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Identification of Metal Reductases and Determination of their Relative Abundance in Subsurface Sedimentary Systems using Proteomic Analysis

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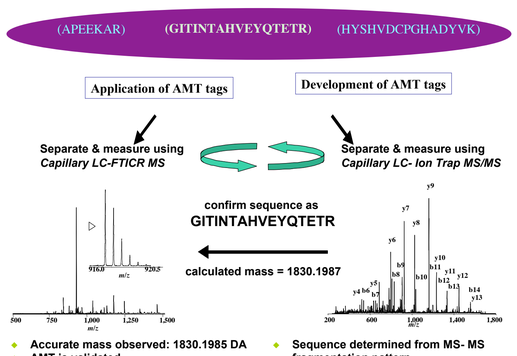
Abstract

Heavy metal and radionuclide contamination at Department of Energy (DOE) sites nationwide constitute a major environmental problem. Of particular interest are U and Tc, as well as Fe and Mn due to their potential direct and indirect effects on contaminant biogeochemical behavior. For the past decade bacteria that utilize metals as terminal electron acceptors have been isolated and identified. These bacteria include members of three major anaerobic groups; the denitrifying, sulfate- and Fe(III)- reducing bacteria. The electron transfer pathways within these bacteria are still not well understood. Moreover, this lack of information substantially impedes efforts to increase *in situ* bioremediation efficiency. Hence, identification of metal reductases, and determination of their similarity between these bacterial groups is essential for understanding these mechanisms and assessing bioremediative potential at DOE sites.

We have used cell fractionation techniques to resolve sub-cellular protein fractions and quantify the purity of proteins within each enriched fraction. Additionally, we have applied classical biochemical separations of fractions to enrich for specific proteins responsible for metal reduction activity. The application of advanced proteomics techniques allows for the identification of all the proteins in the enriched fractions eliminating the need for purifying each protein to homogeneity. We are utilizing a orthogonal purification approaches in both series and parallel to created fractions containing different complements of proteins. As each fraction exhibits the metal reduction activity, the proteins common to all the fractions are the most likely targets for further study by molecular biological techniques.

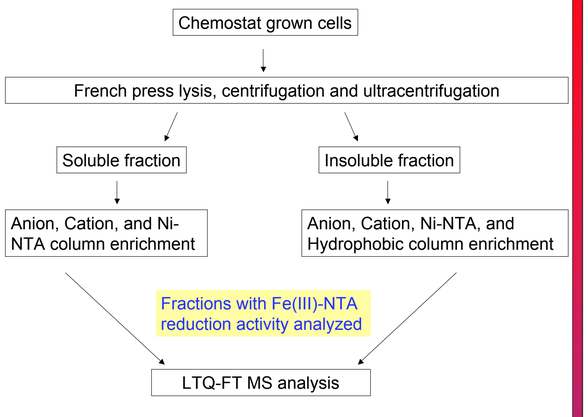
Accurate Mass and Time tag (AMT) Approach



- Accurate mass observed: 1830.1985 DA
- AMT is validated
- Separation, analytical conditions established
- Sequence determined from MS- MS fragmentation pattern
- Potential mass tag (PMT) identified from *D. radiodurans* proteome

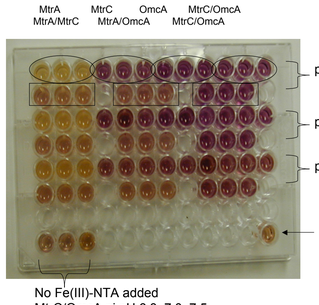
Mass tag = Protein marker

Concept of Experiments



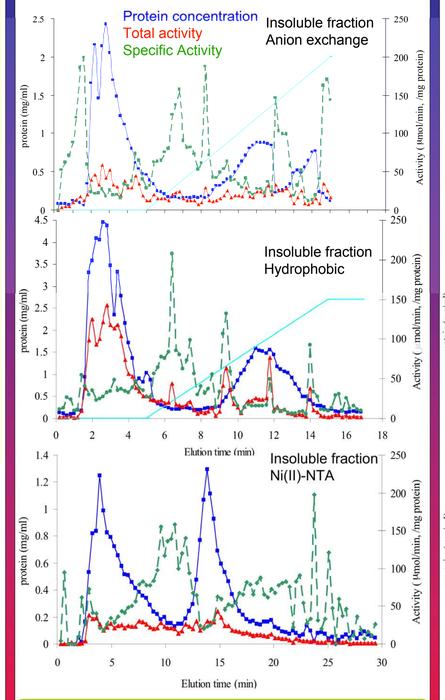
- Protein detection was valid if 2 peptides unique to the parent protein were present in all 3 MS analyses and passed strict filters.
- Proteins from all column fractions of each matrix were then pooled and queried against other matrix results to find proteins in common for insoluble or soluble subcellular fractions.

