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In Search of Anaerobic Ammonia Oxidation by Planctomycete-like Bacteria in Sequencing Batch Reactor Stressed by the Addition of Free Ammonia

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Planctomycetes belong to an unique anaerobic ammonia oxidation (anammox) member of the bacteria. They have a special organelle, anammoxosome, for anaerobic ammonia oxidation. Since they grow extremely slowly, there have been some difficulties identifying them. It is only recently that people have discovered planctomycetes might play a role in the process of converting ammonia and nitrite directly into dinitrogen without the usual conversion from nitrite to nitrate and to nitrogen gas. In a sequencing batch bioreactor treated by the addition of free ammonia, the concentration of nitrite initially spiked, and then overtime the nitrite was converted to nitrate by microorganisms. However, during this time, the level of *Nitrobacter* and *Nitrospira* detected was very low so it was speculated that planctomycetes may actually aid in this nitrifying process. DNA was first extracted from the cells, and then the DNA was amplified via PCR. The amplified DNA was cloned and colony hybridization was performed using probes Pla 46 and Amx 820. Fourteen clones hybridized with the probes. Five were sent for sequencing. None of these clones were planctomycetes due to incorrect hybridization temperature and more importantly, the washing temperature. This was likely that two of the clones were *Actinobacteria*, two were *Proteobacteria* (alpha and gamma) and one was *Flavobacteria*. The washing temperatures should have been increased (higher than the melting temperature of the probes) to remove sequences unspecifically hybridized to the target site. However, the result did suggest that the level of planctomycetes in the bioreactor was very low. Planctomycetes thus would not be responsible for the increase in nitrite reduction.

Human beings generate many nitrogenous compounds in our wastewater. If these nitrogenous compounds were to be released into our waterways, serious environmental and medical problems could result (2). In order to remove nitrogenous compounds from wastewater, nitrification is required. The traditional nitrification is a two-step process. The first step is called ammonia oxidation, in which ammonia is converted to nitrite followed by the second step, nitrite oxidation, in which nitrite is converted to nitrate (6). In final denitrification, nitrate is converted to nitrogenous gas which in the end removes the nitrogen from solution (13). Since aerobic autotrophic and heterotrophic bacteria are capable of nitrification, studies of biological nitrogen cycle using the anaerobic ammonium oxidation pathway have been limited. Moreover, the slow growth rate (doubling time of 11 days) of these anammox bacteria has made their identification difficult since they could not be cultured. However, people believed that lithotrophic microorganisms capable of using ammonium as inorganic electron donor for denitrification under anoxic conditions existed (14).

Strous and others discovered that the missing lithotroph was a new planctomycete. These bacteria contribute to the process of anaerobic ammonia oxidation (anammox) which has an advantage over the traditional nitrification because this new process converts ammonia and nitrite directly into dinitrogen gas eliminating conversion from nitrite to nitrate and to nitrogen gas. The planctomycete anammox bacteria, "*Candidatus Brocadia anammoxidans"* and "*Candidatus Kuenenia stuttgartiensis"* from fresh water, and "*Candidatus Scalindua sorokinii"* from Black sea, have been identified (11).

Planctomycetale is a cold-loving division of bacteria whose members share several peculiar characteristics, in particular a single or double membrane surrounding the chromosome. The anammoxosome, which is located in the cytoplasm, has been identified in all these newly discovered planctomycete species. This special organelle is the site of anammox catabolism (11). Moreover, *Planctomycetale* has been shown to be the organism branching from the very base of the phylogenetic tree through the analysis of its 16S rRNA.

It has been observed that in an aerobic Sequencing Batch Reactor (SBR), the nitrite concentration spiked with the addition of free ammonia. After a period of time, the nitrite concentration decreased. This change indicated that

nitrifying organisms such as *Nitrobacter* and *Nitrospira* were probably converting the available nitrite into nitrate. However, when probes Nb 1000 and Ntspa 685 were used to identify these two microorganisms, the level of *Nitrobacter* and *Nitrospira* was barely detectable. This suggested that there might be other novel organisms such as planctomycetes that were consuming the nitrite. Even though the environment in the bioreactor was aerobic, planctomycetes could still survive since there were always aggregates of cells inside a bioreactor, and the interior part of these aggregates made up an anaerobic environment which was suitable for planctomycetes growth.

Bacterial DNA was extracted and PCR was performed to amplify the extracted DNA. The DNA was cloned and colony hybridization was used to probe the colonies to determine whether planctomycetes were present in the bioreactor.

