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TESTING THE SPECIFICITY OF PRIMERS TO ENVIRONMENTAL AMMONIA MONOOXYGENASE (*AMO*A) GENES IN GROUNDWATER TREATED WITH UREA TO PROMOTE CALCITE PRECIPITATION

Stephanie Freeman, David W. Reed, and Yoshiko Fujita

ABSTRACT

Bacterial ammonia monooxygenase (*amo*A) genes in DNA isolated from microorganisms in groundwater were characterized by amplification of *amo*A DNA using polymerase chain reaction (PCR), Restriction Fragment Length Polymorphism (RFLP) analysis, and sequencing. The *amo*A gene is characteristic of ammonia oxidizing bacteria (AOB). The DNA extracts were acquired from an experiment where dilute molasses and urea were sequentially introduced into a well in the Eastern Snake River Plain Aquifer (ESRPA) in Idaho to examine whether such amendments could stimulate enhanced ureolytic activity. The hydrolysis of urea into ammonium and carbonate serves as the basis for a potential remediation technique for trace metals and radionuclide contaminants that can co-precipitate in calcite. The ammonium ion resulting from ureolysis can promote the growth of AOB. The goal of this work was to investigate the effectiveness of primers designed for quantitative PCR of environmental *amo*A genes and to evaluate the effect of the molasses and urea amendments upon the population diversity of groundwater AOB. PCR primers designed to target a portion of the *amo*A gene were used to amplify *amo*A gene sequences in the groundwater DNA extracts. Following PCR, amplified gene products were cloned and the clones were characterized by RFLP, a DNA restriction technique that can distinguish different DNA sequences, to gauge the initial diversity. Clones exhibiting unique RFLP patterns were subjected to DNA sequencing. Initial sequencing results suggest that the primers were successful at specific detection of *amo*A sequences and the RFLP analyses indicated that the diversity of detected *amo*A sequences in the ESRPA decreased with the additions of molasses and urea.

INTRODUCTION

One of the crucial steps in the nitrogen cycle is the oxidation of ammonia to nitrite. The ammonia monooxygenase (*amo*A) gene has been identified as an integral gene in the conversion of ammonia to hydroxylamine, the first step in overall ammonia oxidation [1]. The *amo*A gene codes for the catalytic subunit of the ammonia monooxygenase enzyme. The *amo*A gene is present in all nitrosifying bacteria that take part in this critical step of the global nitrogen cycle [2]. This gene was previously thought to be unique to bacteria, but recent research has identified ammonium oxidizing

archaeal species that have a similar archaeal *amo*A gene and that may play an important role in marine nitrogen cycling [3].

Along with collaborators, Idaho National Laboratory (INL) is currently investigating a remediation technique for radioactive strontium-90 (^{90}Sr) contamination of aquifers based on the incorporation of strontium into calcite (calcium carbonate, $CaCO_3$). In calcite-saturated aquifers such as the Eastern Snake River Plain Aquifer (ESRPA) in Idaho, co-precipitated strontium in calcite is expected to remain stable for hundreds of years. The specific remediation method under investigation is greatly dependent upon the acceleration of calcite precipitation by native groundwater microorganisms' hydrolysis of urea:

$$H_2NCONH_2 + 2H_2O \rightarrow 2NH_4^+ + CO_3^{2-}$$
(Urea + Water → Ammonium + Carbonate)

The carbonate ion reacts with metals to form carbonate minerals, while the ammonium can serve to displace metal cations (such as Ca^{2+} and Sr^{2+}) from ion exchange sites on aquifer solids, making them available for reaction with the carbonate. However, the ammonium could also serve as an electron donor for ammonia oxidizing bacteria (AOB) present in the aquifer.

