

ABSTRACT

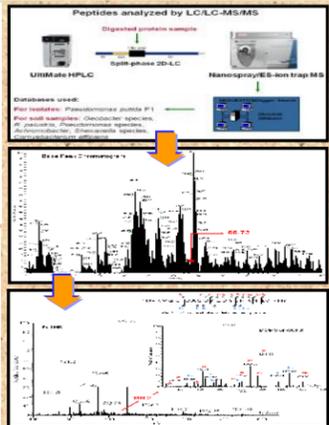
A potential remediation strategy for metal-contaminated subsurface environments is the deployment of microbes for biotransformation of the oxidized, mobile metals to sparingly soluble, reduced forms. Success of such a strategy depends on knowledge of structure and dynamics of the indigenously active microbial community. Mass spectrometry (MS) based proteomics techniques have tremendous potential in unraveling the molecular level protein details of active *in situ* microbial communities in soils or ground-waters. For example, we have conducted proteome MS measurements on three ground-water field samples after acetate amendment at the DOE Rifle Field site in Colorado. We have obtained deep proteomes (~3,000 proteins) that were completely dominated by *Geobacter* species in the ground water samples, which provided information about *Geobacter* physiology during uranium reduction after acetate amendment. Extraction of microbial proteomes from low biomass soil samples in presence of deterrents such as humic acid poses quite a distinct challenge. We have compared several *in-situ* lysis methods for efficient extraction of proteomes from metal contaminated sediment samples (Rifle, Colorado and Hanford, Washington). The use of shotgun MS proteomics yields insight into the proteomic profiles of the soil microbes, providing information about functional activities in these environmental communities. It is well known that bioaugmentation of metal immobilization through reductive precipitation can be accelerated by addition of microbial species such as *Pseudomonas* to soil microbial communities. By using MS proteomics methodologies, we have investigated how *Pseudomonas putida* strain F1 responds to a chromate challenge. Of the ~1700 proteins identified by MS in *P. putida* isolates, about 100 were upregulated under chromate shock. Noteworthy among them were proteins involved in iron transport and metabolism. These studies will help elucidate how to effectively utilize microbes for chromate transformation at field scales.

PROJECT OBJECTIVES

- Extend shotgun mass spectrometry (MS)-based proteomic methods (shown below) as a key molecular-level tool for profiling the metabolic activity and potential of microbial species in the context of complex communities.
- Assess how structure and function in microcosm microbial communities are altered in response to nutrient (glucose, lactate) and chromate amendments.
- Identify microbial molecular indicators (i.e., unique and/or abundant proteins) for metal stress and reduction that might be useful in monitoring bioremediation potential and performance.
- Tackle the challenge of characterizing the proteome dataset of a complex microbial microcosm by pushing the capabilities of current MS methodologies.
- Develop the next generation of microbial ecology tools for use in assessing, and ultimately enhancing, bioremediation performance.
- Expectation:** experimental protocols and results generated will expand quantitative and mechanistic understanding of the *in situ* biological contributions to metal contaminant transformation.

EXPERIMENTAL APPROACHES

- Cells from isolates were sonicated. Proteins were separated into crude and membrane fractions by centrifugation at 100,000 x g for 60 min, followed by trypsin digestion.
- Soil samples were weighed and suspended in Lysis buffer, followed by vortexing and sonication for 20 min and then trypsin digestion.
- Trypsin digestion was accomplished by first reducing the crude or membrane fraction in 6 M guanidine and 10 mM DTT. Trypsin was added at 1:100 (wt/wt) and digested overnight at 37°C. A second trypsin aliquot was added with a final reduction using 20 mM DTT.
- Analysis was performed using a 24-h multidimensional HPLC-MS/MS protocol. Briefly, separation was carried out by 2-D separation using strong cation exchange as the first dimension and C18 reverse phase as the second dimension of separation. An LTQ XL ion trap was operated in the data-dependent mode where a full scan was acquired followed by four tandem mass spectra (MS/MS).
- Peptide identification was completed by the search engine DBDigger/SEQUENT with a protein considered a true "hit" if two unique peptides are identified.



RESULTS

Proteomics of Hanford Site Soils



Figure 1: DOE Hanford Site soil collections. (A) Cr(VI)-free soil from Hanford Background and (B) Cr(VI)-contaminated soil from Hanford 100-D site.