MATERIALS AND METHODS

DNA Extraction. A late phase sample from the sequencing batch bioreactor was provided by Rob Simm from University of British Columbia. Five millilitres of sample was originally removed from the bioreactor and centrifuged to form a pellet for storage. DNA was extracted from the bacteria according to a protocol which could extract DNA and RNA at the same time (16). First, the pellet was resuspended in 1.5 ml of Extraction Buffer (50 mM Tris-BASE at pH 8.0, 5 mM EDTA, and 30% sodium dodecylsulfate). Glass beads (0.1 mm, Sigma) were added to the tubes and beaten for 2.5 minutes at 4800 rpm in a centrifuge (Eppendorf Centrifuge 5415D) and immediately cooled on ice for 1 minute. The sample was centrifuged at 14000 rpm for 3 minutes at 4C. The supernatant was saved in a fresh tube. The whole process was repeated by adding enough extraction buffer to the tube. The two supernatants were pooled, and ammonium acetate was added to make 2M from 10M stock. The mixture was left on ice for 5 minutes, and centrifuged at 14000 rpm for 10 minutes at 4C. The supernatant was saved in a fresh tube. Nucleic acid was precipitated out with 1 volume of isopropanol, and the mixture was centrifuged at 14000 rpm from 15 minutes at 4C. This time, the supernatant was discarded and the pellet was washed with 1ml 70% ethanol and left to air dry for 2 minutes. The pellet was dissolved in 300 µl of TE buffer (10mM Tris-HCl, 0.1mM disodium acetate at pH 7.4) and 33 µl of 3 M sodium acetate at pH 7.0 was added. The mixture was reprecipitated with 2.5 volume of 70% ethanol and centrifuged as above. The supernatant was discarded and the pellet was washed with 1ml of 70% ethanol and left to air dry for 2 minutes. After two minutes, the pellet was dissolved with 50 µl of TE buffer, and 1 µl of 10 mg/ml ribonulcease (Ribonulcease A, Sigma) was added to the sample. The sample was incubated for 15 minutes at 37C. The purity of the DNA was assessed by scanning the sample from 220-340 nm and by running the sample on an agarose gel with ethidium bromide staining.

PCR. For PCR amplification of members of the *Planctomycetales*, an universal primer, 27F, was used as a forward primer in combination with a reverse primer 1390R (*E. coli* positions 1390-1407). PCR reaction was performed via AccuPrime™ SuperMix II (Invitrogen) in a BioRad Gene CyclerTM Thermal Cycler. Reaction mixture was prepared in a total volume of 50 µl with 200 ng of template DNA. Thermal cycling was carried out with an initial denaturation of 4 min at 94C, followed by 30 cycles of denaturation at 94C for 45 s, annealing at 58.5C for 50 s, and elongation at 72C for 3 min (according to Schmid, annealing temperature at 58.6C resulted in specific product formation). Cycling was completed by a final elongation step at 72C for 10 min. The resulting PCR product was visualized via gel electrophoresis.

Cloning, Colony Transfer and Transfer to Nitrocellulose. The PCR products were cloned directly by using the TOPO TA Cloning Kit following the instructions of the manufacturer (Invitrogen). One Shot® Chemically competent *E. coli* cells with pCR®2.1-TOPO® vector were used for this experiment. For colony transferring, the grid template was taped under the plate, and white colonies were transferred onto ampicillin selective plates using sterile toothpicks or inoculating needle (4). The plates were incubated at 37C overnight. Colonies were transferred onto a nitrocellulose (NC) (RioRad) by placing autoclaved NC filter on the plate containing the colonies. The NC filter was carefully removed by picking up its edge. In order to prepare the filter, the NC filter was placed for 5 min on a piece of Whatman 3MM filter paper saturated with 0.5 M NaOH. Then, the filter was placed briefly on a paper towel to blot excess liquid. The NC filters were incubated as above in the following order, 1.5 M NaCl with 0.5 M Tris at pH 7.4, and then, 1.5 M NaCl and 2X SSC at pH 7.0 (from 20X SSC stock: 3 M NaCl, 0.3 M Na_3 Citrate-2H_2O, H_2O to volume) The filters were left to dry on the bench top for 1 hour, and baked for 2 hours at 80C under vacuum (4).