At INL, a quantitative Polymerase Chain Reaction (qPCR) technique is being developed to evaluate the effect of urea additions on native AOB populations present in groundwater. This qPCR method utilizes primers designed to specifically target the *amo*A gene in environmental microbial communities. Methods based on the presence of the *amo*A gene for detection and quantification of AOB species have been previously developed based on conventional Polymerase Chain Reaction (PCR) [4], competitive PCR (c-PCR) [5] and real-time PCR [6]. In this work, we used real-time PCR primers originally developed by others [4,6] with slight modifications in an effort to capture more environmental *amo*A genes and exclude sequences for the similar *pmo*A gene, a subunit of the membrane bound methane monooxygenase gene (pMMO). Parts of the *pmo*A gene have high sequence similarity to *amo*A and non-specific priming is common [6].

The specific objective of the work reported here was to test the effectiveness of these primers in targeting *amo*A genes in environmental samples and to characterize the diversity of genes that could be detected. The primers were applied to genomic DNA extracts collected previously as part of a field experiment in the ESRPA where dilute molasses and urea were added to the aquifer in order to stimulate ureolytic activity. After amplification of genomic DNA with these primers, initial diversity was observed through the use of Restriction Fragment Length Polymorphism (RFLP) analysis. Sequencing was then performed in order to compare these environmental *amo*A gene sequences with *amo*A sequences reported by others for clones and known AOB species.

MATERIALS AND METHODS

Sample Origin: Genomic DNA extracts from microorganisms in groundwater samples taken during the course of a 2002 field experiment using an ESRPA well located at University Place, Idaho Falls, ID, were used as PCR templates during this study. During the first phase of the field experiment, Pre-Treatment, groundwater samples were taken prior to the addition of experimental amendments to establish the background conditions. Dilute molasses (0.00075%) was delivered into the well during the second phase (two weeks in duration), known as Post-Molasses, and urea (and no molasses) was added in the third and final phase (also a two week period), known as Post-Urea. Groundwater samples were collected periodically throughout the experiment and DNA was extracted from cells collected on filters (20 L filtered per extract) [7] using conventional methods. A more detailed description of the field experiment and the DNA extractions is provided in Tyler (2004) [7].

Primer Name	Direction	Sequence	Ref.
A189	Forward	5' - GGHGACTGGGAYTTCTGG - 3'	[8]
A189 DR	Forward	5' - GGNGACTGGGAYTTCTGG - 3'	[9]
*amo*A-2R'	Reverse	5' - CCTCKGSAAAGCCTTCTTC - 3'	[4]
*amo*A-2R' DR	Reverse	5' - CCCCTSBGSRAAVCCTTYTTC - 3'	[9]

Table 1. *amo*A primers. Primers A189 and *amo*A-2R' are shown along with the primers used in this study, A189 DR and *amo*A-2R' DR. Primers were designed from alignments of sequences of *amo*A genes from *Nitrosomonas* and *Nitrosospira genera*. Changes made to the standard *amo*A primers are indicated with bold and underlined typeface. Sequences, given from 5' to 3' end, include degeneracies represented by B, H, K, N, R, S, Y, and V. B represents CGT (implying a C, G, or T at that position). Similarly, H represents ATC, K represents GT, N represents ACGT, R represents AG, S represents CG, Y represents CT, and V represents ACG.

***amo*A Gene Specific Primers**: Primers A189 DR and amoA-2R' DR were used for the PCR amplification (Table 1). The primers were based on primers A189 and amoA-2R' developed in previous studies [4,8]. Primer A189 DR targets both bacterial *amo*A and *pmo*A genes while the amoA-2R' DR primer is designed to target the *amo*A gene only, excluding the *amo*A gene present in *Nitrosococcus* species [9].

