, functional gene primers and recently 454 pyrosequencing. The aim of this paper is to present the role of anammox bacteria in overall global nitrogen cycle and some unique features related to its growth and metabolism. Finally to introduce about the role of recently employed techniques used to expose the hidden mysteries about these bacteria.

KEYWORDS: Anammox bacteria, detection techniques, key players in nitrogen cycle

INTRODUCTION

1. Anammox bacteria

It was believed for a long time that ammonia can only be oxidized under aerobic conditions, in fact, anammox process is more feasible and efficient than aerobic nitrification [1]. The assumptions made by Broda (in which he proposed that oxidation of ammonium with nitrate is energetically more feasible pathway) were basically based on these thermodynamic calculations and were recently confirmed. In the year 1999 the anonymous lithotrophs, which was not identified before was detected as member of order *Planctomycetales*[2].

Anammox process contributes significantly in marine nitrogen production and in part explains the lack of ammonium from water and sediments under anoxic conditions with ammonium oxidation coupled to nitrite reduction (Fig. 1) [3]. Almost equal amount of ammonium and nitrite is consumed by anammox bacteria (usually in a 1:1 ratio) to form N_2

gas. Ammonification of nitrate or mineralization in the water columns can be the process to supply ammonium.

However, nitrite and nitrate can be available for anammox process when these intermediate products are produced by nitrification, denitrification and dissimilatory nitrate reduction processes. Reversible inhibition of anammox process can occur in the presence of oxygen, however the process can be re-activated upon eradication of oxygen. Compared to other processes included in the nitrogen cycle, anammox process has an advantage, in which, nitrogen gas is produced by the consumption of nitrite without releasing N_2O, a major gas producing greenhouse effect, which indeed can be produced by the denitrification process. Unlike nitrification where nitrate is released after aerobic nitrification of ammonia, ammonia can be anaerobically oxidized to nitrogen in the anammox process. Recently, the anammox activity has been documented from several environments, such as Arctic Sea Ice, subtropical mangrove sediments, lacustrine system, estuarine/tidal river sediments deep-sea hydrothermal vents, hot spring etc. Currently in the Netherland in several waste water treatment plants anammox process is being applied successfully instead of nitrification and denitrification processes [4].

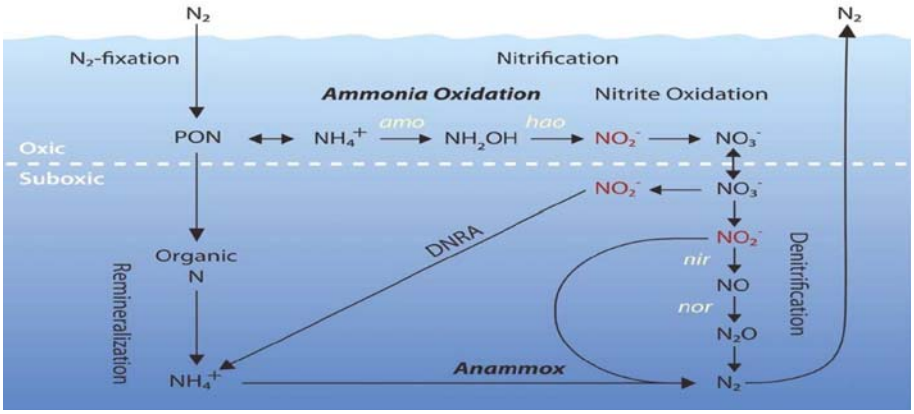

Figure 1 Nitrogen cycle in marine ecosystem**.** Adopted from Francis *et al*., 2007 [5].

In various marine and freshwater ecosystems anammox bacteria are found to be the major or only nitrogen producers [6]. In marine environment almost 40% of nitrogen is produced through microbial processes, however nearly 50% of nitrogen in the atmosphere is released globally by these microbial processes [7].

1.1 Habitat and diversity of anammox bacteria

In marine environment the nitrogen cycle is extremely significant because nitrogen act as limiting part of primary productivity, where anammox bacteria and their activities have been studied in diverse ecosystems around the globe. In the wastewater treatment plant anammox activity was noticed for the first time, whereas anammox bacteria were identified in the study conducted later. The anammox activity was detected in anoxic sediments ranging from 0-67% of the total nitrogen removal. In addition, the other ecosystems where anammox bacterial activity was reported includes marine sponges [8], sediments of estuaries and tidal river, hot spring, freshwater ecosystems and some deep-sea hydrothermal vents. It was found in several investigations that, more than 50% of the marine nitrogen production in these systems were contributed by anammox bacteria, hence the significance of anammox bacteria in the global nitrogen cycle was better understood.