Colony Hybridization. For prehybridization, the NC filters were placed in a hybridization tube. Since there were two NC filters that needed to be hybridized with the same probe, the two NC filters were separated with a piece of membrane divider with the addition of 14 ml of fresh prehybridization solution (3 ml 10% Polyvinylpyrrolidone, 3 ml 10% BSA, 3 ml 10% Ficoll 400, 2.2 ml 10 mg/ml salmon sperm DNA stock, 15 ml 20X SSC, 250 mg yeast RNA and autoclaved H_2O to 150 ml) (4). The tubes were placed in a hybridization incubator for 2-3 hr. Probes Pla 46 and Amx 820 were labeled with [γ^{32}P ATP] via the KinaseMax™ protocol (Ambion). In a 20 ul reaction mixture, 2 pmol of probes were used with 8 pmol of ATP. The radioactive nucleotides were cleaned by following the QIAquick Nucleotide Removal Kit protocol (Qiagen). One microlitre from the first flow-through and eluant was transferred onto a piece of Whatman 3MM filter disk to determine how much radioactivity was incorporated. For hybridization, approximately 30 million CPM of probe was added to the prehybridization solution. The tubes were placed back in the hybridization incubator and incubated overnight at 45C (4). The hybridization solution was carefully removed from the tubes, and the membranes were washed three times with pre-warmed 1X SSC filled to half way up the tube (approximately 40 ml). The first wash was for 10 min and the second and third washes were 30 min. The excess wash solution was removed by blotting against a piece of Whatman 3MM filter paper. Then, the membranes were taped on a piece of plastic mylar membrane. Another piece of plastic mylar membrane was placed on top. The membranes and a piece of X-ray film (Kodak) was placed in an autoradiography cassette for 2 days. After 2 days, the film was taken out and developed while a second film was exposed for 4 days.

Plasmid Isolation and Sequencing. The positive clones were streaked on selective plates to obtain single colonies. Single colonies were inoculated in 5 ml of LB broth and incubated in a 37C water bath overnight with shaking. Gibco Concert High Purity Plasmid Miniprep System was used to isolate the plasmid DNA following the procedures of the manufacturer except that the purified DNA was dissolved in 30 µl of autoclaved H_2O. Purified DNA was sent to University of British Columbia's NAPS Unit (Nucleic Acid Protein Service).

RESULTS

Out of the 200 colonies that were picked from the clones, 14 colonies hybridized with the probes. The planned hybridization was at 45C overnight. However, the temperature had dropped to 28.3C by the next morning. In addition, the divider between the two membranes that were hybridizing with Pla 46 had caused the top membrane to

roll upon itself while rotating. The rolled up membrane rubbed against the membrane beneath it, causing some colonies on the membrane below to disappear. Since we did not know the duration of the hybridization condition at 28.3C, the temperature was adjusted back to 45C and the hybridization continued for 2 more hours. Eleven colonies hybridized with Pla 46 while only 3 hybridized with probe Amx 820. Only 5 colonies, 3 that hybridized with Amx 820, and 2 that hybridized with Pla 46, were sent for sequencing using -21m13 forward and M13R reverse primers.

The sequences with M13R reverse primers were converted to their reverse-complement counterparts so that all sequences were read from the 5' end. The reverse complement sequence and the same sequence with the forward primer were subjected to Emboss Merger (Biological Software, Institut Pasteur) which joined two overlapping nucleic acid sequences into one merged sequence. The probe sequences within the merged sequences were identified via Emboss Matcher which compared two sequences looking for local sequence similarities using a rigorous algorithm. None of the sequences showed complete hybridization with the probes. There were always some mismatches (Table 1). Clone No. 3 hybridized with most of the probe; however, there were 7 mismatches in between. Sometimes the sequences hybridized to only part of the probes. For example, clone No. 1 hybridized to the first 10 bases of the 18-long Pla 46 probe. When compared with the original autoradiograph, all five sequences that were sent for sequencing gave strong signals on the film especially the ones (Clone No. 1 and 2) that were probed with Pla 46. Clone No. 3 showed a weaker signal than that of Clone No.4 and 5. However, the degree of observed signal did not correlate with the percentage of hybridization since none of the sequences completely matched the probes used. Furthermore, Clone No. 3 which had the least signal actually aligned better to Amx 820 than Clone No. 5 which had a stronger signal.

Table 1. Sequences that Hybridized with Probes Pla 46 or Amx 820.