PCR Amplification: The optimized PCR reaction mixture consisted of Taq2000 Polymerase (1U, Stratagene, La Jolla, California, USA), 1X Taq2000 buffer, dNTPs (0.2 mM; Roche Applied Science, Indianapolis, Indiana, USA), BSA (0.2 μg/mL; Roche Applied Science), A189 DR forward primer (900 nM), amoA-2R' DR reverse primer (300 nM), and DNA template (2 μL). Genomic DNA from *Nitrosomonas europaea* was used as template for a positive control reaction. Multiple negative reactions used either no added genomic DNA (water) or genomic DNA extracts from *Escherichia coli* and *Methylococcus capsulatus*. The reactions were performed on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, California, USA) using the following thermal program: 94°C (4 mins), then 38 cycles of 94°C (45 secs), 57°C (60 secs), and 72°C (60 secs), with a final hold of 72°C (5 minutes). PCR products were verified for correct size (670bp) and purity by electrophoresis on an ethidium bromide stained 1.3% agarose gel.

Cloning: Prior to cloning, PCR products derived from multiple samples taken from each of the three phases of the field experiment were pooled (five products for Pre-Treatment, three products from Post-Molasses, and two products for Post-Urea) and then purified by affinity column and gel purification (Qiagen PCR Purificaiton

and Q1Aquick Gel Extraction Kits; Qiagen, Valencia, California, USA). Cloning reactions were performed using the TOPO® TA Cloning system (Invitrogen, Carlsbad, California, USA) following the manufacturer's directions. Cloning reactions were performed on the pooled PCR products from the three different phases, as well as a positive control (*N. europaea*) and a negative control (only the reaction with no DNA template), resulting in the generation of 5 separate clone libraries. PCR products were ligated and added to 50 µL of One Shot® TOP10 *Escherichia coli* competent cells (Invitrogen, Carlsbad, California, USA) for transformation. The transformed cells were plated on LB agar plates containing S-Gal, ITPG, and kanamycin (50 µg/mL; Sigma-Aldrich Corp., St. Louis, Missouri, USA), and grown overnight at 37°C. For each treatment phase clone library, 50 colonies were transferred to new LB agar kanamycin plates, grown overnight, and stored for further analysis.

Restriction Fragment Length Polymorphism (RFLP): Clones from each library were analyzed using RFLP. Individual colonies from each library were re-suspended in 10 µL of sterile water and lysed for 10 mins at 99°C. The lysed cells' DNA was used as template for PCR as described previously, after which the PCR product (20 µL) was used for the restriction analysis. The restriction reaction also contained enzymes HinP1 and MsPI (0.4 U each; New England Biolabs, Ipswich, Massachusetts, USA) in 1X NEBuffer 2, and Triton X-100 (1%; Promega, Madison, Wisconsin, USA). The restriction mixture was incubated at 37°C for 4.5 hours. RFLP products were analyzed by electrophoresis on a 3% MetaPhor® agarose (Cambrex, East Rutherford, New Jersey, USA) gel and stained with ethidium bromide.

Plasmid Purification and Sequencing: After RFLP analysis, plasmid purification was performed on the clones selected for sequencing. Plasmids were extracted from the *E. coli* cells using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, California, USA) according to manufacturer's instructions. Each purified plasmid (50-100 ng) was subjected to cycle sequencing (10 µL reaction) with 2 µL 2.5X sequencing buffer and 1 µL BigDye, both part of the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA), and 1 µL primer (T7, M13F, and M13R; Operon Technologies, Huntsville, Alabama, USA). The sequencing reaction products were purified using a MultiScreen$_{384}$-SEQ filter plate (Millipore, Billerica, Massachusetts, USA). The reactions were subsequently washed with 0.3 mM EDTA, pH 7.8 and re-suspended in 0.3 mM EDTA, pH 7.8. Sequencing was performed in an ABI Prism Model 3700 sequencer (Applied Biosystems, Foster City, California, USA).

