Parallel Proteomic Identification of Metal Reductases and Determination of their Relative Abundance in a Series of Metal Reducing Bacteria

Mary S. Lipton

Dwayne A. Elias, Heather M. Mottaz, Carrie D. Goddard and Alexander S. Beliaev

Biological Separations and Mass Spectrometry
Biological Sciences Division
Pacific Northwest National Laboratory

Battelle

Motivation

Historically, identification of proteins with metal reduction activity has been challenging

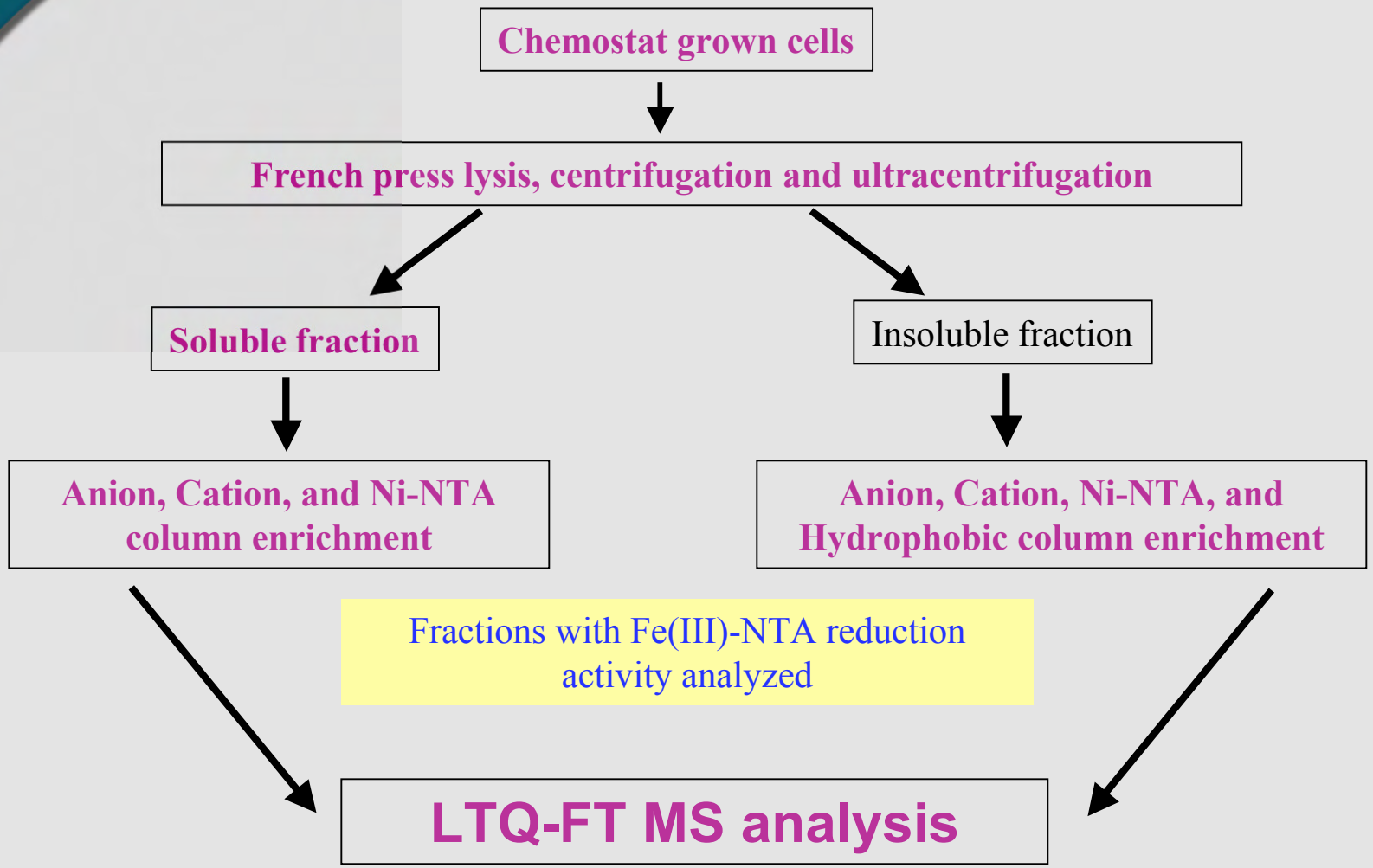
Necessary to purify proteins to homogeneity

Lost metal reduction activity

New advances in by mass spectrometry allowed for the identification of multiple proteins from global lysates and mixtures

This project has applied these mass spectrometry techniques with orthogonal protein purification and enzymology techniques for the preliminary identification of proteins with these activities.