Fe(III) reduction activity measurements



- Activity of each fraction was tested by a method modified from that described (Magnuson et al., (2001) Biochem. J. 359:147-152.) as shown using purified MtrC and OmcA from *S. oneidensis* MR-1.
- Protein concentration was measured using the BCA micro plate method.

S. oneidensis MR-1



MS analysis of fractions

Insoluble fraction

Matrix	Number of Proteins
Cell Extract	*972*
Hydrophobic	515
Anion Exchange	360
Cation Exchange	392
Ni(II)-NTA	845
Common to all Matrices	143
Likely Candidates	**41**

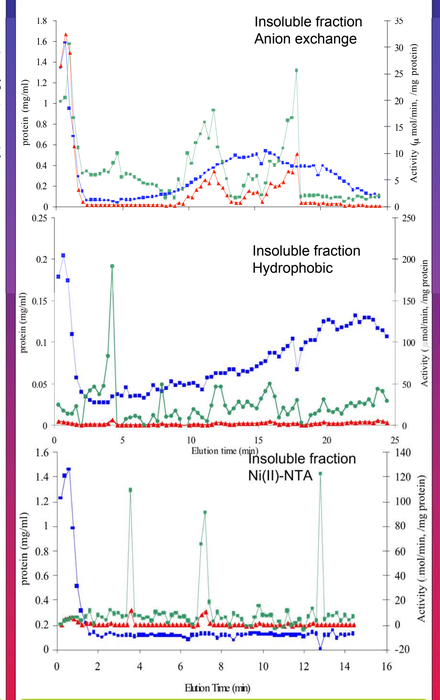
Reference	Protein Description
SO3988	aerobic respiration control protein ArcA
SO4320	agglutination protein
SO1490	alcohol dehydrogenase II
SO1429	anaerobic dimethyl sulfoxide reductase A subunit
SO2756	antioxidant AhpC/Tsa family
SO1637	bacterial surface antigen
SO1112	bacterioferritin subunit 1
SO2882	conserved hypothetical protein
SO3765	conserved hypothetical protein
SO1824	conserved hypothetical protein
SO1518	conserved hypothetical protein
SO1377	conserved hypothetical protein
SO3906	conserved hypothetical protein
SO4134	conserved hypothetical protein
SO2593	conserved hypothetical protein
SO3842	conserved hypothetical protein
SO2264	cysteine desulfurase
SO4666	cytochrome c
SO2361	cytochrome c oxidase cbb3-type, subunit III
SO3286	cytochrome d ubiquinol oxidase subunit I
SO1778	decaheme cytochrome c MtrC
SO1779	decaheme cytochrome c OmcA
SO0970	fumarate reductase flavoprotein subunit precursor
SO4384	hypothetical protein
SO3733	hypothetical protein
SO1521	iron-sulfur cluster-binding protein
SO1519	iron-sulfur cluster-binding protein
SO4349	ketol-acid reductoisomerase
SO1295	major outer membrane lipoprotein putative
SO3740	NAD(P) transhydrogenase alpha subunit
SO1019	NADH dehydrogenase I C/D subunits
SO1103	NADH:ubiquinone oxidoreductase
SO0902	NADH:ubiquinone oxidoreductase
SO4321	OmpA family protein
SO3545	OmpA family protein
SO3896	outer membrane porin putative
SO3904	outer membrane protein TolC
SO4591	tetraheme cytochrome c
SO2907	TonB-dependent receptor domain protein
SO2427	TonB-dependent receptor putative
SO0610	ubiquinol-cytochrome c reductase cytochrome c1

Soluble fraction

Matrix	Number of Proteins
Cell Extract	*275*
Anion Exchange	188
Cation Exchange	212
Ni(II)-NTA	196
Common to all Matrices	120
Likely Candidates	**12**