Challenges of Working with Environmental Samples

- Extraction of microbial proteins from low biomass soil samples in the presence of deterrents such as humic acid poses quite a distinct challenge. We have compared several *in situ* lysis methods for efficient extraction of proteomes from various metal-contaminated sediment samples. Various methods of pretreatment of the soil prior to microbial lysis gave enhanced protein yields.
- Figure 2 and Table 1 demonstrates that our procedure for protein extraction from soil mirrors the spike of *Pseudomonas putida* strain F1 in Hanford soil samples, a sequenced microbe shown to reduce chromate (K. Chourey et al., unpublished data).
- P. putida* F1 is being used as our test organism to evaluate seeding and nutritional amendment effects on microbial community dynamics in microcosms and concomitant Cr(VI) reduction.
- Our MS methods yields very high amounts of peptides most of which are currently unidentified. We will be expanding our proteome databases to confidently profile most of the active subsurface soil microbes. We will be comparing our results with 16S RNA results for the same soil samples.

Table 1: Comparison of number of proteins detected in different samples and the percentage overlap across the different treatments.

| Sample | Proteins | Cr-shocked cells in LB | Cr-shocked cells in MM | Control cells in LB | Control cells in MM | Hanford Pristine soil | Hanford contaminated soil | Soil spiked with Cr-shocked cells | Soil spiked with Control Cells |
|-----------------------------------|----------|------------------------|------------------------|---------------------|---------------------|-----------------------|---------------------------|-----------------------------------|--------------------------------|
| Cr-shocked cells in LB | 2098 | 100.00% | 84.70% | 77.40% | 77.90% | 0.20% | 0.60% | 2.80% | 14.60% |
| Cr-shocked cells in MM | 2426 | 73.30% | 100.00% | 65.60% | 73.80% | 0.20% | 0.50% | 2.20% | 12.80% |
| Control cells in LB | 1887 | 86.00% | 84.30% | 100.00% | 79.40% | 0.10% | 0.30% | 2.80% | 15.40% |
| Control cells in MM | 1976 | 82.70% | 90.60% | 75.80% | 100.00% | 0.10% | 0.60% | 2.70% | 15.50% |
| Hanford Pristine soil | 61 | 6.60% | 8.20% | 3.30% | 3.30% | 100.00% | 11.50% | 24.60% | 31.10% |
| Hanford contaminated soil | 43 | 27.90% | 30.20% | 14.00% | 27.90% | 16.30% | 100.00% | 30.20% | 81.40% |
| Soil spiked with Cr-shocked cells | 179 | 30.20% | 30.20% | 29.10% | 29.60% | 8.40% | 7.30% | 100.00% | 68.70% |
| Soil spiked with Control Cells | 736 | 41.70% | 42.10% | 39.40% | 41.60% | 2.60% | 4.80% | 16.70% | 100.00% |

Figure 2: MS-based proteomic profiles of pristine and contaminated Hanford soil, Hanford soil spiked with *P. putida* F1 cells (Control and Cr-stressed).

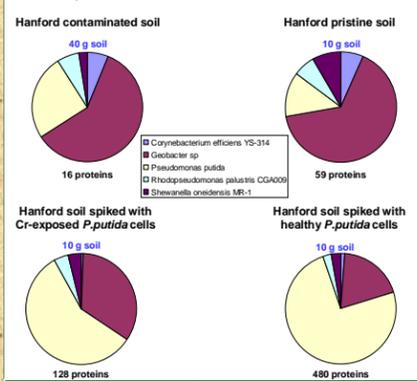


Table 2: A snapshot of unique protein expression in response to chromium shock in (A) *P. putida* F1 cells grown in Minimal media (B) *P. putida* F1 cells grown in LB media