Concept of Experiments

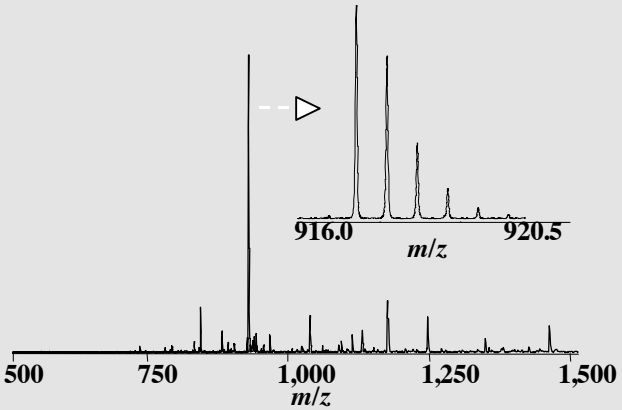


- Protein detection was valid if 2 peptides unique to the parent protein were present in all 3 MS analyses and passed strict filters.
- Proteins from all column fractions of each matrix were then pooled and queried against other matrix results to find proteins in common for insoluble or soluble subcellular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

Accurate Mass Tag (AMT) Concept

(APEEKAR) **(GITINTAHVEYQTETR)** (HYSHVDCPGHADYVK)

Separate & measure using ***Capillary LC-FTICR MS***

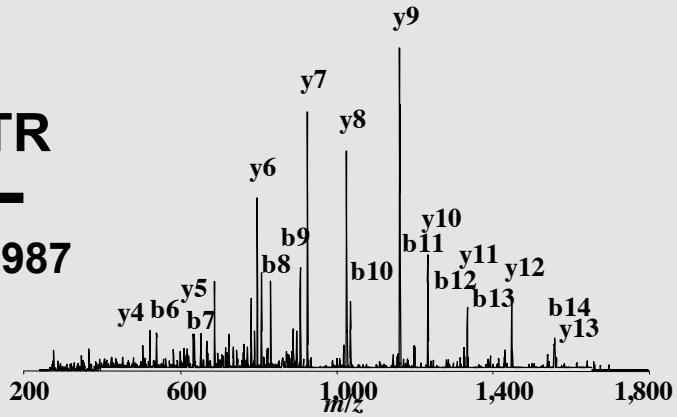


confirm sequence as

GITINTAHVEYQTETR

calculated mass = 1830.1987

Separate & measure using ***Capillary LC- Ion Trap MS/MS***



- 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

PNNL Accurate Mass and Time (AMT) Tag Approach

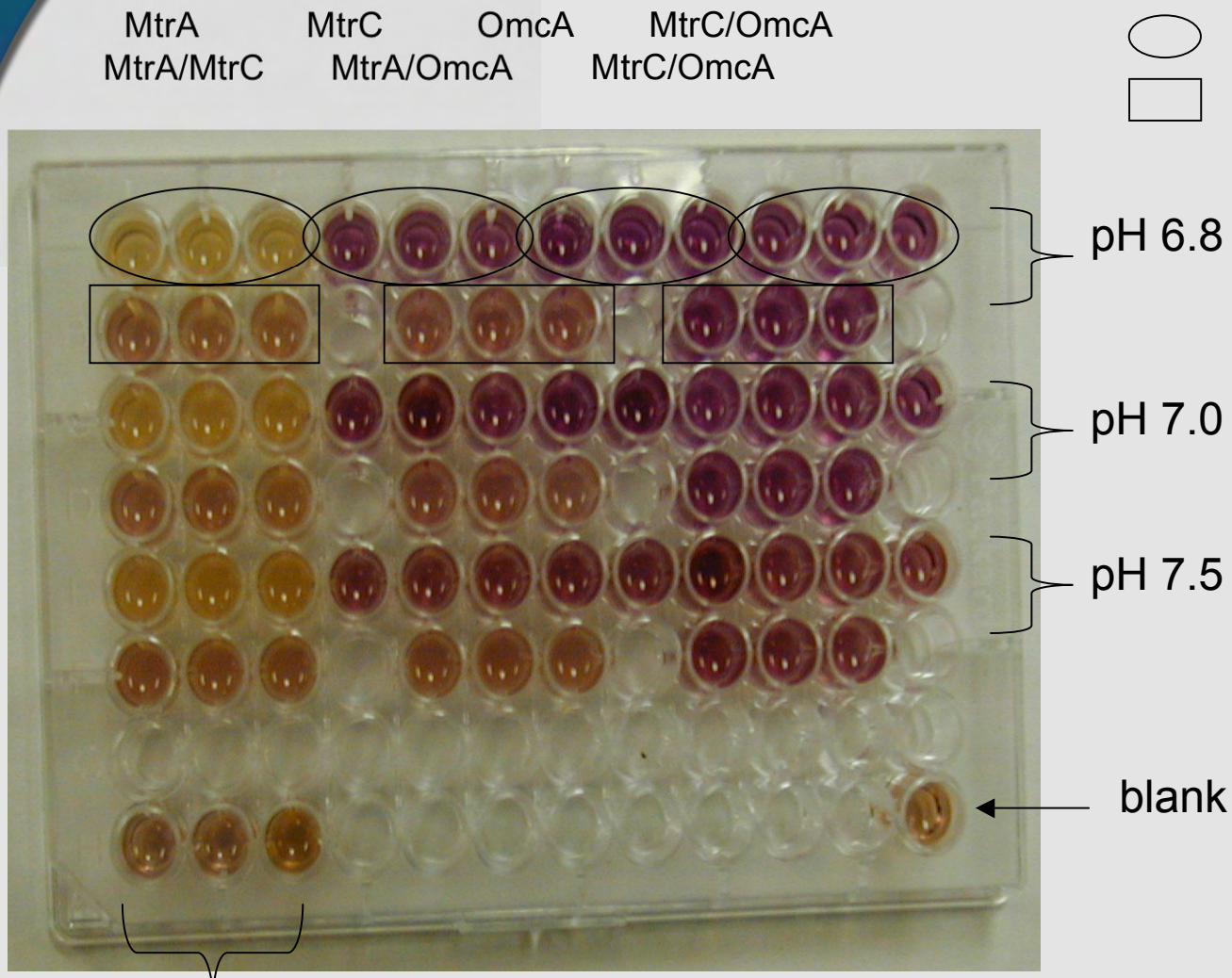
- Peptide generated from proteolytic digest of a protein
- Identified from sequenced genome
- High accuracy mass measurements by FTICR
- Combination of conventional mass spectrometry and FTICR techniques
  - *Higher confidence in peptide identification*
  - *High throughput analyses enabled*