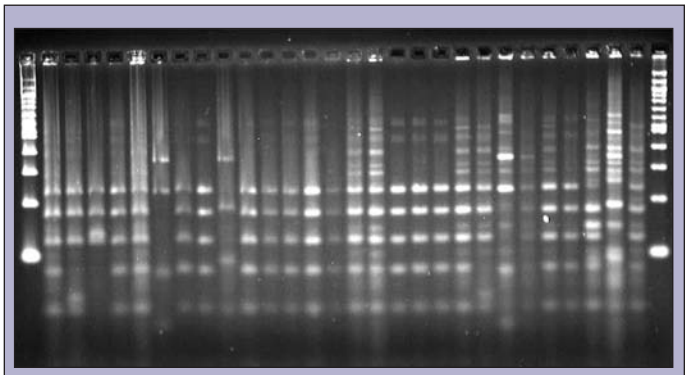

Figure 1. Example of an RFLP gel from Pre-treatment groundwater sample. The outer lanes contain 100 bp DNA ladder (New England Biolabs, Ipswich, Massachusetts, USA) while the inner 28 lanes contain clones from the Pre-treatment groundwater sample. Samples were digested with HinP1 and MsP1 restriction enzymes and were electrophoresed on a 3% agarose gel and stained with ethidium bromide.

RESULTS

The diversity of the *amo*A sequences detected in the UP-1 groundwater samples was characterized using RFLP. The RFLP gels (see for example Figure 1) were analyzed visually to identify unique banding patterns. Initially, PCR products with a common RFLP banding pattern within each library were assigned to the same RFLP group (A through BB; Table 3). Then, patterns were compared between the libraries to see if similar RFLP patterns were found in more than one clone library. In this way, some patterns were found in multiple libraries and resulted in more than one RFLP group label. RFLP analysis of the Pre-Treatment clones showed 12 unique banding patterns from the 50 clones of that library whereas RFLP analysis of the second library, Post-Molasses, and the third library, Post-Urea, showed 8 and 7 unique banding patterns respectively. Two unique banding patterns, namely RFLP Groups A/J_1-J_2/Q and C/L/R, were found in all three samples. This may indicate that the bacterial species represented in these patterns were present throughout the course of the UP-1 experiment. There was also one common pattern found in the Pre-Treatment and Post-Molasses libraries, RFLP Groups G/P, and one pattern present in only the Pre-Treatment and Post-Urea libraries, RFLP Groups E/V. All other banding patterns from each library appeared to be unique.

Primer Name	Direction	Sequence
T7	Forward	5' - TAATACGACTCACTATAGGG - 3'
M13F (-20)	Forward	5' – GTAAAACGACGGCCAG - 3'
M13R	Reverse	5' – CAGGAAACAGCTATGAC - 3'

Table 2. Primers used in sequencing reaction. The oligonucleotide sequences complementing these primers are all found on the TOPO TA vector used during cloning. A majority of the clones chosen for sequencing after RFLP were sequenced with at least two of the primers listed above.

Sequencing was performed on unique RFLP clones and the clones derived from the PCR control reactions. The positive control reaction used a *N. europaea* genomic DNA template while the negative control reaction used no added DNA template in the initial PCR reaction. All of the clones were sequenced using at least two of the three sequencing primers (Table 2) and the majority of clone sequences were visually analyzed to confirm the accuracy of the automatically generated sequence produced by the sequencing software. The sequences were manually fitted if necessary and both the primer sites and vector nucleotides were removed. Since multiple sequences were obtained for each clone, a consensus sequence was generated for each clone. Consensus sequences were compared to the NCBI (National Center of Biotechnology Information) database

RFLP Group	Common RFLP Patterns	Number of clones assigned to each RFLP Group		
		Pre-Treatment	Post-Molasses	Post-Urea
A	J_1-J_2, Q	32	35	41
B		2	0	0
C	L, R	1	7	4
D		2	0	0
E	V	2	0	1
F_1-F_2		3	0	0
G	P	1	1	0
H		1	0	0
I		1	0	0
K	O	0	2	0
M		0	1	0
N		0	1	0
S		0	0	1
T		0	0	1
U		0	0	1
W		1	0	0
X		1	0	0
Y		1	0	0
Z		0	1	0
AA		0	1	0
BB		0	0	1
Number of Unique RFLP Patterns		12	8	7

Table 3. Comparison of RFLP groups found after cloning UP-1 groundwater samples. The three UP-1 clone libraries, Pre-Treatment, Post-Molasses, and Post-Urea all had varying numbers of unique RFLP banding patterns, decreasing in number from Pre-Treatment to Post-Urea. The Common RFLP Patterns column indicates the RFLP patterns that are common between different clone libraries. RFLP groups were first assigned for each library and then compared between the different libraries, resulting in multiple labels in some instances.

using BLAST (Basic Local Alignment Search Tool) [10] to observe how the sequences obtained were related to known *amo*A gene sequences.