| Sequence coverage for the protein (%) | Gene Name and its function | Sequence coverage for the protein (%) | Gene Name and its function |
|---------------------------------------|--|---------------------------------------|--|
| 762 | Put_027 Heavy metal transport/efflux/defense protein | 862 | Put_042 Heavy metal transport/efflux/defense protein |
| 762 | Put_028 Heavy metal transport/efflux/defense protein | 862 | Put_043 Heavy metal transport/efflux/defense protein |
| 762 | Put_029 Heavy metal transport/efflux/defense protein | 862 | Put_044 Heavy metal transport/efflux/defense protein |
| 762 | Put_030 Heavy metal transport/efflux/defense protein | 862 | Put_045 Heavy metal transport/efflux/defense protein |
| 762 | Put_031 Heavy metal transport/efflux/defense protein | 862 | Put_046 Heavy metal transport/efflux/defense protein |
| 762 | Put_032 Heavy metal transport/efflux/defense protein | 862 | Put_047 Heavy metal transport/efflux/defense protein |
| 762 | Put_033 Heavy metal transport/efflux/defense protein | 862 | Put_048 Heavy metal transport/efflux/defense protein |
| 762 | Put_034 Heavy metal transport/efflux/defense protein | 862 | Put_049 Heavy metal transport/efflux/defense protein |
| 762 | Put_035 Heavy metal transport/efflux/defense protein | 862 | Put_050 Heavy metal transport/efflux/defense protein |
| 762 | Put_036 Heavy metal transport/efflux/defense protein | 862 | Put_051 Heavy metal transport/efflux/defense protein |
| 762 | Put_037 Heavy metal transport/efflux/defense protein | 862 | Put_052 Heavy metal transport/efflux/defense protein |
| 762 | Put_038 Heavy metal transport/efflux/defense protein | 862 | Put_053 Heavy metal transport/efflux/defense protein |
| 762 | Put_039 Heavy metal transport/efflux/defense protein | 862 | Put_054 Heavy metal transport/efflux/defense protein |
| 762 | Put_040 Heavy metal transport/efflux/defense protein | 862 | Put_055 Heavy metal transport/efflux/defense protein |
| 762 | Put_041 Heavy metal transport/efflux/defense protein | 862 | Put_056 Heavy metal transport/efflux/defense protein |
| 762 | Put_042 Heavy metal transport/efflux/defense protein | 862 | Put_057 Heavy metal transport/efflux/defense protein |
| 762 | Put_043 Heavy metal transport/efflux/defense protein | 862 | Put_058 Heavy metal transport/efflux/defense protein |
| 762 | Put_044 Heavy metal transport/efflux/defense protein | 862 | Put_059 Heavy metal transport/efflux/defense protein |
| 762 | Put_045 Heavy metal transport/efflux/defense protein | 862 | Put_060 Heavy metal transport/efflux/defense protein |
| 762 | Put_046 Heavy metal transport/efflux/defense protein | 862 | Put_061 Heavy metal transport/efflux/defense protein |
| 762 | Put_047 Heavy metal transport/efflux/defense protein | 862 | Put_062 Heavy metal transport/efflux/defense protein |
| 762 | Put_048 Heavy metal transport/efflux/defense protein | 862 | Put_063 Heavy metal transport/efflux/defense protein |
| 762 | Put_049 Heavy metal transport/efflux/defense protein | 862 | Put_064 Heavy metal transport/efflux/defense protein |
| 762 | Put_050 Heavy metal transport/efflux/defense protein | 862 | Put_065 Heavy metal transport/efflux/defense protein |
| 762 | Put_051 Heavy metal transport/efflux/defense protein | 862 | Put_066 Heavy metal transport/efflux/defense protein |
| 762 | Put_052 Heavy metal transport/efflux/defense protein | 862 | Put_067 Heavy metal transport/efflux/defense protein |
| 762 | Put_053 Heavy metal transport/efflux/defense protein | 862 | Put_068 Heavy metal transport/efflux/defense protein |
| 762 | Put_054 Heavy metal transport/efflux/defense protein | 862 | Put_069 Heavy metal transport/efflux/defense protein |
| 762 | Put_055 Heavy metal transport/efflux/defense protein | 862 | Put_070 Heavy metal transport/efflux/defense protein |