AMT tags provide unique biomarkers for nearly all proteins in a microbe.

FTICR mass spectrometry allows quantitation

- *Stable isotope labeling provides relative abundance changes*
- *Direct peak intensities provides absolute quantitation*

Fe(III) reduction activity measurements

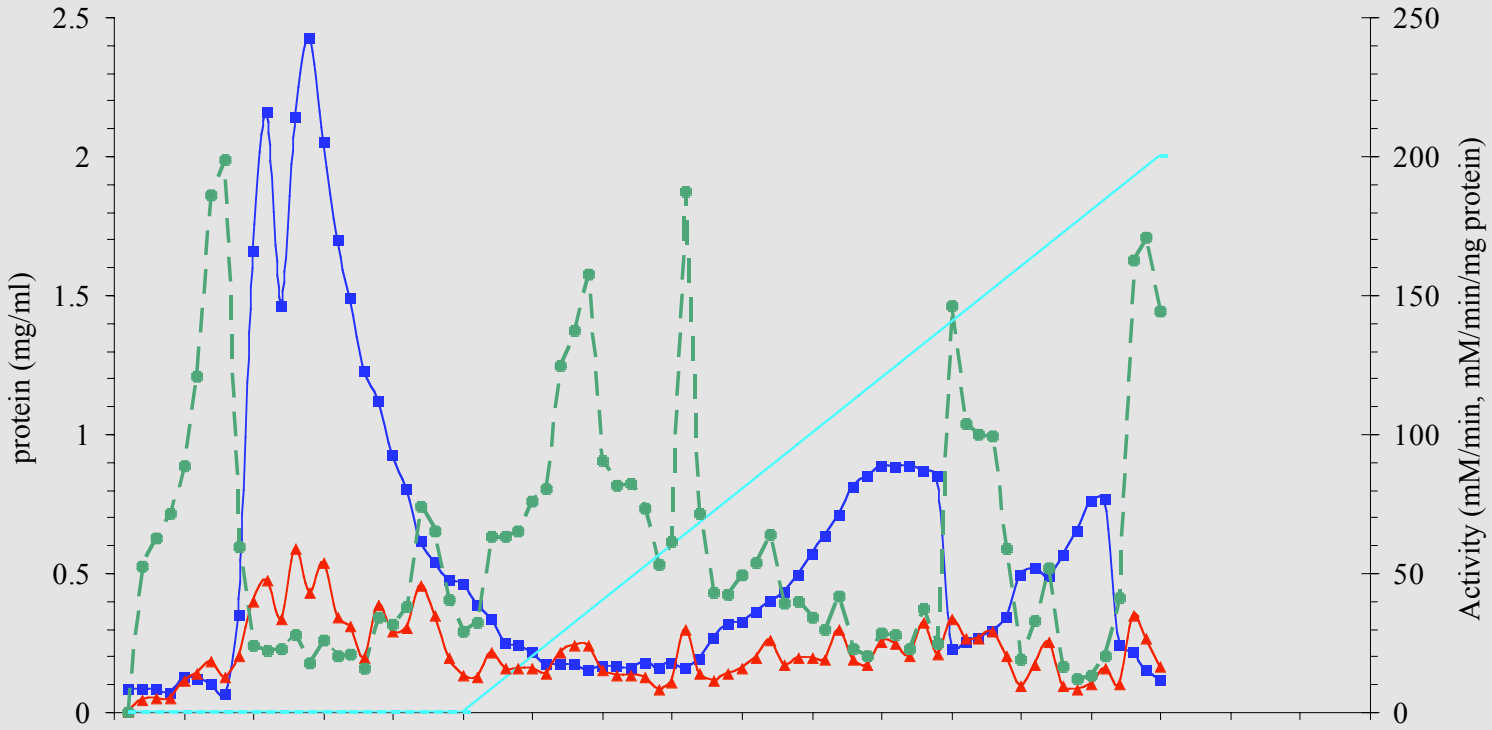


- Activity of each fraction was tested by a method modified from that described (Magnuson et al., (2001) Biochem. J. 359:147-152.) as shown using purified MtrC and OmcA from *S. oneidensis* MR-1.
- Protein concentration was measured using the BCA micro plate method.

