The Effect of Trichloroethene on Minimum Energy Requirement and Gene Expression in a Nutrient Limited Methanotroph

M. E. Delwiche[1], D. T. Newby[1], A. Wood[2*], M. Bingham[3], R. L. Crawford[4], and F. S. Colwell[1]

[1]Idaho National Laboratory, Idaho Falls, ID
[2]Brigham Young University, Provo, UT
[3]Idaho State University, Pocatello, ID
[4]University of Idaho, Moscow, ID
*Pre-Science Teacher Fellow

Abstract

Monitored natural attenuation (MNA) of contaminant plumes requires data for predictive modeling of plume destruction including the rates of microbial contaminant degradation. Methanotrophs are implicated in co-metabolism of trichloroethylene (TCE) in the Snake River Plain aquifer (SRPA) where MNA is the selected method of treatment. Our research aims to: 1) determine realistic activities of these cells when starved, a condition typical of subsurface microbes, and 2) detect the genes that are transcribed when methanotrophs experience stress or starvation related to TCE exposure and conditions in the subsurface. *Methylosinus trichosporium* OB3b (OB3b), a model methanotroph, was starved in a biomass recycle reactor and soluble methane monooxygenase (sMMO) activities determined, with and without TCE exposure (ca. 100 µg TCE/L). Starved methanotrophs, present at 3 x 10^8 cells/mL in the reactor, consumed methane at 0.001 fmoles of methane/cell/day and gradually increased sMMO activities when exposed to higher methane concentrations. sMMO activities of starved OB3b cells exposed to TCE were indistinguishable from cells that were not exposed over brief (one day) periods. The sequences of eight genes, known to code for starvation/stress proteins, were retrieved from phylogenetic relatives (α-proteobacteria) of OB3b. Primers (18-22 bp) were designed from conserved regions in the consensus sequences to obtain OB3b-specific sequences for the eight genes. Primers for the starvation/stress genes successfully amplified all eight genes in OB3b using PCR. Our plan is to clone and sequence these OB3b genes then synthesize oligonucleotides that can be added to a microarray that includes targets for OB3b structural and regulatory gene sequences as a prelude to evaluating gene expression under different nutrient availability conditions and in the presence and absence of TCE.

Incorporation of starvation-based rate estimates into natural attenuation models of contaminant plumes will permit estimates of the fraction of TCE natural attenuation that can be attributed to methanotrophic co-metabolism in a given aquifer system.

Introduction

Monitored natural attenuation (MNA) is a cost effective strategy for destroying chlorinated solvent groundwater plumes. However, the use of MNA depends upon accurate biogeochemical data for the site including a reasonable evaluation of the microbial activity responsible for contaminant mineralization. Methanotrophs are key contributors to natural attenuation of trichloroethylene (TCE) through aerobic co-metabolism. As with most subsurface microbes, methanotrophs are expected to exhibit low metabolic activities when living in an aquifer. In order to simulate these energy-poor subsurface conditions we have used a biomass recycle reactor (BRR) to starve *Methylosinus trichosporium* strain OB3b (a model methanotroph) in order to evaluate methanotrophic activity and gene expression with and without TCE.

Our objectives are to: 1) provide estimates of the activity of methanotrophs in oligotrophic environments, 2) evaluate the effect of TCE on that activity, and 3) develop assays for evaluating gene expression in methanotrophs grown under oligotrophic conditions. These activities, especially as they relate to the co-metabolism of TCE by starved methanotrophs, may be used to improve models that describe the rates of natural attenuation of TCE. In addition, we hope to understand how these essential microbes respond to starvation and TCE in the context of gene expression.

Methods

BRR operation: To simulate conditions of subsurface energy flow, OB3b was cultured in a BRR (Figure 1). The culture was grown with excess energy (5% methane in air) to high cell density (>5 x 10^8 cells/mL) as determined by fluorescence microscopy. Once cell numbers stabilized, culture medium (nitrate minimal salts or NMS) was replaced by cycling through a laminar flow filtration device (Millipore) to remove metabolic wastes while retaining biomass. Methane was then reduced (>1%) to poise the culture at starvation conditions. Once stationary phase culture conditions were attained, the BRR was operated in fed batch rather than continuous mode. Effective dilution rate was 0.012 h^{-1}. Methane concentration was monitored by three methods: metered feed rate, direct measurement by gas chromatograph (GC) of headspace composition, and measurement of dissolved concentration in permeate by bubble extraction GC analysis.