In freshwater and terrestrial ecosystems the significance of anammox bacteria have also been determined including freshwater marshes, rivers, lakes and river estuaries, several soil types, such as permafrost soils, agricultural and reductised soil, peat soils and ground water. Molecular analysis reveals that terrestrial anammox diversity is quite higher than the diversity of anammox bacteria in marine ecosystems [9]. The first report of anammox bacteria in the lake ecosystem came from the Lake of Tanganyika, the world second largest lake. The ecological significance of freshwater ecosystems such as rivers, lakes, wetlands and their role as natural habitat for anammox bacteria cannot be ruled out [10].

The marine anammox bacteria i.e., *Ca.* Scalindua sorokinii was first documented from the Black Sea. Thereafter the other member of *Ca.* Scalindua lineage have also been identified from the marine ecosystems, includes Ca. Scalindua arabica in the sediments of Arabian Sea [11]. Both *Ca.* Brocadia and *Ca.* Kuenenia greatly dominate the waste water treatment system, whereas *Ca.* Scalindua are usually found predominant and ubiquitous in natural environment such as anoxic marine environments. The high diversity of anammox bacteria have been reported in tidal river estuaries and freshwater ecosystems with comparison to anoxic marine environment. From the sediments of Cape Fear River estuary with relatively high salinity gradient the anammox bacterial diversity was noticed high, hence *Ca.* Scalindua was found highly halo-tolerant in nature, whereas *Ca.* Brocadia and *Ca.* Kuenenia were found less tolerant to salinity.

1.2 Anammox bacterial phylogeny

The first anammox bacteria detected with 16S rRNA gene sequence was *Ca.* Brocadia anammoxidans, which showed its affiliation to the phylum *Planctomycetes*. Now anammox bacteria are differentiated from the rest of *Planctomycetes* and are separated as a monophyletic cluster deeply branched within *Planctomycetes*, which are composed of five genera [12] (**Fig. 2**). So far the identified genera of anammox bacteria include *Brocadia*, *Jettenia*, *Scalindua*, *Kuenenia* and *Anammoxoglobus*.Because none of the anammox bacteria could be obtained in a pure culture, so all of them have the status as *Candidatus*. Generaof *Ca.* Brocadia and *Ca.* Kuenenia are common anammox bacteria in the waste water treatment plants [13].

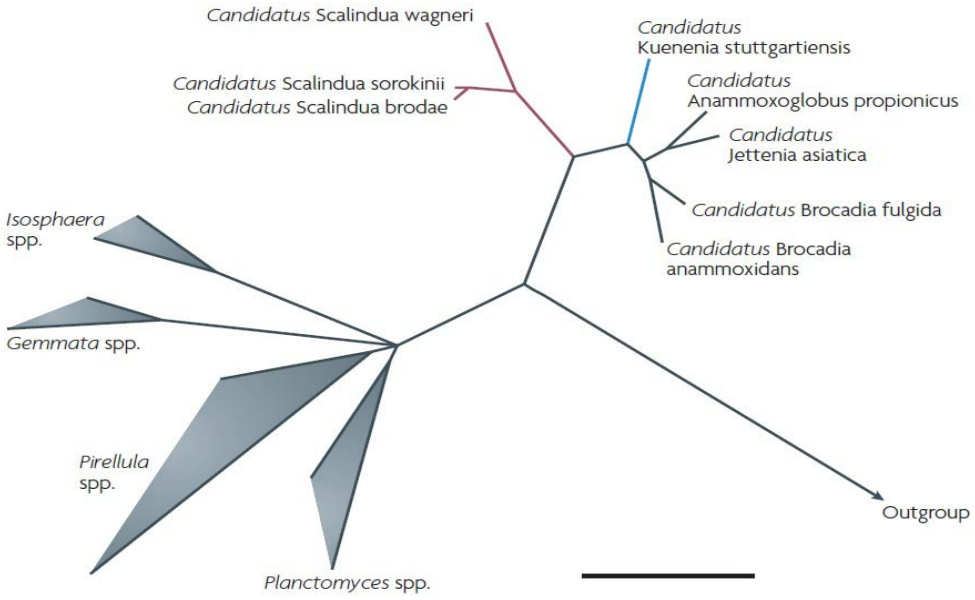

Figure 2 Based on 16S rRNA gene the phylogenetic analysis of the phylum *Planctomycetes* and five anammox families and genera of anaerobic ammonium oxidizing bacterial. On the right bottom of the tree the bar represents 10% sequence divergence and the sequence divergence from other Bacteria is high (Out group). The figure is adapted from Kuenen, 2008 [14].