Shewanella oneidensis

Protein concentration
Total activity
Specific Activity

Insoluble fraction
Anion exchange



Shewanella oneidensis

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

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

Shewanella oneidensis candidates

"Insoluble proteins"

SO1778 decaheme cytochrome c MtrC

SO1779 decaheme cytochrome c OmcA

OM protein, functional purified protein shows Fe(III)-reduction; mutants deficient in Fe(III)-reductio

SO4591 tetraheme cytochrome c

high homology to tetraheme cytochrome c_3; classically involved in metal reduction

SO3286 cytochrome d ubiquinol oxidase

SO0610 ubiquinol-cytochrome c reductase cytochrome c_1

SO4666 cytochrome c

classically involved in mediating electron transfer

SO4321 OmpA family protein

SO3545 OmpA family protein

OM protein, likely redox reactive

SO2361 Cytochrome c oxidase cbb_3 type

[low O_2] expressed terminal reductase and so may also be reactive with oxidized metals.

SO1429 anaerobic dimethyl sulfoxide reductase A subunit

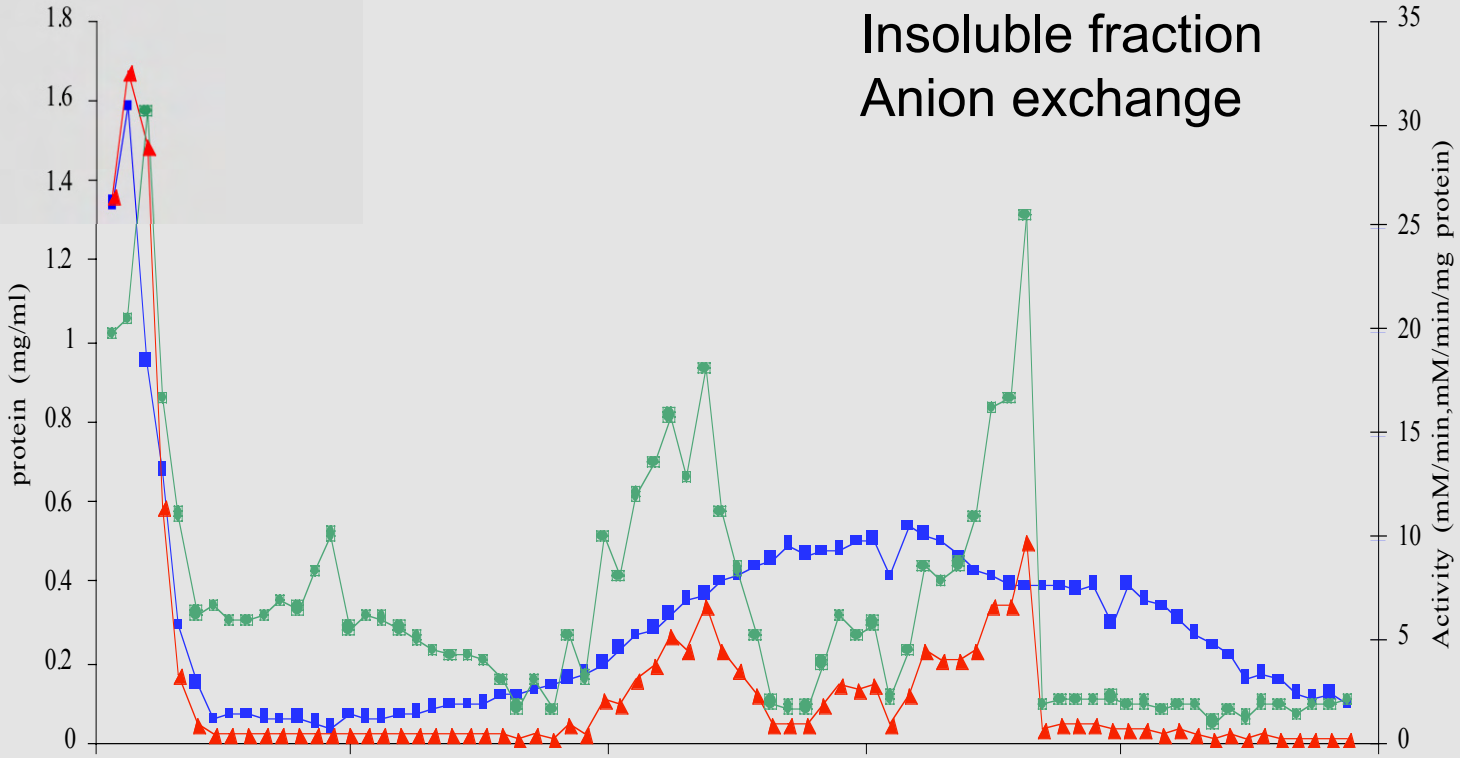
terminal reductase for anaerobic respiration of DMSO; likely redox reactive

"Soluble Proteins"

SO1776 outer membrane protein MtrB

loosely OM associated protein; mutants deficient in Fe(III)-reduction

Dsv. desulfuricans G20



Dsv. desulfuricans G20

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

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

Dsv. desulfuricans G20 candidates

"Insoluble proteins"

Bacterioferritin (cytochrome b_1) *detected in both fractions

likely involved in electron transfer pathway

Ni,Fe-hydrogenase I large and small subunit *detected in both fractions

redox reactive protein and known to be involved in Tc(VII)-reduction

Coenzyme F_{420}-reducing hydrogenase *detected in both fractions

redox reactive protein, may be involved in Tc(VII)-reduction(?) similar to the NiFe-Hydrogenase.