As expected, BLAST analysis of the gene sequences obtained for the positive controls (clones Pos1 and Pos2) indicated a very close relationship to *N. europaea* (complete genome, NCBI ascension number |BX321863.1|). Although only a very faint band of DNA was visualized in the ethidium bromide gel from the negative control sample, the DNA was cloned (clones Neg1 and Neg2), and the sequences closely matched a portion of the *N. europaea* genome, indicating that contamination of the negative control from the positive control might have occurred during PCR amplification. For the clones derived from environmental samples, data was not obtained for 5 groups (RFLP pattern A, L, S, T, and X clones) because of inefficient sequencing reactions that produced unreadable outputs. The remaining 25 clones were submitted to BLAST for analysis. A majority of the sequenced clones, 14 in total (representing RFLP groups B, C, F1, F2, G, I, J1, J2, N, P, Q, R, Z, and BB), matched most closely to the *amo*A gene from two unidentified bacteria (isolate E-207M, NCBI ascension number |AJ317947| and clone W10 682 7 |DQ008439|). With respect to known isolated bacteria, the output from the BLAST search indicated that the sequence from the clones matched most closely to the *amo*A gene of *Nitrosomonas sp*. AL212 (NCBI ascension number |AF327918|).

Three additional clones were very similar to those obtained for the positive control. The BLAST analysis of these clones E, H, and V indicated that the highest similarity was to the *amo*A gene region of *N. europaea*,. The remaining 8 clone sequences (D, K, M, O, U, W, Y, and AA) were similar in size to the expected *amo*A PCR products yet the sequencing suggested improper priming during the sequencing reaction.

DISCUSSION AND CONCLUSIONS

Analysis of the RFLP screening results showed differences between the banding patterns of the clones obtained from the three UP-1 experimental treatment phases. The RFLP diversity of the *amo*A gene fragments apparently decreased over the course of the experiment with the first experimental period, Pre-Treatment, having the greatest number of unique banding patterns. It is possible that some species might have been more responsive than others in this changing environment and dominated the AOB community during the different phases of the experiment. Only two banding patterns were common in all three clone libraries, while only two other patterns were found in multiple clone libraries. The other banding patterns were unique to the sample library in which each was found. As a majority of the banding patterns were changing with each sample, with only a few being maintained throughout the various samples, the RFLP analysis seems to indicate that the molasses and urea amendments have a large impact on the subsurface microbiological community.

Sequence data for three of the four RFLP groups with the most common RFLP pattern (J_1, J_2, and Q; unfortunately the sequencing reaction for the clone representing group A did not provide a usable sequence) matched closely with the sequence of the *amo*A gene of known AOB species. Additionally, clones that represented eleven other RFLP banding patterns indicated a close match with this same, known AOB species. Because a majority of the clone sequences were found to closely match known AOB species, it appears that our primers successfully detected bacterial *amo*A genes in the environment. The fact that at least 14 clones shared a similar BLAST output suggests that one particular species or closely related AOB may dominate the nitrosifying subsurface microbial community of the ESRPA. However, potential biases in DNA extraction, PCR, and cloning mandate caution in extrapolating results from clone libraries to the extant microbial communities [11].

The correlation of the gene sequences of RFLP groups E, H, and V to that of *N. europaea* and the finding of this same sequence in the negative control suggested that contamination might have occurred during PCR. Another possibility is that the subsurface community in the ESRP aquifer did in fact naturally contain *N. europaea* or closely related organisms.