Currently a novel propionate oxidizing species of anammox lineage was identified from the propionate degrading enrichment and was given the name *Ca.* Anammoxoglobus propionicus, which has a 91% similarity level of 16S rRNA gene sequence with the rest of anammox bacteria [15]. The unique internal cellular structure called as anammoxosome is the characteristic of anammox bacteria and some other members of *Planctomycetes*. Anammoxosome is oval shape portion surrounded by membrane containing ladderane lipid and anammox process is believed to occur inside it [16] (**Fig. 3**). The rigidity of

anammoxosome membrane help to resists toxicity of hydrazine and thereby, protects the cell from these toxic intermediates.

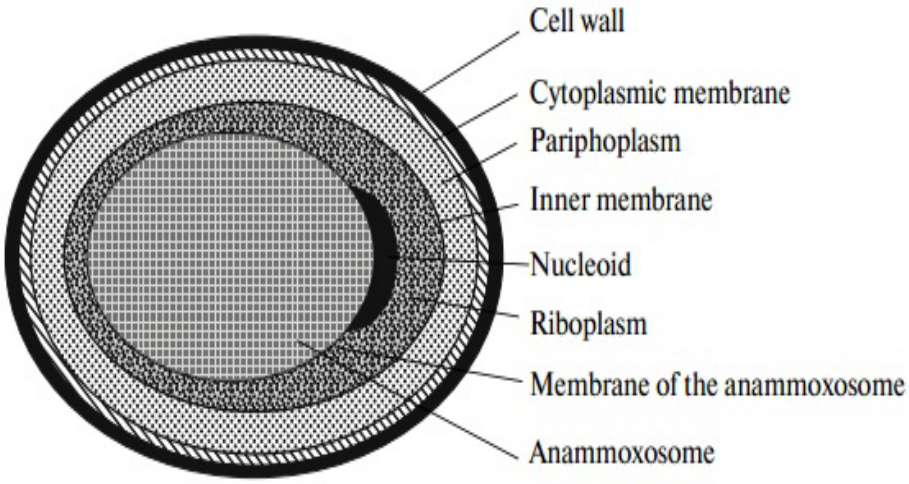

Figure 3 Diagrammatic arrangement of the anammox cell structure. The figure is adapted fromNozhevnikova *et al*., 2012[17].

Although morphological similarities of anammox bacteria were found with other members of *Planctomycetes*, they differ genetically in terms of rRNA operon organization. The 16S and 23S rRNA genes are unlinked in member of genera *Planctomycetes*, *Pirellula*, *Planctomyces* and *Gemmata*, but are linked in the genus *Isosphaera* and anammox bacteria. It has also been documented that rRNA genes of anammox bacteria are connected with an intergenic spacer regions of 450 bases [18].The high divergence in the 16S rRNA sequence (below 80%) of anammox bacteria to rest of *Planctomyces* genera proposed that branch of anammox bacteria might be a subsequent order within the *Planctomycetes*[19].

1.3 Cell growth, physiology and metabolism

The anammox bacteria are coccoid with variable sizes ranging from 800 to 1,100 nm in diameter [20]. The slow growth rate of anammox bacteria (7-11 days) might be the reason to create hurdles to grow them in pure culture from various environmental samples. The limited amount of nitrite and ammonium in the environment is believed to be responsible to slow down the metabolic activity and consequently prolong the doubling time of bacteria. The pH and temperature are the most significant physiological features for the best possible growth of anammox bacteria ranging from 6.5-9.0 and 20-45ºC respectively. It is believed

earlier that anammox bacteria are obligate anaerobes in nature. However some recent studies have validated that some species can tolerate limited amount of dissolved oxygen [21].

The typical anammox bacterial cell is differentiated into three discrete sections bounded by a specific membrane. Almost 30% volume of the anammox bacterial cell is occupied by membrane bounded anammoxosome. The anammoxosome is bounded by riboplasm-cytoplasm comprising ribosome particle along with nucleoid. The cytoplasmic membrane surrounds the final cytoplasmic section called as paryphoplasm. The cell wall lacking peptidoglycan is the last membrane found in the anammox bacterial cell [22].

The membrane of anammoxosome is composed of ladderane lipids which is a unique feature in anammox bacteria[23]. The compact structure and distinctive composition of membrane may contribute to shield the cell of anammox from the toxic intermediates such as hydrazine and hydroxylamine or expand the area to increase their activity by metabolic pathways [24].