Reference	Protein Description
SO4054	5,10-methylenetetrahydrofolate reductase
SO3988	aerobic respiration control protein ArcA
SO0958	alkyl hydroperoxide reductase C subunit
SO2882	conserved hypothetical protein
SO3842	conserved hypothetical protein
SO2264	cysteine desulfurase
SO1140	dihydrodipicolinate reductase
SO1937	ferric uptake regulation protein
SO0970	fumarate reductase
SO3855	malate oxidoreductase
SO1776	outer membrane protein precursor MtrB
SO2935	oxidoreductase

Dsv. desulfuricans G20



MS analysis of fractions

Insoluble fraction

Matrix	Number of Proteins
Cell Extract	*326*
Hydrophobic	122
Anion Exchange	164
Cation Exchange	198
Ni(II)-NTA	175
Common to all Matrices	26
Likely Candidates	**11**

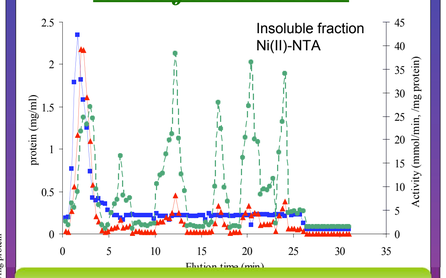
Reference	Protein Description
gi|23473865|ref|ZP_00129160.1|	Anaerobic selenocysteine dehydrogenase
gi|23475856|ref|ZP_00131126.1|	Bacterioferritin (cytochrome b1)
gi|23475073|ref|ZP_00130363.1|	Coenzyme F420-reducing hydrogenase
gi|23475394|ref|ZP_00130681.1|	Dissimilatory sulfite reductase (desulfoviridin)
gi|23475395|ref|ZP_00130682.1|	Dissimilatory sulfite reductase (desulfoviridin)
gi|23473599|ref|ZP_00128895.1|	FAD/FMN-containing dehydrogenases
gi|23475074|ref|ZP_00130364.1|	Heterodisulfide reductase, subunit A
gi|23475071|ref|ZP_00130361.1|	Membrane protein involved in colicin uptake
gi|23474623|ref|ZP_00129916.1|	Ni,Fe-hydrogenase I
gi|23473161|ref|ZP_00128457.1|	Proline dehydrogenase
gi|23473601|ref|ZP_00128897.1|	Pyruvate:ferredoxin oxidoreductase

Soluble fraction

Matrix	Number of Proteins
Cell Extract	*836*
Anion Exchange	ND
Cation Exchange	ND
Ni(II)-NTA	657
Common to all Matrices	N/A
Likely Candidates	**71**

Reference	Protein Description
gi|23474600|re	COG0059: Ketol-acid reductoisomerase
gi|23475184|re	COG0062: Uncharacterized conserved protein
gi|23475800|re	COG0141: Histidinol dehydrogenase
gi|23473865|re	COG0243: Anaerobic selenocysteine dehydrogenases
gi|23473249|re	COG0247: Fe-S oxidoreductase
gi|23473599|re	COG0277: FAD/FMN-containing dehydrogenases
gi|23474001|re	COG0370: Fe2+ transport system protein B
gi|23474626|re	COG0374: Ni,Fe-hydrogenase I large subunit
gi|23476244|re	COG0378: Ni2+-binding GTPase, hydrogenase expression
gi|23475512|re	COG0479: fumarate reductase, Fe-S protein
gi|23474201|re	COG0492: Thioredoxin reductase
gi|23474074|re	COG0535: Predicted Fe-S oxidoreductases
gi|23475197|re	COG0569: K+ transport systems, NAD-binding component
gi|23474087|re	COG0607: Rhodanese-related sulfurtransferase
gi|23473167|re	COG1014: Pyruvate:ferredoxin oxidoreductase, alpha subunit
gi|23475406|re	COG0731: Fe-S oxidoreductases
gi|23473995|re	COG0735: Fe2+/Zn2+ uptake regulation proteins
gi|23473199|re	COG0739: Membrane proteins related to metalloendopeptidases
gi|23475776|re	COG0778: Nitroreductase
gi|23473738|re	COG0822: NifU homolog involved in Fe-S cluster formation
gi|23473165|re	COG1014: Pyruvate:ferredoxin oxidoreductase, b subunit
gi|23473601|re	COG1014: Pyruvate:ferredoxin oxidoreductase, g subunit
gi|23474512|re	COG1034: NADH:ubiquinone oxidoreductase
gi|23475493|re	COG1035: Coenzyme F420-reducing hydrogenase, beta subunit
gi|23475494|re	COG1145: Ferredoxin
gi|23475077|re	COG1146: Ferredoxin
gi|23475074|re	COG1148: Heterodisulfide reductaseand related polyferredoxins
gi|23473631|re	COG1271: Cytochrome bd-type quinol oxidase, subunit 1
gi|23473400|re	COG1454: Alcohol dehydrogenase, class IV
gi|23473386|re	COG1529: Aerobic-type carbon monoxide dehydrogenase
gi|23473320|re	COG1538: Outer membrane protein
gi|23474371|re	COG1538: Outer membrane protein
gi|23475532|re	COG1538: Outer membrane protein
gi|23474624|re	COG1740: Ni,Fe-hydrogenase I small subunit
gi|23475073|re	COG1908: Coenzyme F420-reducing hydrogenase, delta subunit
gi|23473162|re	COG1979: Fe-dependent alcohol oxidoreductases/dehydrogenase
gi|23473640|re	COG2033: Desulfoferrodoxin
gi|23475490|re	COG2048: Heterodisulfide reductase, subunit B
gi|23475856|re	COG2193: Bacterioferritin (cytochrome b1)
gi|23474929|re	COG2249: Putative NADPH-quinone reductase
gi|23473856|re	COG2414: Aldehyde:ferredoxin oxidoreductase
gi|23474145|re	COG2414: Aldehyde:ferredoxin oxidoreductase
gi|23473649|re	COG2885: Outer membrane protein
gi|23475536|re	COG3672: Predicted periplasmic protein
gi|23474732|re	COG3681: Uncharacterized conserved protein
gi|23474430|re	COG4231: Indolepyruvate ferredoxin oxidoreductase, a and b su.
gi|23475858|re	COG4624: Iron only hydrogenase large subunit
gi|23473580|re	COG4656: Predicted NADH:ubiquinone oxidoreductase, su. RnfC
gi|23475449|re	COG4659: Predicted NADH:ubiquinone oxidoreductase, su. RnfG
gi|23475818|re	COG4775: Outer membrane protein/protective antigen OMA87