Coumarin oxidation assay: The metabolic state of the starved culture was monitored by measuring the rate at which soluble methane monooxygenase (sMMO) oxidizes coumarin, an analog for methane. Samples (10 mL) were removed from the BRR, the cells pelleted by centrifugation, resuspended in fresh methane-free NMS, and then provided with 25 mM sodium formate (reductant) and 10 mM coumarin and immediately placed on the fluorimeter for reading. Coumarin oxidation was generally linear during the 45-min assay. Coumarin oxidation produces a fluorescent product measured on a spectrophotometer. The rate of coumarin oxidation was compared to the rate of methane oxidation by testing a split sample from the BRR. One half of the sample was subjected to the coumarin assay, while the other half was monitored for disappearance of headspace methane. Rates of coumarin and methane oxidation (moles/cell/day) were found to be equivalent.

TCE experiment: To determine the influence of TCE on starved cells, samples were removed from the BRR, treated as described above and then exposed to 0.0015 mg TCE/L NMS (150 ppb) in serum vials. Coumarin oxidation rates were measured after 0, 5, and 24 h of TCE exposure and compared to rates obtained for cultures of OB3b simultaneously extracted from the BRR but not exposed to TCE. At each sampling time, headspace in the vials was tested for TCE by GC analysis. Controls consisting of 10 ml sterile NMS medium with 150 ppb TCE were used to account for TCE loss due to adsorption and leakage.

Starvation genes: Because the OB3b chromosome has not been sequenced, many of its gene sequences are not available in public databases. Eight gene sequences coding for proteins related to housekeeping, cell stress, and key metabolic activities were retrieved from databases with data on phylogenetic relatives of OB3b in the taxa α-proteobacteria (Table 1). Oligonucleotide probes (50-60 bp) for a microarray need to be specific, but analysis of the sequence alignment of these genes showed low sequence conservation, so probes designed from consensus sequences were highly degenerate. Therefore, OB3b-specific sequences for the eight genes needed to be determined. First, primers (18-22 bp) were designed from shorter regions that were conserved in the consensus sequences. The amplicons will be cloned and sequenced. OB3b specific oligonucleotides will be synthesized from this sequence information, and added to a microarray that includes sequences of structural and regulatory gene sequences for *mmo* and *pmo* targets for later analysis of gene expression under different nutrient availability conditions and in the presence and absence of TCE.

PCR conditions: We used Invitrogen SureStart Taq polymerase. Some of the primers required optimization with $MgCl_2$. The thermocycler program: 95° C, 9 min hot start; 3 temperature, 40 cycles- 94° C 1 min, 48° C 1 min, 72° C 2 min, extend 72° C for 15 min, hold at 4° C.

Figure 1. The BRR consists of a 2 L culture vessel, feed and recirculation pumps, gas supply and vent tubing, laminar flow recycle filter, and connecting tubing. The sparged reactor vessel contains 1200 ml of culture, is stirred, and maintained at 30° C.

Table 1. Candidate methanotroph genes of interest, the function of the gene product, the expected amplicon size, and the respective primers that are conserved in consensus sequences for the α-proteobacteria.

Gene	Gene product function	Expected amplicon size (bp)	Primer sequences (5' – 3')
ntrA	Encodes σ^{54}; controls mmoX expression	700	L:AACTGATGCAGTCGATCCAG R: GCCAGGAGATCGAGGTTG
rpoS	encodes σ^{s}; subunit of RNA polymerase	606	L:BTTCTCGACCTAYGCCACCT R:GAACARCGACTCRCTGGACA
relA	Amino acid restriction	617	L:YTCCGAGGAGACSATGGAMA R:GCCAGGAATAGGCRTTSGAT
spoT	Starvation protein, caloric restriction	680	L:CAGGATATGCGYGAVGAGYT R:GCCTTTBGGMGTRAARCAGA
uspA	Universal Stress Protein (UspA); ancient, conserved proteins found in bacteria, archaea, flies, fungi, plants	604	L:AATGDHACGAABGGCTGAAG R:GTBACDCCACCAAAGACGAG
*gro*EL	Heat-shock protein	950	L:CGTCACCAAYSCBGANAAGA R:CTTCSGTSGTGABCAGCAGN
*gro*ES	Heat-shock protein	327	L:GGSATCATCATYCCCGAYAC R:CCATSACGTCGSTTTCCTTC
tuf	Encodes elongation factor Tu; housekeeping gene	507	L:TCACTACGCVACGTYGACT R:TCBTCRCCRACCTTVACGAT