In the process NH_4^+ is oxidized to N_2 under anaerobic conditions whereas NO_2^- is used as terminal electron acceptor[25]. Almost 90% (NH_4^+ and NO_2^-) is usually transformed into N_2, however only the remaining 10% is converted into NO_3^-[26]. Herebicarbonate is used as a source of carbon, thereby proving that anammox bacteria can consume CO_2 and act as a chemolithoautotrophs.It has been reported that when lacking nitrate production and cell material, the energy generating process can be progressed [27].

Anammox process is a complex mechanism in which nitric oxide (NO) and hydrazine (N_2H_4) is used as intermediate by anammox bacteria. During the whole anammox reaction four important enzymes i.e., hydrazine synthase, hydrazine dehydrogenase, nitrite reductase and nitrate reductase are used to catalyze the different products (**Fig. 4**) [28].

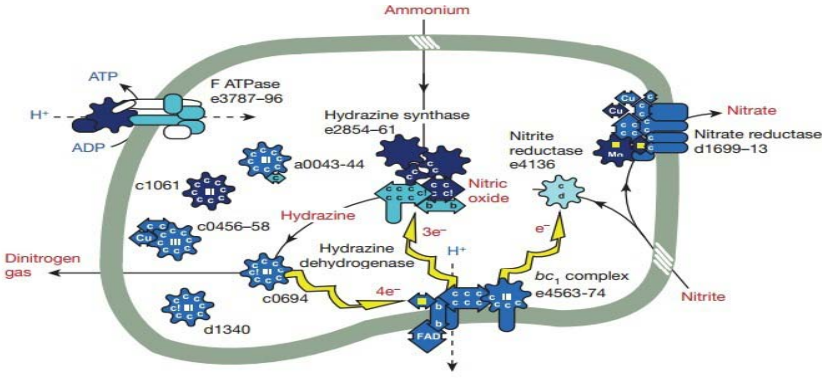

Figure 4 The anammox process presented in the diagram shows the enzymatic mechanism involved in the process, Kuenenia stuttgartiensis was the model organism. The figure is adapted from Kartal *et al*., 2011 [28].

According to the study hydrazine synthase is responsible to form N-N bond in N_2, whereas nitrite reductase enzyme reduces the nitrite into nitric oxide, which further along with ammonium is converted to energy rich and toxic intermediate hydrazine by hydrazine synthase enzyme. The hydrogen proton is removed by the hydrazine dehydrogenase enzyme to create nitrogen gas [29]. The proton released in the anammox mechanism is further added to the acetyl-CoA pathway for the fixation of bicarbonate [30]. The nitrate which is an important fraction of bicarbonate fixation in cell biomass is generated by nitrate reductase by nitrite oxidation. The hydrazine oxidoreductase, a major enzyme in this reaction resides inside the anammoxosome and combine nitrite and ammonium in equal ratio.

2.0 Detection methods for anammox bacteria

The slow growing anammox bacteriahave made it difficult to identify and further taxonomicallyassign them to confirm identity because still no success for microbiologist to get pure culture. The long incubation period/doubling time of anammox bacteria has been documented to be about 11-30 days [31]. Recently a number of enrichment culturing techniques have been used to grow anammox bacteria from different environmental samples.

2.1 Enrichment of anammox bacteria

Due to slow growth rate and lack of pure culture, several enrichment techniques are preferred for the cultivation of anammox bacteria. The most widely used sequencing batch reactor (SBR) technique is the method of choice to achieve maximum enrichment and quantitative analysis of the slow growing anammox bacterial community [31]. The 15-liter anoxic SBR provided with ammonia and nitrite mineral media in connection with the percoll density centrifugation was implementedwhich successfully get *Ca.* Brocadia anammoxidans enriched with a density of 10^{10}- 10^{11}cells/ml [32]. Moreover, several other enrichment techniques have also been usedsuch as gas lift reactors etc for the culturing of anammox bacteria. In the following,we will briefly describe some non-culturing techniques and chiefly molecular methods used for studying anammox bacterial community.