G. metallireducens and *G. sulfurreducens*



MS analysis of fractions

G. metallireducens

Insoluble fraction

Matrix	Number of Proteins
Cell Extract	*237*
Hydrophobic	76
Anion Exchange	43
Cation Exchange	51
Ni(II)-NTA	64
Common to all Matrices	**9**

Soluble fraction

Matrix	Number of Proteins
Cell Extract	*560*
Anion Exchange	229
Cation Exchange	174
Ni(II)-NTA	154
Common to all Matrices	**14**

G. sulfurreducens

Insoluble fraction

Matrix	Number of Proteins
Cell Extract	*414*
Hydrophobic	228
Anion Exchange	207
Cation Exchange	234
Ni(II)-NTA	227
Common to all Matrices	87
Likely Candidates	**38**

Reference	Protein Description
GSU1644	ABC transporter, ATP-binding protein
GSU3401	periplasmic amino acid-binding protein
GSU1013	chemotaxis MotB protein, putative
GSU0384	conserved hypothetical protein
GSU0095	conserved hypothetical protein TIGR00103
GSU3259	cytochrome c family protein
GSU3259	cytochrome c family protein
GSU0274	cytochrome c family protein
GSU2811	cytochrome c Hsc (hsc)
GSU2813	cytochrome c551 peroxidase (ccpA-2)
GSU0509	Fe(III) reductase, alpha subunit (sfrA)
GSU0510	Fe(III) reductase, beta subunit (sfrB)
GSU1932	hypothetical protein
GSU2090	hypothetical protein
GSU2496	hypothetical protein
GSU1467	iron-sulfur cluster-binding protein
GSU1468	keto/oxoacid ferredoxin oxidoreductase, a s.u.
GSU1469	keto/oxoacid ferredoxin oxidoreductase, b s.u.
GSU1470	keto/oxoacid ferredoxin oxidoreductase, g s.u.
GSU0644	KH domain protein
GSU0872	lipoprotein, putative
GSU2940	lipoprotein, putative
GSU0343	NADH dehydrogenase I, F subunit (nouF)
GSU0344	NADH dehydrogenase I, G subunit, putative
GSU0346	NADH dehydrogenase I, I subunit (nuoI-1)
GSU0350	NADH dehydrogenase I, M subunit (nuoM-1)
GSU1700	NADP-dependent malic enzyme (maeB)
GSU0024	OmpA domain protein
GSU0360	OmpA domain protein
GSU1609	outer membrane efflux protein
GSU0814	outer membrane efflux protein, putative
GSU2939	outer membrane porin FmdC, putative
GSU0073	outer membrane protein, putative
GSU2268	outer membrane protein, putative
GSU2267	outer membrane protein, putative
GSU2305	peptidoglycan-associated lipoprotein
GSU0097	pyruvate ferredoxin/flavodoxin oxidoreductase
GSU3291	V-type H(+)-translocating pyrophosphatase