"Soluble proteins"

Predicted NADH:ubiquinone oxidoreductase, RnfC and RnfG s.u.
Putative NADPH-quinone reductase
NADH:ubiquinone oxidoreductase
Cytochrome bd-type quinol oxidase, subunit 1

classically involved in mediating electron transfer

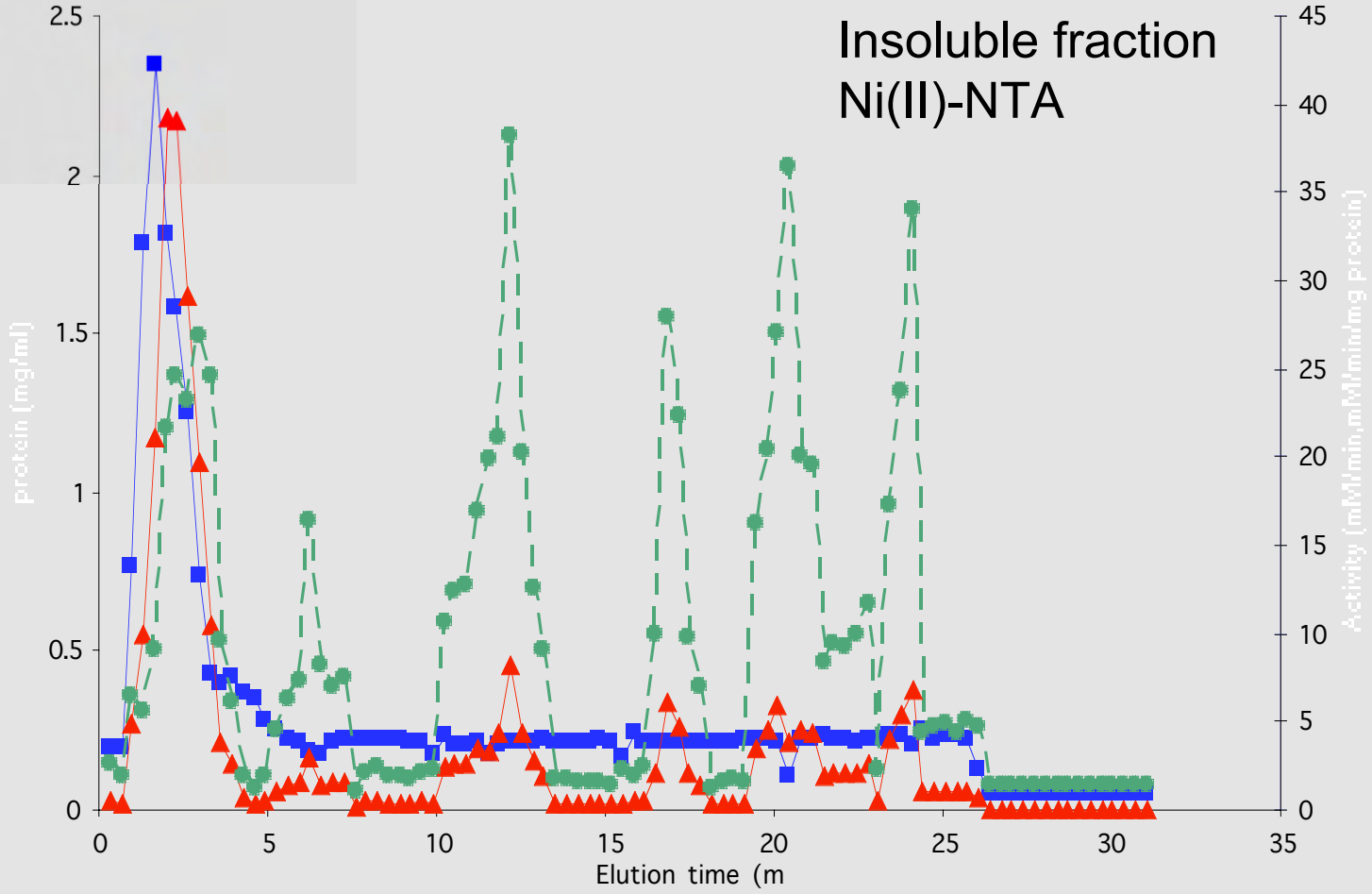
Predicted Fe-S oxidoreductases (X3)
Fe-S oxidoreductases (X2)

redox reactive proteins apparently in the periplasm

Outer membrane protein (X4)

OM protein, possibly redox reactive

Geobacter sulfurreducens



Geobacter sulfurreducens

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

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

Geobacter sulfurreducens candidates

"Insoluble proteins"

GSU0509/ GSU0510 **Fe(III) reductase, a and b subunit (sfrA, sfrB)**

annotated as Fe(III) reductase

GSU3259/ GSU0274 **cytochrome c family protein**

GSU2811* **cytochrome c Hsc (hsc)**

GSU2813 **cytochrome c551 peroxidase (ccpA-2)**

heme containing; classic electron transfer proteins and may function as terminal reductase.