| 762 | Put_056 Heavy metal transport/efflux/defense protein | 862 | Put_071 Heavy metal transport/efflux/defense protein |
| 762 | Put_057 Heavy metal transport/efflux/defense protein | 862 | Put_072 Heavy metal transport/efflux/defense protein |
| 762 | Put_058 Heavy metal transport/efflux/defense protein | 862 | Put_073 Heavy metal transport/efflux/defense protein |
| 762 | Put_059 Heavy metal transport/efflux/defense protein | 862 | Put_074 Heavy metal transport/efflux/defense protein |
| 762 | Put_060 Heavy metal transport/efflux/defense protein | 862 | Put_075 Heavy metal transport/efflux/defense protein |
| 762 | Put_061 Heavy metal transport/efflux/defense protein | 862 | Put_076 Heavy metal transport/efflux/defense protein |
| 762 | Put_062 Heavy metal transport/efflux/defense protein | 862 | Put_077 Heavy metal transport/efflux/defense protein |
| 762 | Put_063 Heavy metal transport/efflux/defense protein | 862 | Put_078 Heavy metal transport/efflux/defense protein |
| 762 | Put_064 Heavy metal transport/efflux/defense protein | 862 | Put_079 Heavy metal transport/efflux/defense protein |
| 762 | Put_065 Heavy metal transport/efflux/defense protein | 862 | Put_080 Heavy metal transport/efflux/defense protein |
| 762 | Put_066 Heavy metal transport/efflux/defense protein | 862 | Put_081 Heavy metal transport/efflux/defense protein |
| 762 | Put_067 Heavy metal transport/efflux/defense protein | 862 | Put_082 Heavy metal transport/efflux/defense protein |
| 762 | Put_068 Heavy metal transport/efflux/defense protein | 862 | Put_083 Heavy metal transport/efflux/defense protein |
| 762 | Put_069 Heavy metal transport/efflux/defense protein | 862 | Put_084 Heavy metal transport/efflux/defense protein |
| 762 | Put_070 Heavy metal transport/efflux/defense protein | 862 | Put_085 Heavy metal transport/efflux/defense protein |
| 762 | Put_071 Heavy metal transport/efflux/defense protein | 862 | Put_086 Heavy metal transport/efflux/defense protein |
| 762 | Put_072 Heavy metal transport/efflux/defense protein | 862 | Put_087 Heavy metal transport/efflux/defense protein |
| 762 | Put_073 Heavy metal transport/efflux/defense protein | 862 | Put_088 Heavy metal transport/efflux/defense protein |
| 762 | Put_074 Heavy metal transport/efflux/defense protein | 862 | Put_089 Heavy metal transport/efflux/defense protein |
| 762 | Put_075 Heavy metal transport/efflux/defense protein | 862 | Put_090 Heavy metal transport/efflux/defense protein |
| 762 | Put_076 Heavy metal transport/efflux/defense protein | 862 | Put_091 Heavy metal transport/efflux/defense protein |
| 762 | Put_077 Heavy metal transport/efflux/defense protein | 862 | Put_092 Heavy metal transport/efflux/defense protein |
| 762 | Put_078 Heavy metal transport/efflux/defense protein | 862 | Put_093 Heavy metal transport/efflux/defense protein |
| 762 | Put_079 Heavy metal transport/efflux/defense protein | 862 | Put_094 Heavy metal transport/efflux/defense protein |
| 762 | Put_080 Heavy metal transport/efflux/defense protein | 862 | Put_095 Heavy metal transport/efflux/defense protein |
| 762 | Put_081 Heavy metal transport/efflux/defense protein | 862 | Put_096 Heavy metal transport/efflux/defense protein |
| 762 | Put_082 Heavy metal transport/efflux/defense protein | 862 | Put_097 Heavy metal transport/efflux/defense protein |
| 762 | Put_083 Heavy metal transport/efflux/defense protein | 862 | Put_098 Heavy metal transport/efflux/defense protein |
| 762 | Put_084 Heavy metal transport/efflux/defense protein | 862 | Put_099 Heavy metal transport/efflux/defense protein |
| 762 | Put_085 Heavy metal transport/efflux/defense protein | 862 | Put_100 Heavy metal transport/efflux/defense protein |