2.2 Isotope pairing technique

Currently isotope pairing technique is an ideal method after enrichment to examine the anammox activity. The incubation with stable isotope ^{15}N is the most common method in an environmental sample such as anoxic water and sediment samples for quantification of anammox activity. The stable isotope ^{15}N labeled ammonium experiment was first used to

examine the anammox activity and nitrogen loss in a bioreactor system. Thus different isotopic forms of nitrate, nitrite and ammonium are used for this purpose and ^{15}N labeled nitrate or ^{15}N labeled ammonium and ^{14}N labeled nitrite could be used to detect anammox activity. As described in previous studies, anammox bacteria consume NH_4^+ and NO_2^- in a 1:1 ratio to generate N_2 whereas denitrifiers utilize two $^{15}NO_3^-$.Thus denitrification will produce$^{30}N_2$ by the combination of two $^{15}NO_3^-$ when $^{15}NO_3^-$ and $^{14}NH_4^+$ will be incubated. At the same time $^{29}N_2$ could be obtained by the combination of each of the nitrogen source as a result of anammox reaction. Similarly, denitrification reaction can consume two $^{14}NO_3^-$ molecules which would be resulted in $^{28}N_2$ production, and when $^{14}NO_3^-$ and $^{15}NH_4^+$was used anammox reaction can result in $^{29}N_2$. The use of ^{15}N-labeled nitrate not only disclosed the anammox activity during examination but also revealed the diverse nature of anammox bacteria to contribute in DNRA [33]. Moreover, the inhibition mechanism of anammox in marine sediments was also examined by using ^{15}N tracer system [34]. The occurrence and activity of anammox bacteria can be determined by a modified version of isotope labeling technique in which $^{13}CO_2$ and/or ^{15}N labeled inorganic N species were used for stable isotope probing. In combination with molecular techniques stable isotope probing would become more effective, by which anammox community composition and relevant microbial activity can be determined in any environmental samples[35].

2.3 Lipid analysis for anammox detection

Unlike other bacteria, anammox bacteria have a unique structure called anammoxosome. The outer membrane of this intracellular structure is composed of specialized lipids. The typical impermeable nature of this ladderane lipid containing membrane is actually due to composition of cyclobutane/cyclohexane ring systems which are not found in any known non-anammox bacterial membranes. A high quantity of glycerophopholipids are present along with the ladderane lipids. In comparison to ladderane core lipid, ladderane IGPs (intact ladderane glycerophopholipids), such as C20-[3]-ladderane monoalkylether–phosphocholine can precisely indicate about the living biomass, due to which they are believed to be more specific for identifying the living anammox bacteria. Hitherto, the ladderane lipids are not only utilized to investigate the existence of anammox bacteria, but their relations to the ecological events in the environmental samples.

Currently the high performance liquid chromatography/atmospheric pressure chemical ionization-MS/MS method, a highly sensitive technique, is used for the detection of relatively little quantity of ladderane lipids even in composite mixture such as sediments, and by the use

of this technique very low quantity can be detected as little as around 35pg of ladderane lipids. Therefore, the sensitivity of this technique is far better than other methods such as GC/MS detection systems [36]. In fact, for the detection of anammox bacteria ladderane lipids are suitable biomarkers, in spite of some limitations on environmental samples: (1) methods of extraction are complex (2) due to inadequate existence and abundance in the water and sediment samples, high amount of samples are required to obtain desirable results (3) the presence of various inhibitors such as humic acid and fulvic acid may create difficulty in purification (4) similarly non-living organic matter may also contain these lipids therefore producing biologically false positive results related to the incidence of metabolically active anammox bacteria [37].

2.4 Microsensors

Microsensors is another powerful tool for the determination of anammox reaction either in marine sediments or biofilms at a low level. The manipulated biofilm is used to detect different nitrogen types such as nitrite, nitrate and ammonium ions, with ion-selective microelectrodes based on Ag/AgCl [38]. The commercially available Clark-type dissolved oxygen microelectrode can be used to measure oxygen (http://www.unisense.com/). The productions of nitrate with gradual decrease of nitrite and ammonium concentration in the same location specify the confirmation of anammox reaction in a biofilm [39]. The use of combined nitrate/nitrite and dissolved oxygen microelectrodes in the biofilm can be used to determine the complete autotrophic nitrogen removal by partial nitritation and anammox reaction, in mangroves and in estuary sediments. In addition, FISH with microsensor techniques can be used together, to identify the presence and activity of anammox bacteria [40].

2.5 Confocal Raman microscopy

The bacteria can be differentiated at the strain level and Confocal Raman microscope makes it possible in microbiological studies. The aggregated anammox bacteria usually found in biofilm can be identified by three dimensional distribution patterns. *Ca.* Brocadia from the enrichment culture have been recognized by performing noninvasive technique of Raman vibrational signature. The more specific, target version and modified confocal resonance Raman microscopy can be used to target cytochrome c, which is widely distributed in anammox bacteria and accounted for almost 10% of the total cellular proteins. Therefore, anammox bacteria can be identified without damaging them [41].