In summary, preliminary sequencing results indicate that the primers used in this study were selective for the *amo*A gene region of various AOB. This finding supports the use of these primers to gauge the effect of urea addition on both the quantity and composition of the AOB community of the subsurface. However, further work is needed to elucidate the relationships between the gene sequences obtained here and the *amo*A gene sequences of known AOB species.

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REFERENCES

[1] T. C. Hollocher, M. E. Tate, and D. J. D. Nicholas, "Oxidation of ammonia by *Nitrosomonas europea*: definitive 18O-tracer evidence that hydroxylamine formation involves a monooxygenase," in *The Journal of Biological Chemistry*, Vol. 256, No. 21, pp. 10834-10836, Nov. 1981.

[2] C. D. Sinigalliano, D. N. Kuhn, and R. D. Jones, "Amplification of the amoA Gene from Diverse Species of Ammonium-Oxidizing Bacteria and from an Indigenous Bacterial Population from Seawater," in *Applied Environmental Microbiology*, Vol. 61, No. 7, pp. 2702-2706, July 1995.

[3] C. A. Francis, K. J. Roberts, J. M. Bemen, A. E. Santoro, and B. B. Oakley, "Ubiquity and Diversity of Ammonia-oxidizing Archaea in Water Columns and Sediments of the Ocean," in *PNAS*, Vol. 102, No. 41, pp. 14683-14688.

[4] J. H. Rotthauwe, K. P. Witzel, W. Liesack, "The Ammonia Monooxygenase Structural Gene amoA as a Functional Marker: Molecular Fine-Scale Analysis of Natural Ammonia-Oxidizing Populations," in *Applied Environmental Microbiology*, Vol. 63, No. 12, pp. 4704-4712, Dec. 1997.

[5] G. Harms, A. C. Layton, H. M. Dionisi, I. R Gregory, V. M. Garrett, S. A. Hawkins, K. G. Robinson, and G. S. Sayler, "Real-Time PCR Quantification of Nitrifying Bacteria in a Municipal Wastewater Treatment Plant," in *Environmental Science and Technology*, Vol. 31, No. 2, pp. 343-351, 2003.

[6] A. J. Holmes, A. Costello, M. E. Lidstrom, and J. C. Murrell, "Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related," in *FEMS Microbiology Letters*, Vol. 132, No. 3, pp. 203-208, October 1995.

[7] T. L. Tyler, "Detection and Characterization of Urease Genes in Groundwater Bacterial Communities," Ph.D. thesis, Department of Biology, Idaho State University, Pocatello, Idaho, 2004.

[8] Y. Okano, Y. R. Hristova, C. M. Leutenegger, L. E. Jackson, R. F. Denison, B. Gebreyesus, D. Lebauer, and K. M. Scow, "Application of Real-Time PCR To Study Effects of Ammonium on Population Size of Ammonia-Oxidizing Bacteria in Soil," in *Applied Environmental Microbiology*, Vol. 70, No 2, pp. 1008-1016, Feb. 2004.

[9] D. W. Reed, S. Freeman, L. M. Petzke, and Y. Fujita, "Quantification of Ammonia Oxidizing Bacteria in Groundwater Treated With Urea to Promote Calcite Precipitation," poster presented at the *2005 International Symposia for Subsurface Microbiology and Environmental Biogeochemistry*, August 14th-19th, 2005, Jackson Hole, Wyoming, USA.

[10] "BLAST Database (Basic Local Alignment Search Tool)", Online document, Version BLAST 2.2.11 released, National Center for Biotechnology Information, National Library of Medicine, and National Institutes of Health. http://www.ncbi.nlm.nih.gov/BLAST/

[11] F. von Wintzingerode, U. B. Gobel, and E. Stackebrandt, "Determination of microbal diversity in environmental samples: Pitfalls of PCR-based rRNA analysis", in *FEMS Microbiology Reviews*, Vol. 21, pp. 213-229, November 1997.