EXTRACTING AND IDENTIFYING ENVIRONMENTAL PROTEIN MARKERS FROM TCE-CONTAMINATED GROUNDWATER

Ravindra Paidisetti, Ronald Crawford, and Andrzej Paszczynski
Environmental Biotechnology Institute, University of Idaho, Moscow, ID, USA.

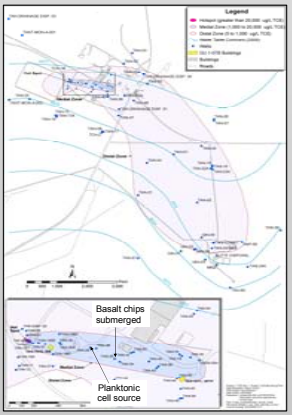
University of Idaho

Abstract

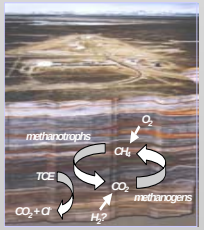
Proteomics-based techniques for monitoring pollutant degradation processes are rapidly developing as excellent tools for improved management of environmental remediation technologies. Recently, tandem mass spectrometry has been used to measure gene expression at the protein level to provide real-time information about environmental interactions between pollutants and microbial communities. In the present study, we applied environmental proteomics using MS/MS to investigate the co-metabolic degradation of trichloroethylene (TCE), a widely-occurring and toxic groundwater contaminant. Our goal was to identify biomarkers (signature peptides) within methanotrophic bacterial proteins such as methane monooxygenase (MMO) that are active in degradation of TCE. We have developed a new method for extracting total proteins from environmental samples. Utilization of the method in combination with MS/MS resulted in successful extraction and identification of MMO-based biomarker peptides from both pure cultures and environmental samples.

Introduction

Proteomics is one of the fastest developing research areas contributing to the understanding of the metabolic dynamics of biological systems. There are now a number of new approaches to isolate and sequence proteins extracted directly from environmental samples. By directly analyzing the metaproteomes of environmental samples, the contributions of microorganisms to biodegradation of a given pollutant can be studied (1, 2). One contaminant of environmental concern is trichloroethylene (TCE), an anthropogenic compound that currently contaminates many aquifers in the U.S. TCE has been used as an additive in adhesives, paint removers, and typewriter correction fluids as well as in degreasing operations and radionuclide processing (3). Our current investigations were focused on co-metabolic degradation of TCE in groundwater sources at the Test Area North (TAN) site on Idaho National Laboratory (INL) land near Idaho Falls, ID. Groundwater at the TAN site is contaminated with TCE that was used as a solvent for radionuclide processing. The removal of TCE by physical procedures such as pump-and-treat and vacuum extraction have been largely unsuccessful at sites like TAN. There has been growing interest in bioremediation for TCE contamination because it results in effective degradation of chemical compounds with lower human exposure risk and minimal environmental disturbance. Monitoring of the TCE plume at the TAN site indicates diminution of TCE through natural attenuation processes. However, confirmation of co-metabolic degradation of TCE by natural attenuation at TAN requires proof of the metabolic involvement of methanotrophs in the degradation process. Methanotrophs produce a set of proteins called methane monooxygenases (MMO) that oxidize methane as their natural substrate, thereby providing a carbon source for the organisms (5). MMOs are also capable of co-metabolically transforming chlorinated solvents like TCE into nontoxic end products such as CO_2 and Cl^- (3). MMO exists in two forms, a membrane-bound particulate form (pMMO) and a cytoplasmic soluble form (sMMO). The particulate MMO form has been observed in almost all methanotrophs, whereas the soluble form has been observed in only a few (4). pMMO form consists of two components, pMMOH (a hydroxylase comprised of 47, 27, and 24-kDa subunits) and pMMOR (a reductase comprised of 63 and 8-kDa subunits). The active center of pMMO consists of a trinuclear copper cluster and a mononuclear Fe^{2+} in an octahedral environment (4, 5). sMMO consists of three components: a hydroxylase (protein A-250 kDa), a dimer of three subunits ($\alpha_2\beta_2\gamma_2$), a regulatory protein (protein B-15.8 kDa), and a reductase (protein C-38.6 kDa). The active form of sMMO contains a unique di-iron non-heme site that binds dioxygen, methane, and other substrates (5).



This map shows Test Area North (TAN) in the Snake River Plain Aquifer, the TCE plume and the wells used to monitor groundwater conditions. TCE and other liquid wastes were injected into well TSF-05 (near the TCE hot spot) from 1952 to 1972. The contamination has spread southeast via groundwater flow. We have sampled microbial communities within the medial zones of wells TAN 29 (planktonic samples) and TAN 35 (basalt chip biofilms).



Conceptual model of TCE co-metabolism in the Snake River Plain aquifer showing a hypothetical cross-section of the layered, fractured basalts. Biogenic methane is dissolved in the groundwater and sustains methanotrophs, a key groundwater community that actively expresses the enzymes required to dechlorinate the TCE.