Probe	Specificity	Sequence	Clone No.	Part of sequence that matches probe	% Identity of sequence aligned	% Identity between probe and sequence
Pla 46	*Plancto-mycetales*	5'-GACTTGCATGCCTAATCC-3'	1	1 10 GACTTGCATGCCTAATCC GACTTGCATG……. 1400	100% (10/10)	55.6% (10/18)
		3'-CCTAATCCGTACGTTCAG-5'	2	2 16 CCTAA**T**C**CG**TA**C**GTTCAG ...CTAA**CC**A**TT**AA**G**TTC…. 930	73.3% (11/15)	61.1% (11/18)
Amx 820	Anaerobic Ammonium Oxidizer	5'-AAAACCCCTCTACTTAGTGCCC-3'	3	3 22 AAAACCCCT**C**TA**C**TTAGT**GC**CC …..AACCCCT**G**T**TG**TTAGT**TG**CC 1180 1190	75% (15/20)	68.2% (15/22)
			4	3 22 AAAACCC**C**T**C**TA**C**TTAGT**GC**CC …..AACCC**TT**G**T**C**C**TTAGT**TG**CC 1160 1170	75% (15/20)	68.2% (15/22)
		3'-CCCGTGATTCATCTCCCCAAAA-5'	5	1 18 C**C**CGTGA**TT**C**A** **T**CT**C**CCCAAAA C**A**CGTGA**G**T**AA**C**C**T**G**CCC…… 200 210	72.2% (13/18)	59.0% (13/22)

Table 2. Blast Results for Five Selected Clones

Probe Used	Clone No.	Blast Result	% Identity
Pla 46	1	Alpha-proteobacteria	98
	2	*Actinobacteria*	94
Amx 820	3	*Flavobacteria*	95
	4	Gamma-proteobacteria.	93
	5	*Actinobacteria*	95

Blast search was performed on all five sequences to find the identity of the sequences. Since the probes did not bind specifically to planctomycetes, the result of the blast search was predicted to yield other bacteria presented in the sample instead of planctomycetes (Table 2). A general rule of thumb for Blast search is that when the percent identity is greater than 30%, we consider that two sequences are homologous. Blast results for all five sequences in this experiment gave a percent identity above 90% which was much greater than 30%. This suggests that the query sequences and the Blast hits are homologous (Table 2). For Clone No.1, which was probed with Pla 46, the blast search showed a 97-98% identity with alpha-proteobacteria with 0 E-value. Alpha-class of Proteobacteria had the highest 'species richness' followed by the Planctomycetes, the beta-class of Proteobacteria, and the GNS-bacteria in nitrifying-denitrifying activated sludge of an industrial wastewater treatment plant (9). Blast search for Clone No 2. and 5 revealed *Actinobacteria*, gram-positive bacteria with high DNA G+C contents (7). *Actinobacteria* and *Proteobacteria* beta subclass were the second major microbial species in SBR sludge for biological phosphate removal (11). Clone No. 3 was identified as *Flavobacterium limicola* and *Flavobacterium psychrophilum* that belonged to the *Flavobacteria* member. Cytophaga-flavobacteria, as a group, is important in mediating the liberation of inorganic orthophosphate (Pi) from phosphomonoesters of detrital organic phosphate (organic-P) in the aerobic activated sludge process of wastewater treatment (10). Clone No. 4 was homologous to Gamma-proteobacteria. This finding was not surprising since the seed sludge of a nitrite-oxidizing bioreactor was dominated by 18% of gamma subclass of proteobacteria (3).

In Bergey's map of all prokaryotic 16S sequences, planctomycetes, proteobecteria (alpha, beta and gamma) and flavobacteria belong to the same group but actinobacteria belong to a more distant group. Since there was no specific pattern of binding for the probes, we could not conclude that the probe bound to bacteria that were somewhat similar to planctomycetes in 16S sequences even though there were some mismatches (5).

DISCUSSION

Even though *Planctomycetales* were not detected in this experiment, the result of this experiment is still important because it demonstrates the importance of optimum hybridization and washing temperature. According to Bergey's Manual of Systematic Bacteriology, hybridization may occur between two strands that are not fully complementary. As a result, the mismatches are stabilized by the matches but the resulting hybrid is less stable than a fully complementary hybrid. Both the hybridization temperature and the washing temperature have a strong influence on the kinetics and the specificity of hybridization. The optimum hybridization temperature should be close to but below the dissociation temperature (T_d) which is similar to the melting temperature (T_m) for double stranded DNA. T_m is defined as the temperature at which 50% of the DNA is dissociated into single strands (12). On one hand, if the hybridization temperature is much lower than T_d, probe specificity would be compromised meaning that more mismatches would result. On the other hand, if the hybridization temperature is much higher than T_d, the sensitivity of the hybridization assay would decrease greatly. The T_d can be approximately determined by % GC content (1). Washing temperatures that are higher than T_m of the probe will wash away any unspecific sequences that are bound before the specific sequences get disassociated because helix stability is decreased by mismatched pairs.

