Detection and Enumeration of Bacterial Urease mRNA by Quantitative Reverse-Transcription PCR

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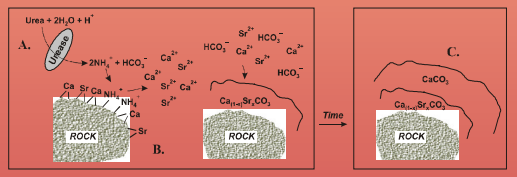
ABSTRACT

The ureolytic capability of microorganisms plays an integral role in a novel remediation scheme for immobilization of trace metals and radionuclides (e.g., ^{90}Sr) in the subsurface by co-precipitation in calcite. Calcite precipitation is accelerated by the indigenous microbial hydrolysis of urea, which results in increased pH and alkalinity.[1] We hypothesize that the rate at which urea hydrolysis occurs in the environment is directly correlated to the amount of urease messenger RNA (mRNA) produced by the native environmental Bacterial community because there is a direct relationship between mRNA copy number and active enzyme units. In order for this hypothesis to be tested, a method is needed to quantify Bacterial urease mRNA. We developed a quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay for transcripts of Bacterial *ure*C, the gene coding for the large catalytic subunit of urease. Degenerate PCR primers were designed based on alignments of known ureolytic organisms and ureolytic isolates that were collected from the Snake River Plain Aquifer in Idaho, USA. SYBR Green I was used to measure fluorescence in real-time PCR. *Bacillus pasteurii*, a constitutive ureolytic organism, and *Escherichia coli*, a non-ureolytic organism, were used as positive and negative qRT-PCR controls, respectively. Standard curves were generated using purified PCR product amplified from *B. pasteurii* cDNA. The standard curve was linear over 7 orders of magnitude with a detection limit at 3.6 fg cDNA per reaction. All PCR products were analyzed by gel electrophoresis to ensure the correct amplicon size; previous work with closely related primers had shown that amplified products from environmental DNA had the expected sequence homology with *ure*C. We evaluated different preservation methods for actively growing *B. pasteurii* cells prior to RNA isolation by comparing quantities of *ure*C qRT-PCR products. The most effective preservation methods for *ure*C mRNA processing were freezing cell pellets at -80°C or storing them in RNAlater (Ambion) solution. We also investigated the most effective method for the isolation of RNA from Bio-Sep beads (a composite of aramid polymer and powdered activated carbon) that have been previously colonized by bacteria in the environment. Bio-Sep beads were incubated in groundwater amended with molasses and urea. While many types of RNA isolations were tested, total RNA was most successfully extracted from the beads using Qiagen RNeasy chemistry with silica-based-gel columns, high salt conditions, and quick processing time. While cDNA transcribed from the mRNA from the groundwater enrichments was amplified by the qRT-PCR assay for *ure*C, it was below quantifiable levels. In summary, the optimization of each part of the qRT-PCR assay (cell preservation, RNA isolation, and qRT-PCR) is essential for accurately measuring mRNA in the environment.

MOTIVATION FOR RESEARCH

UREOLYTICALLY DRIVEN CALCITE PRECIPITATION AND CO-PRECIPITATION OF ^{90}Sr

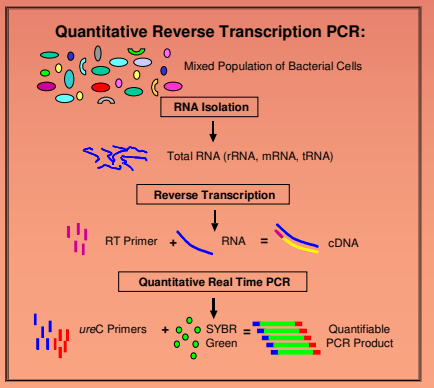


A. Urea hydrolysis produces NH_4^+ and HCO_3^-, and raises pH.

B. NH_4^+ exchanges with metals on mineral surfaces. HCO_3^- promotes calcite precipitation and co-precipitation of the strontium-90 (^{90}Sr).