GSU1468/69/70* **keto/oxoacid ferredoxin oxidoreductase, a,b,g subunits**

GSU0343/44/46/50 **NADH dehydrogenase I; F,G,I,M subunits (nouF,l-1,M-1)**

classically involved in mediating electron transfer

GSU0024/0360 **OmpA domain protein (X2)**

GSU0073/2267/2268 **outer membrane protein, putative (X3)**

OM proteins, possibly redox reactive

"Soluble proteins"

GSU2504/1397 **cytochrome c family protein**

heme containing; classic electron transfer proteins and may function as teminal reductase.

GSU2731 **polyheme membrane-associated cytochrome c (ferA)**

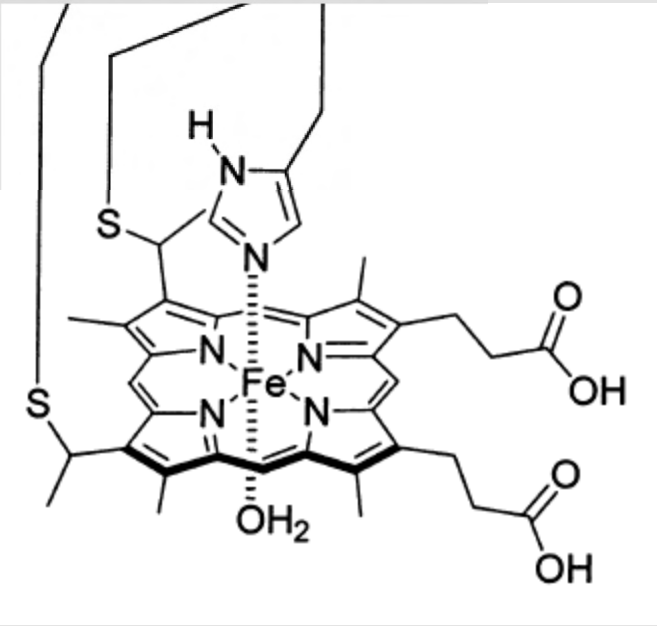
fer system well-known to be involved in Fe(III)-reduction

GSU1177/1178 **fumarate reductase, flavoprotein iron-sulfur subunits (frdA,B)**

redox reactive protein

Identification of *c*-type heme-containing peptides in *Shewanella oneidenis*

-C-X-Y-C-H-



- Under-represented
- 2D gel analysis:

Giometti, C. S. et. al. Proteomics ***2003****, 3, 777-785.*

Fumarate reductase

both abundance and p*I* changes

p*I*: 7.6 under aerobic condition

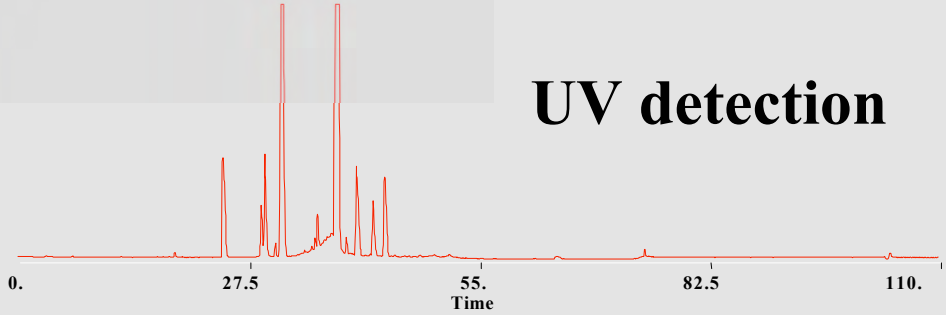
p*I*: ~6.5 under low oxygen condition

potential modification changes

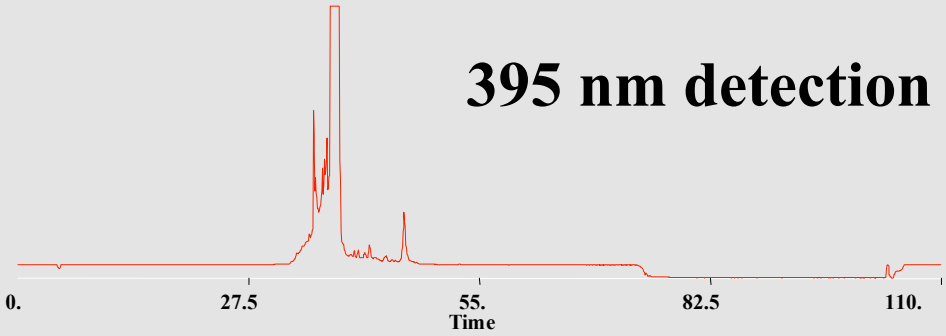
- **Charge state of Fe in gas phase - critical to determine the heme modification mass**
- **Fragmentation pattern of heme-containing peptides**