2.6. Fluorescent in situ hybridization (FISH)

FISH is considered to be the suitable method for cultivation-independent in situ identification of microbes from environmental samples. Similarly, this technique has been used in various studies for the quantitative and qualitative detection of anammox from environmental samples. The use of fluorochrome-labeled DNA oligonucleotide probes significantly contributes to the success fulimplementation of this method in practice [42]. The fluorescent tagged probes bind to the targeted sites while both unbounded and excessive probes are washed out. Furthermore, the probe attached to the bacterial cells can be identified by epi-fluorescent microscope.

The intensity of fluorescence varied based on variable excitation/emission wavelengths, similarly the use of different fluorochrome on each probe allows immediate and precise identification of dissimilar bacteria. Nevertheless, to insure the exact results some preemptive measures should be adopted to avoid any signal interference between unrelated probes, although the protocol has already been generalized for confirmation. The highly targeted probes are available for anammox bacteria such as from phylum-specific [43] to family-specific and genus-specific probes, and these are selected on the basis of research goal. Currently various oligonucleotides probes have been used and designed to target different anammox genus/species, some of the commonly used probes along with other relevant data are available. Majorly the available probes designed for the anammox bacterial detection are based on 16S rRNA genes of anammox bacteria. Some probes are also designed on the basis of 23S rRNA gene like L-*-Amx-1900-a-A-21. In future, it is possible to develop more specific probes for identification of anammox bacterial communities due to rapid developments in research, particularly genomic analysis of anammox bacteria. Nevertheless, one of the common difficulty in finding novel anammox bacterial species is high divergence (<87.1% identity) between diverse anammox genera [44].

All these limitations can be reduced by adopting different approaches to improve the FISH procedure for the detection of anammox bacterial abundance. For example the signaling intensity of probe can be improve by polynucleotide FISH and catalyzed reported deposition FISH (CARD-FISH), or decreasing access issues of probe mounting productivities of hybridization of the probes with different chemistries, such as peptide nucleic acid FISH and locked nucleic acid FISH [53]. In addition, the application of FISH can be expanded by combination with other research technique to get more detail information about the anammox bacterial metabolic activities.

2.7 Molecular methods for anammox bacterial detection

Culture independent approaches, such as molecular based techniques like 16S rRNA or functional genes are the methods of choice and have been widely used for anammox detection due to great hurdles in their cultivation. The 16S rRNA gene based PCR amplification is the most commonly used methods to detect anammox community in environmental samples, facilitating further quantitative and phylogenetic analyses. Besides the commonly used 16S rRNA gene, some other biomarker genes can also be used to obtain more precise information about the anammox bacteria because these biomarker genes are associated with metabolic reactions of the anammox bacteria, therefore providing more appropriate information about the relevant bacteria. The PCR based approaches such as single stranded conformation polymorphism, density gradient gel electrophoresis and terminal restriction fragment length polymorphism [45]can be used to determine a diverse microbial community via universal primers or specific microbes viaspecialgenes. All these techniques are routinely used in different molecular labs for microbial detection as well as anammox bacteria.

2.7.1 Phylogenetic analysis by 16S rRNA gene

For the phylogenetic analysis of different microbial communities 16S rRNA genes are the ideal biomarkers used in various studies. The study of anammox bacteria with 16S rRNA genes indicated that they branched within the phylum *Planctomycetes*[46]. The detection of anammox bacteria from natural environments is majorly carried out by using 16S rRNA genes. The amplification of anammox bacterial 16S rRNA genes with bacterial universal primers is not very successful, mainly due to thenon-specific amplification of anammox bacteria caused by their high level of divergence between the 16S rRNA genes sequences (<87.1% identity). However such primers are not available with diverse range of PCR amplification for different anammox bacterial genera, particularly when the abundance of anammox bacteria is relatively lower than other bacterial communities (<1%). The targeted primers used for anammox bacteria have low specificity, due to which non-specific or unwanted bacterial amplification may occur for example*Vibrio* species. Recently nested PCR is used to amplify anammox bacteria from environmental samples with increased specificity. In this technique, double pair of primers are used, and the initial DNA fragment is amplified with the first set of primer while the product obtained is used as the template for a second round of PCR with the second set of primers. The nested PCR method is comparatively more targeted for anammox bacteria for any environmental samples. It has also been found that

some primers are available with more specific amplification to anammox bacteria. Therefore, by using these primers sufficient information can be obtained about the phylogenetic diversities, community composition and quantitative distribution of anammox bacteria in different environments.