C. Continued precipitation of calcite isolates ^{90}Sr from contact with groundwater.

METHODS



Preservation of Cells

Five treatments of actively growing *B. pasteurii* cells were tested:

1. Extraction of fresh cell pellet
2. Extraction of cell pellet frozen at -80°C
3. Extraction of cell pellet suspended in 50 µl (1:5 ratio) RNAlater (Ambion)
4. Extraction of cell pellet suspended in 50 µl (1:5 ratio) of RNAprotect (Qiagen)
5. Extraction of cells preserved in a 1:2 ratio of cell suspension to RNAprotect.

Cell pellets for treatments 1-4 were obtained by centrifuging at 15,000 x *g* for 5 min. Treatments 3 and 4 were incubated at 4°C overnight then transferred to -80°C prior to extraction. Treatment 5 was incubated at room temperature for 5 min after addition of RNAprotect followed by centrifugation at 5,000 x *g* for 10 min, and the resulting pellet was frozen at -80°C prior to extraction.

Groundwater Enrichments

100 ml water from the Snake River Plain Aquifer was added to Bio-Sep beads previously soaked in an autoclaved 1% molasses solution. *B. pasteurii* cells ($1x10^9$) were added to 2 enrichments which were placed at 12°C or 25°C. One enrichment, containing only molasses soaked Bio-Sep beads, was placed at 25°C.

qRT-PCR

RNA was isolated with the RNeasy Isolation Kit (Qiagen) and treated with DNase I (Ambion). Reverse Transcription was performed with the Thermo-X Reverse Transcriptase (Invitrogen) using random hexamers. Degenerate primers 385iF and 733R[2] designed to target all ureolytic bacteria were used in quantitative real-time PCR with the Lightcycler-FastStart DNA Master SYBR Green I (Roche) chemistry.

RESULTS

A.

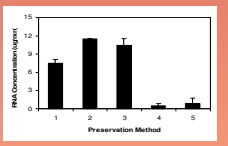


Figure 1. Total RNA measured by UV absorbance based on 3 replicates. Preservation methods 1-5 are listed in the Method section of this poster.

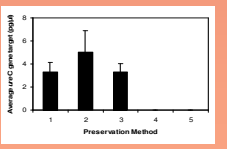


Figure 2. Quantification of ureC gene targets measured by qRT-PCR based on 3 replicates. Preservation methods 1-5 are listed in the Method section of this poster.

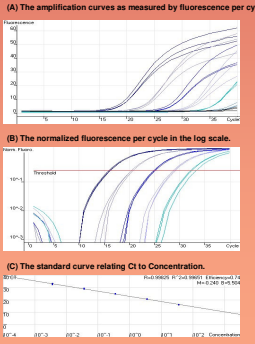


Figure 3. Standard curve amplification of *B. pasteurii* for the RNA preservation analysis.

B.

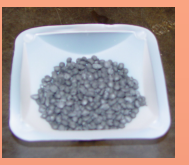


Figure 4. Bio-Sep beads prior to incubation in laboratory enrichments.

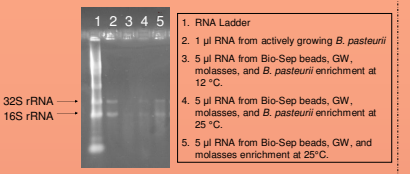


Figure 5. Agarose gel containing RNA isolated from laboratory enrichments. Control enrichment did not contain visible rRNA (data not shown)

CONCLUSIONS

▪The most effective preservation methods for preserving *ure*C mRNA are freezing a cell pellet at -80˚C and storing a cell pellet in RNAlater. The -80˚C method may be useful for freezing soil and other biomass-containing solids, while the RNAlater method may be most suitable for use in the field.

▪Total RNA was isolated (using Qiagen RNeasy chemistry) from Bio-Sep beads incubated in groundwater enrichments. Improved sensitivity of this isolation method will allow *ure*C mRNA to be quantified by the qRT-PCR method.

REFERENCES

[1]Fujita, Y., Ferris, F. G., Lawson, R. D., Colwell, F. S., and R. W. Smith. 2000 Geomicrobiol. J. 17: 305-318.

[2]Petzke, L.M. PhD Dissertation. Idaho State University. In preparation.

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