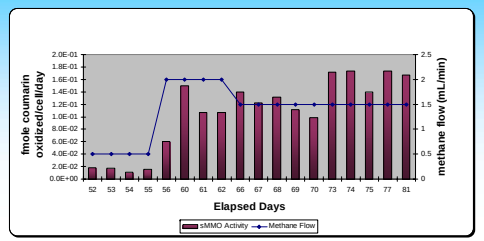

Figure 2. Relationship of sMMO activity (coumarin oxidation rate) to methane feed rate for *M. trichosporium* OB3b cells grown in a BRR. Total gas feed was the methane rate shown plus 50 ml/min air.

Results and Discussion

For cells derived from the BRR, OB3b responds slowly to changes in availability of methane (Figure 2). With stable methane concentrations the coumarin oxidation rates of the cells remain constant. If methane supply is interrupted for a prolonged period (several hours to several days), the ability to oxidize coumarin drops to near zero. When methane feed is re-established, that ability slowly recovers. For BRR-starved cells, coumarin oxidation was not detectable when dissolved methane concentration was below 2 nM. Excess methane (>1% in feed) did not result in any change to coumarin oxidation rate.

TCE was transformed by OB3b cells grown in the BRR (Figure 3). TCE disappeared from the headspace of these cultures more rapidly than it did from abiotic controls although loss of TCE also occurred in vials without cells. OB3b cells from the BRR held for 5 and 24 h showed a gradual decrease in sMMO activity regardless of whether TCE was present or not.

Representative stress genes (Table 1) proved to have enough sequence conservation across members of the α-proteobacteria to allow the design of primers. When PCR was conducted with DNA extracted from OB3b, these primers enabled successful amplification of bands that were of appropriate size (except for the tuf gene) to match the targeted gene sequences (Figure 4). All eight primer sets for the genes of interest resulted in amplification of products of the expected size.

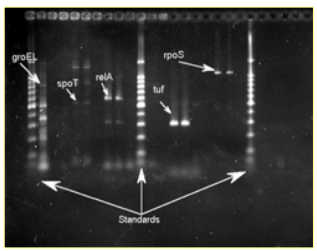

Figure 4: Five of the eight genes amplified from DNA extracted from *Methylosinus trichosporium* OB3b by using the primers as described in Table 1.

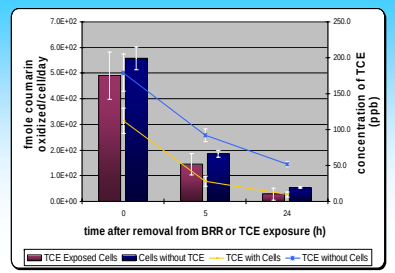

Figure 3. sMMO activity and TCE concentrations for *M. trichosporium* OB3b cultures from the BRR when the cells are either exposed and not exposed to TCE.

Conclusions

Methane levels in the BRR dictate the level of sMMO activity in OB3b cells from the BRR. Cells from the BRR respond slowly (>one day) to changes in the methane concentration.

Extended exposure of starved OB3b cells to TCE caused sMMO rates to decrease. However, the rate of sMMO activity decreased in these cells even when they were not exposed to TCE. OB3b cells from the BRR showed an ability to decrease the levels of TCE present in the aqueous culture.

Next Steps

We are comparing coumarin oxidation rates (and TCE co-metabolism) in BRR-sustained cells to cells that are rapidly growing under excess methane concentrations. We are also considering how TCE co-metabolism by BRR-sustained cells varies according to differences in TCE concentration and methane concentration.

Related to the stress genes we are cloning and sequencing the amplicons obtained through the use of our primers to confirm that the sequences are related to genes that code for proteins known to be expressed under conditions of cell stress. Once confirmed, these sequences will be used to design probes for microarrays used to discover gene function under different metabolic conditions, in our case, starved cells, or cells in the presence of TCE. In anticipation of development of these microarrays, cells from the BRR, with and without TCE exposure, have been preserved for extraction of RNA for reverse transcriptase PCR.

Acknowledgements

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