Studies on *P. putida* F1 isolate show that its mechanism for chromate reduction depends on the medium it is surrounded with. Experience gained by studying effects of amendments on *P. putida* in soil microcosms will help evaluate the conditions in which *P. putida* may reduce chromate efficiently in soil.

Proteomics Of Rifle Site Soil



Figure 3: DOE Rifle Site, Colorado

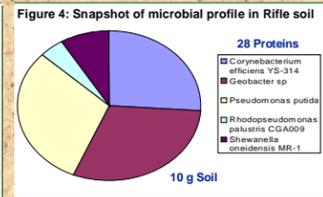


Figure 4: Snapshot of microbial profile in Rifle soil

Figure 5: Strain variation in groundwater sample

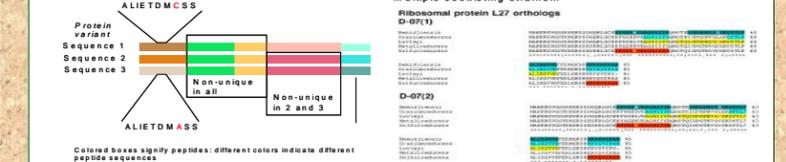


Figure 6: Experimental setup for Acetate injections and sampling timeline following the amendment.

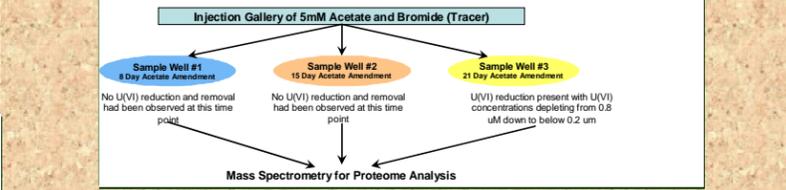


Table 3: Profile of *Geobacter* species in ground water samples at 8 days, 15 days and 21 days following acetate amendment.