Objectives

Our general project goal is to develop a proteomics-based process to monitor natural attenuation of TCE at the TAN site by targeting the identification of signature peptides derived from pMMO and from the evolutionarily conserved *alpha* subunit of the sMMO hydroxylase component.

Our specific objectives for the effort described here were to:

1)Extract and identify signature peptides from the reference culture *Methylosinus trichosporium* OB3b and

2) Extract and identify signature peptides from environmental samples (planktonic samples).

Materials and methods

***Methylosinus trichosporium* OB3b cultures:** OB3b cultures were prepared by growth in nitrogen mineral salt media (NMS Media) at 30˚C and shaking at 200 rpm. Liquid cultures were grown in 150-mL serum vials supplied with a headspace of methane and air (3:7). For the expression of sMMO, cultures were grown in copper-free NMS media. Copper (4 μM) was added to the media for expression of the pMMO. The cultures were harvested at mid-log phase by centrifugation at 5,000 rpm for 10 min. The pellets were suspended in NMS media with 5% DMSO and stored at -80˚C until further analysis.

Planktonic cell samples: Planktonic bacteria from 600 L of water from the TAN 29 well (see map) were concentrated by filtration (0.05-μm filter). Approximately 1,300 mL of the filtrate was transported on ice to the University of Idaho. The filtrate was further centrifuged at 7,000 g for 20 min to concentrate the cells into a final volume of 20 mL. The total number of bacterial cells was 6 x 10^8/mL. Cell concentrates were preserved at -80˚C prior to proteomic analysis. Correlation between the amounts of proteins being expressed with the numbers of methanotrophs and MMO genes was studied using RT2PCR.

Proteomic analysis: We developed a method using tandem mass-spectrometry to analyze trypsinized whole-protein preparations of both *M. trichosporium* OB3b (internal control) and field samples of planktonic cells from the TAN aquifer (see Fig. 1).

Pure culture (OB3b) or environmental samples
↓
Cells suspended in equal volume of 40% formic acid boiled for 15 min
↓
Vacuum dry to evaporate formic acid
↓
Resuspend the cell pellets in 100mM ammonium bicarbonate (pH 8)
↓
Add 10 mM DTT to the supernatant and incubate at 50˚C for 15 min
↓
Add 20 mM IAM and incubate in dark at room temp. for 15 min
↓
Add trypsin (100 ng/μl) to reduced and alkylated protein (1:100 ratio) and incubate at 37˚C for 15 h
↓
Centrifuge for 5 min at 10,000 g
↓
Sample for UPLC ESI MS/MS analysis (see Fig. 5)

Fig. 1: Extraction and digestion of total protein from OB3b cultures and subsequent sample preparation for tandem mass spectrometry (MS).

MS Data generation: The digested protein samples were separated using a Nanoacquity ultra performance liquid chromatograph (UPLC) and delivered to the tandem MS for analysis. Samples were loaded into a trapping column attached in tandem to an eluting/separation column. Samples were trapped onto the trapping column for 5 min using 100% A (0.1% formic acid in 100% DDH_2O). Separation of the samples was achieved by using a gradient of solutions A & B (0.01% formic acid in 100% ACN). The eluted sample fractions were directed to the QTof Premiere nanospray ion source for mass spectral analysis. MS data was analyzed using Mass Lynx Windows XP software (version 4).

MS Data analysis: Analysis of MS/MS spectra and sequencing and identification of protein and peptides were completed by using ProteinLynx Global Server Version 2.2. The software identifies proteins by comparing probability scoring and cross-correlation algorithms for the MS/MS data with known databases (protein identification by peptide mass fingerprinting, http://www.waters.com for probability scoring and http://www.thermo.com for cross-correlation algorithms). The identification of proteins and peptides was performed by searching the MS spectra against the Swissprot non-redundant and/or MMO databases.

Results

- We showed that it is possible to successfully apply and optimize a formic acid-heat lysis procedure to extract total protein from both OB3b cultures and environmental samples.
- MMO proteins in OB3b cells and planktonic sample were expressed in high quantities in the presence of methane and/or TCE. They were among the most numerous source of peptides identified from both pure cultures and environmental samples.
- MMO peptide signatures were detected at cell concentrations as low as 1 mg (or 4 x 10^5 cells) and at sMMO protein concentrations of 10-50 pg, which represents 10^4-10^5 methanotrophs.

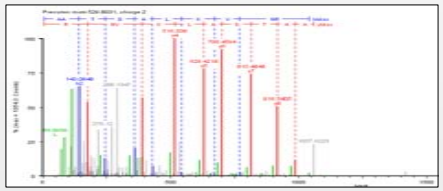


Fig. 2: MS/MS spectrum of a peptide sequence from the sMMO hydroxylase component of the alpha subunit obtained from the OB3b culture. The two series marked Y and B represent the single amino acid lost.

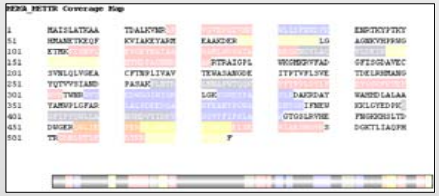


Fig. 3: The hydroxylase alpha subunit of the sMMO protein sequence showing 52.4% sequence coverage that is contributed by 24 peptides (shown in different colors).