Purification of heme-containing peptides from Horse Cyt.*C*

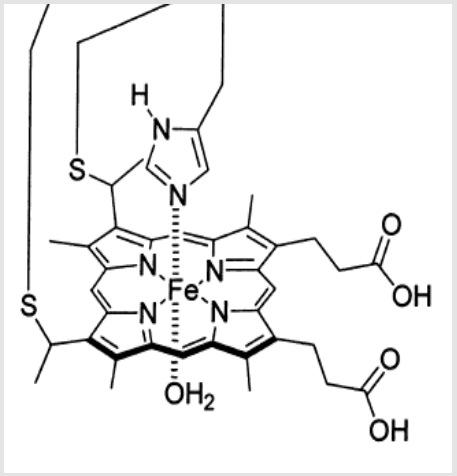
UV detection



395 nm detection

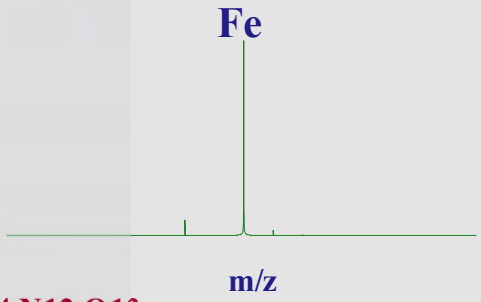


-C-X-Y-C-H-

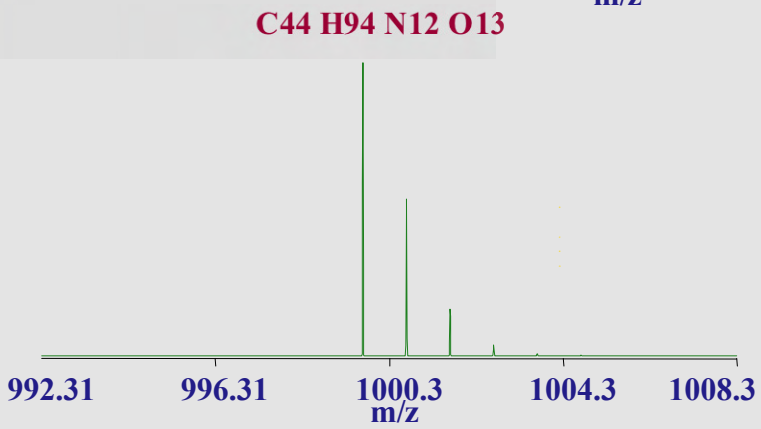


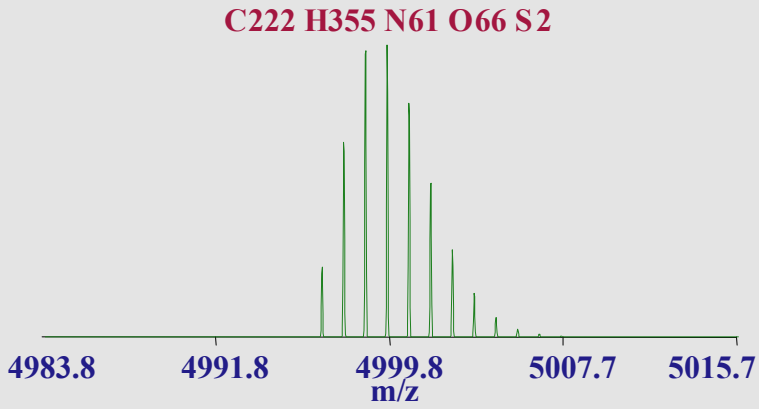
Soret band absorption

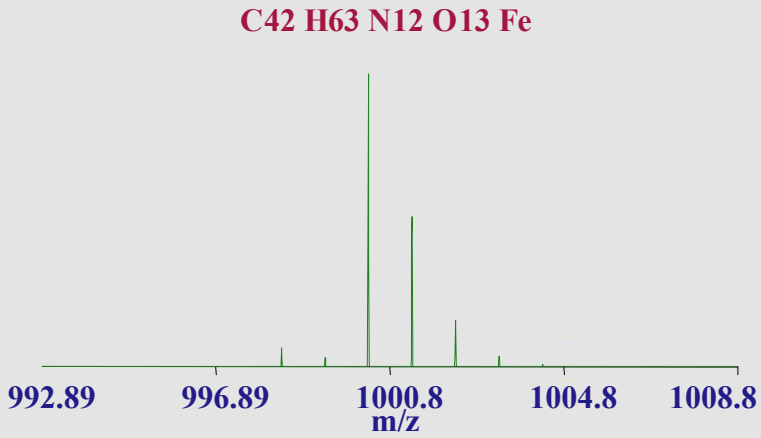
Heme signature isotopes due to Fe naturally occurring isotopes

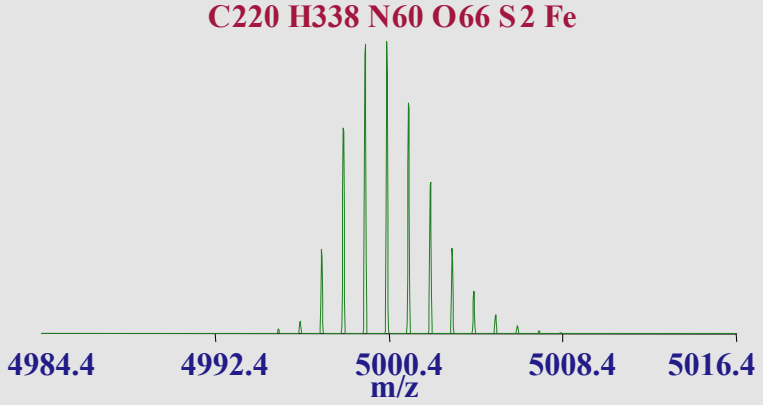


^{54}Fe: 5.845%
^{56}Fe: 91.754%
^{57}Fe: 2.119%
^{58}Fe: 0.282%









Heme-containing peptides (one heme modification on cysteine in CXYCH motif) identified from MtrA