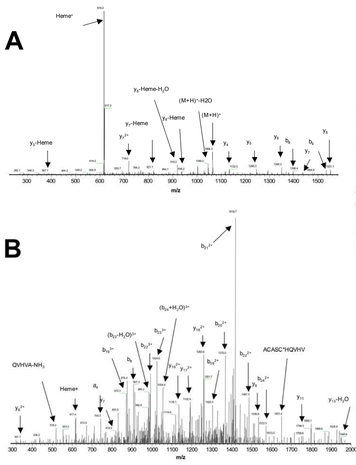
Soluble fraction

Matrix	Number of Proteins
Cell Extract	*398*
Anion Exchange	119
Cation Exchange	137
Ni(II)-NTA	360
Common to all Matrices	91
Likely Candidates	**20**

Reference	Protein Description
GSU3289	conserved hypothetical protein
GSU2504	cytochrome c family protein
GSU1397	cytochrome c family protein, putative
GSU2811	cytochrome c Hsc (hsc)
GSU0509	Fe(III) reductase, alpha subunit (sfrA)
GSU1177	fumarate reductase, flavoprotein subunit (frdA)
GSU1178	fumarate reductase, iron-sulfur protein (frdB)
GSU2496	hypothetical protein
GSU1468	keto/oxoacid ferredoxin oxidoreductase, a s.u.
GSU1469	keto/oxoacid ferredoxin oxidoreductase, b s.u.
GSU1470	keto/oxoacid ferredoxin oxidoreductase, g s.u.
GSU1909	ketol-acid reductoisomerase (ilvC)
GSU2263	oxidoreductase, Gfo/Idh/MocA family
GSU2731	polyheme membrane-associated cytochrome c (ferA)
GSU0097	pyruvate ferredoxin/flavodoxin oxidoreductase
GSU3294	rubredoxin-oxygen oxidoreductase, putative
GSU2814	rubrerythrin
GSU2612	rubrerythrin/rubredoxin protein, putative
GSU3246	thioredoxin peroxidase
GSU3453	uroporphyrinogen decarboxylase (hemE)

Heme Containing Peptide Identification via MS/MS

Our lab has developed a procedure for the high-throughput detection of heme-containing peptides using a search for a mass of 615.1694 Da that accounts for the formal charge of the *c*-type heme. This is valid for peptides 700-7000 Da with a 2% false positive rate. (F. Yang et al. (2005) J. Proteome Res. *In press).*



MS/MS fragmentation spectra of heme-containing peptides (A) K. GADSCLMC*HK.K and (B) R.MSWNGGHHDNADV ACASC*HQVHVAK.D from tryptic digest of MtrA. *indicated the heme attachment site.

Corroborating Lines of Evidence

Working closely with other groups, a number of candidate proteins in *S. oneidensis* MR-1 have been verified:

1. MS proteomic data generated with the *Shewanella* federation have shown the importance of MtrABC and OmcA.
2. K.O. mutants of MtrABC and OmcA generated through the EMSL Grand Challenge show decreased Fe(III)-reduction in all but MtrA; (see Y. Gorby presentation and J. Fredrickson poster).
1. Purified MtrC and OmcA reduce Fe(III)-NTA and HFO *in vivo* but not MtrA; (L. Shi et al, submitted *J. Biol. Chem.*).

Discussion and Future Directions

- Subcellular fractions of *S. oneidensis* MR-1, *Dsv. desulfuricans* G20, *G. metallireducens* and *G. sulfurreducens* have been enriched by multiple matrices and the proteins from fractions displaying Fe(III)-reduction activity identified as putatively being involved in metal-reduction in each of these organisms.
- The combination of reductase enrichment with high-throughput, comprehensive MS analysis yields more information without lengthy purification of each protein and the possible loss of activity during purification.
- These amino acid sequences will be used to reannotate genes in these organisms against newer sequencing efforts, thus improving their functional categorization.
- These sequences will also be used together with our heme-containing peptide detection methods to identify organisms from sediment extracts of DOE sites including FRC, Rifle and Hanford.

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