| Sample-Set1(Run1) | Species | Unique count | Sample-Set1(Run2) | Species | Unique count | Sample-Set1(Run3) | Species | Unique count |
|---------------------------------|---------------------------------|--------------|---------------------------------|---------------------------------|--------------|---------------------------------|---------------------------------|--------------|
| Geobacter MZ1 | Geobacter MZ1 | 241 | Geobacter MZ1 | Geobacter MZ1 | 202 | Geobacter MZ1 | Geobacter MZ1 | 290 |
| Geobacter bemidjensis | Geobacter bemidjensis | 394 | Geobacter bemidjensis | Geobacter bemidjensis | 434 | Geobacter bemidjensis | Geobacter bemidjensis | 396 |
| Geobacter uranumreducens RF4 | Geobacter uranumreducens RF4 | 378 | Geobacter uranumreducens RF4 | Geobacter uranumreducens RF4 | 382 | Geobacter uranumreducens RF4 | Geobacter uranumreducens RF4 | 396 |
| Geobacter sp. FCC-32 | Geobacter sp. FCC-32 | 354 | Geobacter sp. FCC-32 | Geobacter sp. FCC-32 | 380 | Geobacter sp. FCC-32 | Geobacter sp. FCC-32 | 393 |
| Geobacter sulfurreducens | Geobacter sulfurreducens | 204 | Geobacter sulfurreducens | Geobacter sulfurreducens | 235 | Geobacter sulfurreducens | Geobacter sulfurreducens | 233 |
| Geobacter lovleyi SZ | Geobacter lovleyi SZ | 196 | Geobacter lovleyi SZ | Geobacter lovleyi SZ | 239 | Geobacter lovleyi SZ | Geobacter lovleyi SZ | 192 |
| Geobacter metallireducens GS-15 | Geobacter metallireducens GS-15 | 190 | Geobacter metallireducens GS-15 | Geobacter metallireducens GS-15 | 191 | Geobacter metallireducens GS-15 | Geobacter metallireducens GS-15 | 166 |
| Sample-Set2(Run1) | Species | Unique count | Sample-Set2(Run2) | Species | Unique count | Sample-Set2(Run3) | Species | Unique count |
| Geobacter MZ1 | Geobacter MZ1 | 394 | Geobacter MZ1 | Geobacter MZ1 | 344 | Geobacter MZ1 | Geobacter MZ1 | 317 |
| Geobacter bemidjensis | Geobacter bemidjensis | 451 | Geobacter bemidjensis | Geobacter bemidjensis | 446 | Geobacter bemidjensis | Geobacter bemidjensis | 432 |
| Geobacter uranumreducens RF4 | Geobacter uranumreducens RF4 | 590 | Geobacter uranumreducens RF4 | Geobacter uranumreducens RF4 | 502 | Geobacter uranumreducens RF4 | Geobacter uranumreducens RF4 | 519 |
| Geobacter sp. FCC-32 | Geobacter sp. FCC-32 | 336 | Geobacter sp. FCC-32 | Geobacter sp. FCC-32 | 273 | Geobacter sp. FCC-32 | Geobacter sp. FCC-32 | 289 |
| Geobacter sulfurreducens | Geobacter sulfurreducens | 263 | Geobacter sulfurreducens | Geobacter sulfurreducens | 245 | Geobacter sulfurreducens | Geobacter sulfurreducens | 209 |
| Geobacter lovleyi SZ | Geobacter lovleyi SZ | 212 | Geobacter lovleyi SZ | Geobacter lovleyi SZ | 196 | Geobacter lovleyi SZ | Geobacter lovleyi SZ | 170 |
| Geobacter metallireducens GS-15 | Geobacter metallireducens GS-15 | 176 | Geobacter metallireducens GS-15 | Geobacter metallireducens GS-15 | 162 | Geobacter metallireducens GS-15 | Geobacter metallireducens GS-15 | 170 |
| Sample-Set3(Run1) | Species | Unique count | Sample-Set3(Run2) | Species | Unique count | Sample-Set3(Run3) | Species | Unique count |
| Geobacter MZ1 | Geobacter MZ1 | 465 | Geobacter MZ1 | Geobacter MZ1 | 399 | Geobacter MZ1 | Geobacter MZ1 | 377 |
| Geobacter bemidjensis | Geobacter bemidjensis | 536 | Geobacter bemidjensis | Geobacter bemidjensis | 533 | Geobacter bemidjensis | Geobacter bemidjensis | 503 |
| Geobacter uranumreducens RF4 | Geobacter uranumreducens RF4 | 517 | Geobacter uranumreducens RF4 | Geobacter uranumreducens RF4 | 502 | Geobacter uranumreducens RF4 | Geobacter uranumreducens RF4 | 500 |
| Geobacter sp. FCC-32 | Geobacter sp. FCC-32 | 313 | Geobacter sp. FCC-32 | Geobacter sp. FCC-32 | 276 | Geobacter sp. FCC-32 | Geobacter sp. FCC-32 | 297 |
| Geobacter sulfurreducens | Geobacter sulfurreducens | 273 | Geobacter sulfurreducens | Geobacter sulfurreducens | 239 | Geobacter sulfurreducens | Geobacter sulfurreducens | 230 |
| Geobacter lovleyi SZ | Geobacter lovleyi SZ | 510 | Geobacter lovleyi SZ | Geobacter lovleyi SZ | 438 | Geobacter lovleyi SZ | Geobacter lovleyi SZ | 471 |
| Geobacter metallireducens GS-15 | Geobacter metallireducens GS-15 | 217 | Geobacter metallireducens GS-15 | Geobacter metallireducens GS-15 | 202 | Geobacter metallireducens GS-15 | Geobacter metallireducens GS-15 | 216 |

CONCLUSIONS

- We have developed a protocol for protein extraction from metal-contaminated soil samples (Hanford, WA and Rifle, CO). Overcoming this challenge will help us identify active subsurface microbial communities existing at these DOE sites and study how they react to soil amendments which will help enhance bioremediation efforts at contaminated DOE sites.
- Proteomics of Hanford soil shows that subsurface microbial community is made up of many microbes, which include *P. putida* strains. Active microbes are quite sparse in contaminated soil.
- We have also shown that "shotgun" proteomics can be applied to the complex microbial communities in ground-water field samples and effluent column tubing in the Rifle Field site in Colorado.
- Acetate amendments to Rifle soil site leads to a 'Geobacter species' bloom which does help in uranium reduction. We could confidently identify ~3000 proteins from the ground water samples which reflect differential expression of *Geobacter* species following acetate amendment and culminating in uranium reduction.
- When a dominant microbial species is present with a reference genome, moderate depth in proteome coverage can be achieved. Strain variation within dominant species can also be identified in these complex microbial communities.
- Large numbers of quality MS/MS spectra are acquired for which no correct database entries exist. Clearly, metagenome data from the same samples would greatly enhance the depth of proteome coverage at high confidence.

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