2.7.2 Phylogenetic analysis by functional gene biomarkers

One of the disadvantages of 16S rRNA gene as a molecular biomarker is that it does not give any information about the physiology of the anammox bacteria due to irrelevance to the function. When quantitative reverse-transcription PCR is used with functional gene biomarkers along with presence of anammox bacteria, the information about the activity of anammox can also be determined. In addition, functional biomarker genes can improve the sensitivity of the detection of anammox as low as <1% in the whole bacterial community and could screen very low amount of anammox presence in the environment. Hence, the existing development in the genomics, biochemistry and physiology of anammox bacteria will certainly help in identifying more functional genes related to anammox bacteria, which will allow to identify more anammox bacterial communities. Until now, four different anammox functional biomarker genes are used and all these are core component of their metabolic activity found in entire anammox candidates including: nitrite and nitrate reductases, hydrazine hydrolase, and hydrazine dehydrogenase (HZO). Therefore, it is suitable to design additional primers for anammox bacterial detection more specifically. At this time HZO hydrazine oxidoreductase also known as hydroxylamine oxidoreductase-like protein (HAO), has been widely used in many studies, hence large number of sequences are available in the public database. Almost ten primers encoding this gene have been available so far which is the highest number of any anammox bacterial primer used, however, each primer target three different clusters of HZO/HAO proteins [47].

Anammox bacteria were detected effectively with *hzo* gene primers from samples related to various ecosystems including wastewater treatment plants, mangrove sediment, estuaries, coastal and deep-ocean sediments hydrothermal vents, and oil reservoirs [48]. It has been elucidated through various studies that *hzo* gene is the most suitable candidate functional biomarker for selective identification of anammox bacteria from any environmental samples under different conditions due to its high specificity towards this group of organism as compare to 16S rRNA gene primers. The cd1 nitrite reductase (NIRS) gene is another commonly used functional biomarker used for the identification of anammox bacteria and recently for the amplification of *Ca.* Scalindua spp, some primers sets are designed [49].

The activity of anammox bacteria was detected from the Peruvian upwelling zone and the *nirS* genes were detected at the transcriptional level. Recently in few studies it has been validated that two sets of new PCR primers (AnnirS) and (ScnirS), gave better results for the detection of anammox bacterial *nirS* genes from the marine sediments, however the retrieved results showed that the ScnirS primer is targeted towards the *ScalinduanirS* gene whereas AnnirS primer set is perhaps specific for other genera of anammox bacterial *nirS* genes [49]. However no primer is available hitherto for the two other functional biomarkers, nitrate reductase and hydrazine hydrolase, in part due to this limited sequences obtained recently. Therefore, still more options are available in the research on these aspects.

2.7.3 Denaturant gradient gel electrophoresis (DGGE)

The DGGE is a reliable and appropriate technique to study the diversity and abundance of anammox bacteria from environmental samples. In addition, it is a suitable method to describe phylogenetic relationships between bacteria. Similarly this technique is preferred and considered to be the power tool to investigate variation patterns in community structure of selected bacterial lineage [50]. Variable length of nucleotide sequences can be separated from about (200 to 700bps) by using DGGE method of detection. The low and high denaturant gradient of amplified gene product of rDNA obtained by PCR after electrophoresis on polyacrylamide gel contribute majorly in separation in DGGE when mixed with denaturant mixture [51], hence, as compare to other genetic fingerprinting techniques DGGE is used more frequently. On the other hand, temperature gradient gel electrophoresis also has analogous mechanism, however, instead of denaturant, temperature act as a gradient. Both these techniques are widely used to detect anammox bacteria [52].

2.7.4 Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is used to analyze microbial community with restriction digest of double stranded fluorescently dye end-labeled PCR fragments. Compared with other molecular fingerprinting techniques such as DGGE and SSCP, it has advantages due to higher throughput, faster analysis, but has disadvantages with respect to some factors such as time-consuming, expensive and underestimation of microbial diversity. Despite of these drawbacks, still T-RFLP has been widely used in the research both in large-scale plants and laboratory-scale reactors.

In fact, DGGE is more widely employed than T-RFLP, because it has both longer applications history, cost-effective and is appropriate for obtaining sequence information by

separating DGGE bands. Also, T-RFLP needs more steps such as achieving fluorescently dye PCR products and using restriction enzymes. However, studies about the application of T-RFLP indicated functional microbial communities in research wastewater such as AOB, NOB, and anammox bacteria. At the same time, these studies focused on the species composition of microbial communities and variations [50].