In this experiment, oligonucleotide probes Pla 46 (18 bp long), and Amx 820 (22 bp long) were used. Both Pla 46 and Amx 820 have % GC of 50 but the T_d of Amx 820 is 55C while T_d for Pla 46 is 48C. There is a difference between the T_d because the length of Amx 820 is longer than that of Pla 46. In the actual experiment, the membranes were supposed to be hybridized and washed at 45C. However, the hybridization temperature dropped from 45C to 18C sometime during the overnight incubation period so there was a possibility that the membranes had been incubating at 18C overnight. If this was the case, the 18C incubation would have contributed to the lack of specificity. At this temperature, the probe binds to unspecific sequences, resulting in many mismatches. Furthermore, the membranes should be washed at higher temperature in order to wash away probes that were unspecifically hybridized. However, our membranes were washed at 45C, which was the same temperature used for hybridization so any unspecific sequences bound to the probes produced a signal under autoradiography.

The use of two probes was intended to increase the precision of hybridization. Mutations can occur through evolutionary process so a good signature site might cover only 95% of all members of the planctomycetes group thus leaving behind the 5% that may contain a few mismatches (1). Moreover, due to high diversity of microorganisms, there might be bacteria that have the identical probe target site but do not belong to the members of planctomycete. Pla 46 and Amx 820 were chosen because the former hybridizes to *Planctomycetales* and the latter hybridizes to anaerobic ammonium oxidizer. As a result, Amx 820 is more specific in identifying anaerobic ammonia oxidizer than Pla 46 since Amx 820 hybridized to only anaerobic ammonia oxidizer members of *Planctomycetales* while Pla 46 hybridizes to all *Planctomycetales.* However, in this experiment, the two probes did not bind to the colonies corresponding to the same clone. Therefore, the increased efficiency of using two probes in

parallel was not achieved due to the low hybridization and washing temperatures so other bacteria presented in the bioreactor were detected instead of *Planctomycetales.*

Microorganisms other than planctomycetes were detected so we can conclude that there were probably very few planctomycetes in the bioreactor. A total of 200 clones were subjected to hybridization and 14 out of 200 colonies actually hybridized with the probes. Five out of these 14 colonies were sent for sequencing but not one was identified as planctomycetes. If planctomycetes make up 1% of the total microorganisms in the bioreactor, 2 out of 200 which should have been among the 14 clones should be planctomycetes. Statistically, the chance of selecting 5 colonies from the 14 colonies and having one of them to be planctomycetes is quite high but the five clones that were selected were not planctomycetes. This suggests that the percent population of planctomycete was probably lower than 1%. According to Table 2, we see that out of the 5 colonies, two were *Proteobacteria,* and two were *Actinobacteria*. This may suggest that these bacteria dominate the population of microorganisms in the bioreactor. It does not mean that planctomycetes were not present in the bioreactor. There were probably too few to be detected. For this reason, we can conclude that planctomycetes are not the responsible for the high conversion of ammonium to nitrite and nitrate even when the level of *Nitrobacter* and *Nitrospira* was low. In other words, planctomycetes were not the novel organism responsible for increased nitrification in the SBR.

FUTURE EXPERIMENT

In the future, it is strongly recommended that the optimum hybridization temperature be implemented. Moreover, multiple probes can be used to increase the degree of confidence. Application of both Pla 46 with Amx 820 probe *in situ* demonstrated that bacteria detected with Amx 820 made up more than 99% of the cells stained by Pla46, but there were some Pla 46-positive bacteria that did not hybridize with probe Amx 820 (14). Therefore, new target sites should also be considered since there might be other anaerobic ammonium oxidizers presented in the nitrifying reactors that could not be detected via Pla 46 or Amx 820. The washing temperature is also very important. The membranes should be washed at increasing temperature until the sequences unspecifically bound to the target sites are washed away. The washing temperature can be determined by looking at the T_m for the probes. The optimum washing temperature would be one which is somewhat higher than the T_m of the probes in order to dissociate mis-pairs.

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