Peptide (heme motif in bold)	CS	Heme %	Xcorr	ΔCn
K.NTEMEVCTSC*HTK.Q K.NTEMEVC*TSCHTK.Q	2 3	100 42	2.3306 1.2992	0.1803 0.2986
K.SPMAGLQCEAC*HGPLGQHNK.G	3	12	3.4542	0.0160
K.GADSCLMC*HK.K K.GADSCLMC*HKK.S	2 3	100 43	2.0053 1.7164	0.0324 0.2529
K.QNSVC*MSCHQDDKR.M K.QNSVCMSC*HQDDKR.M K.QSTLSADKQNSVC*MSCHQDDKR.M	2 3 3	100 77 28	1.8044 1.6546 2.6833	0.2683 0.2198 0.1195
R.MSWNGGHHDNADVACASC*HQVHVAK.D	3	12	3.4852	0.0799
R.SC*LNCHSQVHGSNHPSGK.L	2 3	70 40	2.7004 3.9437	0.1118 0.0187
R.APQL C*QQCH ASDGHASNA.Y	3	20	3.9667	0.0090
K.LWEHAPVTENCVTC*HNPHGSVNDGMLK.T	3	4.6	1.7809	0.0727

Heme-containing peptides identified from SO0970 and SO2727 proteins

ORF	Peptide (heme attachment motif in bold)	Charge state	FTICR MS		LCQ MS/MS
			Error (ppm)	Signature isotopes	
SO 0970	K.GGVTNDNLTHENGQCVSCHGDLK.E	3	−0.6	Yes	Yes
	VSPHKSHLIGEIACTSCHK	3	−4.6	No[a]	No
	K.SHLIGEIACTSCHKGHEK.S	2,3	−0.6, −0.4	Yes	Yes
	K.SHLIGEIACTSCHK.G	2,3	−1.5, −0.6	Yes	Yes
	K.SVAYCDACHSFGFDMPFGGK.W	2,3	−2.6, −1.6	Yes	Yes
	A.APEVLADFHGEMGGCDSCHVSDK.G	3	No	No	Yes
SO 2727	K.PTCESCHDDGR.T	2,3	−1.5, −0.5	Yes	Yes
	K.LSDFHAESGGCESCHK.D	2,3	−1.8, −1.1	Yes	Yes
	LSEMDAVHKPHDGNLVCADCHAVHDMNVGQK	3,4	−3.0, −2.2	Yes	No
	PHDGNLVCADCHAVHDMNVGQK	3	−2.8	No[a]	No
	LSEMDAVHKPHDGNLVCADCHAVHDMNVGQKPTCESCHDDGR	4,5	−3.5, −3.0	No	No
	K.DGTPSADGAFEFAQCQSCHGK.L	2,3	−3.1, −2.0	Yes	Yes
	LSDFHAESGGCESCHKDGTPSADGAFEFAQCQSCHGK	4	−2.7	Yes	No

[a] Due to low intensity and S/N

Conclusions

- **Combination of protein purification, enzymology and advanced mass spectrometry schemes has yielded a method for identification of metal reduction proteins**
- **Eliminates the need to purify each protein to homogeniety**
- **Enrichment of subcellular fractions of *S. oneidensis* MR-1, *Dsv. desulfuricans* G20, and *G. sulfurreducens* by achieved by multiple purification schemes**
- **Proteins isolated in fractions displaying Fe(III)-reduction activity in all schemes are identified as putatively being involved in metal-reduction activities**
- **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.**
- **Further use of characterization of heme containing proteins allow more comprehensive understanding of these proteins.**

Acknowledgements

Dwayne A. Elias, Heather M. Mottaz, Carrie D. Goddard and Alexander S. Beliaev

Biological Systems Analysis and Mass Spectrometry

Richard D. Smith

Separations

Yufeng Shen
Rui Zhao
Alex Shvartsburg
Kostas Petritis
David Simpson

Mass spectrometry

Ljiljana Paša-Tolić
David Anderson
Harold Udseth
Aleksey Tolmachev
Weijun Qian
Jason Page
Keqi Tang
Ron Moore
Tao Liu
Rui Zhang
Andre Vilkov
Therese Clauss

Shewanella Federation

Margie Romine
Yuri Gorby
Jim Fredrickson
Matt Marshall

Data processing, software development and statistics

Gordon Anderson
Sam Purvine
Dave Clark
Gary Kiebel
Ken Auberry
Matt Monroe
Angela Norbeck
Eric Strittmatter
Nikola Tolic

Sample processing, etc.

Mary Lipton
Heather Motaz
Marina Gritsenko
David Camp
Eric Livesay
Kim Hixson
Carrie Goddard
Josh Adkins*
Jon Jacobs

$$ **NABIR program: Office of Biological and Environmental Research** $$