2.7.5 Quantitative PCR Analysis

The classical PCR amplification lacks the ability to measure the quantity of the target gene, however, the quantitative PCR (qPCR) and competitive PCR techniques can be used to detect the abundance of target genes or cell density and the principles applied in these techniques have already been described in previous studies. Due to less accurate results and expensive reagents competitive PCR is used less frequently compared to qPCR technique. The qPCR approach has been used in various researches either by using 16S rRNA or functional gene. The high sensitivity, better results and reliability of the qPCR method make it more popular in quantification analyses over other technique such as FISH. Similarly by measuring the growth curve of the bacteria the maximum growth rate of anammox bacteria can be determined more accurately [53]. Initially, the documented doubling time of the anammox bacteria was estimated to be 11 days, however, by the use of qPCR different range of anammox bacterial growth was estimated and found to be 3.6 to 17 days [53]. The anammox bacteria can be measured by qPCR technique from various environmental samples such as waste water treatment reactors, marine oxygen minimum zones and marine sediments. The activity of anammox bacteria could also be detected by using RT-PCR of the functional genes such as *hzo* and *Scalindua-nirS* gene. Nevertheless, the results obtained by qPCR about anammox bacteria may not be similar with the results retrieved by FISH technique [54]. The incongruent results might be due to various reasons including diverse detection efficacies of different probes and primers used for the various anammox species, the detection limit of the probes used in FISH method and the interference of various organic matters in the bacterial RNA or DNA gene by q-PCR is the most operative and appropriate method used in numerous studies.

2.7.6 The use of Metagenome

Metagenomics has emerged as a powerful tool that is developed by DNA sequencing technology in which genomic content of any microbial communities can be studied [55]. Currently, only 1% of microorganisms in marine environments are culturable, metagenomics

tools are therefore of great advance to obtain the genomic content of any microbial communities in a natural habitat.

Due to the lack of pure culture and genetic information of anammox bacterium, metagenomics offers wonderful guidelines to create innovations about the anammox bacteria from enrichment and naturalsystems. In 2006 the enrichment culture of '*Ca.* Kuenenia stuttgartiensis' was used for the first time to study the metagenome data of this anammox bacteria to investigate gene responsible for biosynthesis of ladderane and biological metabolism of hydrazine and some unpredicted metabolic versatility. For these kind of metagenomics studies reference data with high quality is essentially required, however, only one almost complete reference genome of anammox bacteria is availablei.e., 4.2 MB with 5 contigs and 98% of *Ca.* Kuenenia stuttgartiensis as a model organism [56]. Beside the dataset of Kust genome, some recent datasets comprising drafts genome of both "*Ca.* Scalindua profunda and anammox bacterium *Ca.* Kuenenia stuttgartiensis KSU1 and a rough dataset comprising a draft genome of "*Ca.* Brocadia fulgida" are available. Hereafter, the metagenome study of "*Ca.* Jettenia asiatica" was also performed from the enrichment culture [57]. The genomic dataset of *Ca.* Kuenenia anammox strain KSU1 was used as a model organism to detail study of key genes in the dataset were identified. The metagenome analysis of *Ca.* Jettenia asiatica was employed by sequencing technologies Illumina and 454 pyrosequencing. A set of 25 different vital genes for anammox metabolism were detected compared to the protein sequences of "*Ca.* Kuenenia stuttgartiensis". A gene encoding copper containing nitrite reductase NirK was detected in the genome by these techniques, in addition with the use of MetaCluster analysis, 16S rRNA gene analysis and read mapping the community structure was examined, which indicated the existence of some important microbial communities including methanogens, denitrifying methanotroph "*Ca.* Methylomirabilis oxyfera" and methanogens[57].

The metagenome study of *Ca*. Scalindua profunda was observed by construction of fosmid and shotgun libraries and sequenced by Sanger method, whereas community DNA was sequenced by 454 Titanium technology. The noticeable variations in the gene expression and organization of some key anammox enzymes including nitrite reductase (NirS), hydrazine synthase (HzsAB) and inorganic nitrogen transport proteins were analyzed by metagenome techniques based on *Ca*. Scalindua profunda metagenome and environmental metagenome data as model. These accessible genome data which provide information about the diversity of anammox bacteria will certainly help us to explore about these fascinating microbes.

Moreover, metagenomics will support the comprehensive study of genome of closely associated species or strains [58].

Conclusion

From few decades enormous work have been done to better understand the anammox process and bacteria by employing different enrichment techniques along with the other indirect detection methods such as, molecular techniques and cell structural analysis methods etc. This study is focused on critical understanding of anammox bacterial cell structures, metabolism, its pervasiveness and role in nitrogen cycle. Similarly along with other previously utilizing methodologies few latest techniques have also been discussed in this paper to find better solutions for some of the questions related to this bacteria.

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