Table 1: The top 10 peptide hits of the total protein extracted from OB3b cultures (as identified by the Protein Lynx Global Server).

Accession	Description	mW	Peptides	Coverage (%)
P27353	MMO A alpha chain	59,916	24	52.471
P27354	MMO A beta chain	44,992	17	46.446
P27355	MMO A gamma chain	19,314	9	44.970
P22869	MMO A alpha chain	60,609	7	11.764
P75022	Elongation factor Tu EF Tu	42,599	5	11.764
P35862	60-kDa Cpn60 3 groEL protein	57,618	5	9.174
P27356	MMO regulatory protein B	14,873	4	42.753
Q930X9	10-kDa Cpn10 3 groES protein	11,165	4	27.619
P33253	ATP synthase beta chain	51,512	3	11.252
P12293	Methanol dehydrogenase subunit	69,753	3	3.962

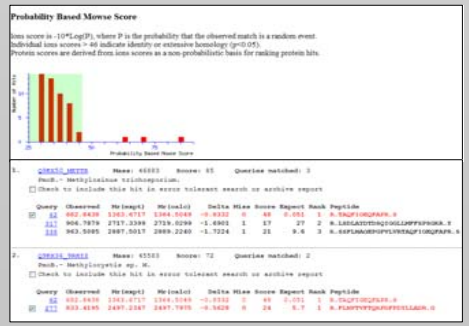


Fig. 4: pMMO peptides identified in the TAN 29 planktonic sample (results obtained using MASCOT).

Table 2: Proteins and bacterial species identified in the TAN 29 planktonic biomass sample.

Accession	Protein identified	Bacterial species	Group
Q9KX50	PmoB	*Methylosinus trichosporium*	Alphaproteobacteria
Q9KX36	PmoB	*Methylocystis sp. M*	Alphaproteobacteria
JQ0706	Alcohol dehydrogenase alpha chain precursor	*Methylobacterium extorquens*	Alphaproteobacteria
Q605M5	Hypothetical protein	*Methylococcus capsulatus str. Bath*	Gammaproteobacteria
Q8RMH5	Large subunit of methanol dehydrogenase	*Methylobacillus sp. SK-5*	Betaproteobacteria
Q8XZY7	Glutamine synthetase	*Ralstonia solanacearum*	Betaproteobacteria
Q7WZR9	Ribosomal protein L15	*Candidatus Portiera aleyrodidarum*	Gammaproteobacteria
Q6N5A3	30S ribosomal protein S18	*Rhodopseudomonas palustris*	Alphaproteobacteria
F95407	Formyltetrahydrofolate deformylase	*Sinorhizobium meliloti*	Alphaproteobacteria
Q7MQ77	Hypothetical protein VV0131	*Vibrio vulnificus*	Gammaproteobacteria
E83151	Hypothetical protein PA3949	*Pseudomonas aeruginosa PAO1*	Gammaproteobacteria
Q6HF43	Hypothetical protein	*Bacillus thuringiensis*	Firmicutes
Q7P4J1	Hypothetical protein	*Fusobacterium nucleatum*	Fusobacteria
Q7X3V8	CtpE gene product (unknown function)	*Erwinia sp. Ejp 556*	Gammaproteobacteria
Q84BQ4	Arthrofactin synthetase C	*Pseudomonas sp. MIS38*	Gammaproteobacteria
Q7UDL8	Putative lipoprotein	*Shigella flexneri*	Gammaproteobacteria
Q6ALQ9	5' to 3' DNA helicase	*Desulfotalea psychrophila*	Deltaproteobacteria
AAD06018	Unknown function	*Helicobacter pylori J99*	Epsilonproteobacteria
Q6MP21	Hypothetical protein precursor	*Bdellovibrio bacteriovorus*	Deltaproteobacteria
Q8F2E1	Two-component hybrid sensor and regulator	*Leptospira interrogans*	Spirochaetes
Q70C90	Hypothetical protein	*Streptococcus thermophilus*	Firmicutes
Q64YE3	Putative outer membrane protein	*Bacteroides fragilis*	Bacteroidetes
Q6R3I4	Conserved protein	*Gemmata sp. Wa1-1*	Planctomycetes
Q5YTH3	Hypothetical protein	*Nocardia farcinica*	Actinobacteria
Q84BI0	Vls recombination cassette Vls4 protein	*Borrelia afzelii*	Spirochaetes

Conclusions

- The formic acid-heat lysis procedure results in successful extraction of total protein from pure and environmental samples, and the method should be broadly applicable for consistent and reliable protein extraction from environmental samples.
- The pMMO peptides observed in the planktonic samples are consistent with the hypothesis that environmental copper concentrations initiate expression of the particulate MMO form and suppress expression of the soluble form.

Future directions

The methods developed will be further optimized for identification of both particulate and soluble methane monooxygenases from basalt chip biofilms from the TAN 35 well and other environmental samples.

Fig. 5: Waters® Nanoacquity UPLC and nanospray quadrupole-time of flight (QTof) Premiere used for sample analysis.

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