Proteome Profiling of *Shewanella oneidensis* MR-1 *fur*, *etrA*, and *furletrA* Mutants

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Overview:

Purpose:

Make a preliminary comparison of semi-quantitative proteomic data and microarray data using *Shewanella oneidensis* MR-1 *fur*, *etrA*, and *etrA/fur* knockouts with wild type strain. Studying gene knockout strains by whole transcriptome and whole proteome methods were used is an attempt to understand the regulation of iron acquisition (*fur)* and anaerobic energy metabolism (*etrA*) in this microbe.

Methods:

- S. oneidensis MR-1 WT, *fur, etrA*, and *etrA/fur* mutants were grown to mid-log phase under anaerobic and aerobic conditions.
- Mutant transcriptomes were compared with WT using microarrays covering ~99% of the predicted open reading frames from *S. oneidensis*.
- The entire proteome from each growth state was digested with sequencing grade trypsin.
- For proteomic analysis WT and mutant samples were analyzed by 1-D LC-MS/MS employing 5 mass ranges and with automated nano switching 2D LC-MS/MS (SCX-RP C18) system with 1 or 2 mass range scans.
- MS/MS spectra was searched with SEQUEST against all predicted ORFs from *S. oneidensis* MR-1.
- Output files were filtered and sorted with DTASelect and compared with Contrast.

Results:

- Triplicate whole proteome analysis of WT and *fur* mutant by nano switching 2D LC-MS/MS and 1D-LC-MS/MS resulted in the identification of 837 proteins from WT, 963 from *fur*, for a total of 1102 proteins from both.
- A qualitative comparison between WT and *fur* was made using the Contrast software. Proteins showing dramatic change in identification level (4 or more unique peptides and/or 30% sequence coverage) were compared with microarray data.
- 31 proteins from proteome data showed significant change between WT and *fur*. These proteins were compared with microarray data and of these 17 agreed with microarray data, 10 did not, and 4 have no microarray data.
- A follow up experiment involved an small scale initial analysis of WT, *fur/etrA* and *etrA* were by 1D-LC-MS/MS with multiple mass range scanning. From duplicate analysis a total of 605 proteins were identified in the *etrA/fur* mutant, 595 in *etrA mutant*, and 654 in WT. The total proteins identification from all three samples was 842.

Introduction:

Shewanella oneidensis MR-1 is a facultative aerobic Gram-negative bacterium (figure 1). It is unique in its capability of using a wide variety of oxidized metals, fumarate, nitrate, and other chemical species as well as oxygen as terminal electron acceptors during respiration. During this process, heavy metals are reduced by the microbe rendering them insoluble thus making this microbe a primary candidate for bioremediation. While the exact mechanism of this microbe's ability to reduce these chemicals is currently widely studied, the regulation of metal transport and reduction as well as the switch between aerobic and anaerobic growth are not well understood. A putative ferric uptake regulator (*fur*) gene (figure2) and a putative electron transport regulator (*etrA*) gene have been identified in *S. oneidensis* by sequence annotation. Recently, the *S. oneidensis* genome was fully sequenced and fully annotated (Heidelberg et al. Nature Biotechnology 2002) allowing for whole transcriptome and whole proteome analysis (figure 3). Studying gene knockout strains of both of these potential global regulators by whole transcriptome and whole proteome methods should greatly increase our understanding of these regulatory mechanisms.

Figure #1 Shewanella oneidensis (Glausser et al. *Science* **2002** *295*, 117-119)

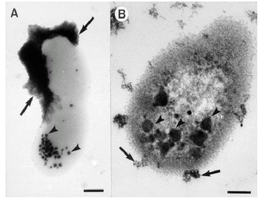


Figure # 2 Typical State of Iron uptake genes under High Iron Conditions

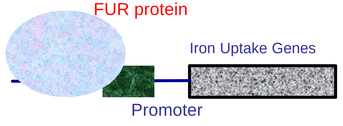
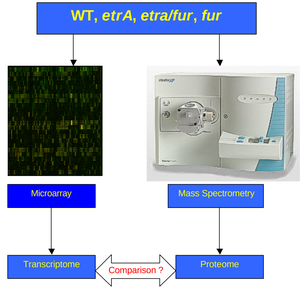


Figure #3 Comparison of microarray and mass spectrometry



Experimental:

Preparation of Microarray:

- **RNA Procedure**: WT and mutant cells were collected and put into Trizol directly. RNA was extracted according to Invetrogen protocol and purified using RNeasy kit (Quiagen). 3 biological replicates were used each having Cy5 and Cy3 complementary pairs.
- **cDNA Preparation**: 10ug RNA and 3.3ug of random primers were used to obtain cDNA. 1uM cy3/5 dUTP was used to label the cDNA. Reaction incubated at 42⁰ C for 2 hours.
- **Hybridization Procedure**: *the cDNA was mixed with 50% formide and 10g/ul Herring sperm DNA, and then hybridized with the slides. The slides were hybridized at 50⁰C overnight.*

1-D-LC-MS/MS:

- One-dimensional LC-MS/MS experiments were performed with an Ultimate HPLC (LC Packings, a division of Dionex, San Francisco, CA) coupled to an LCQ-DECA or LCQ-DECA XP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an electrospray source (figure 4). Injections were made with a Famos (LC Packings) autosampler onto a 50ul loop. Flow rate was ~4ul/min with a 240min gradient.
- A VYDAC 218MS5.325 (Grace-Vydac, Hesperia, CA) C18 column (300µm id x 15cm, 300Å with 5µm particles) or a VYDAC 238EV5.325 monomeric C18 (300µm id x 15cm, 300Å with 5µm particles) was directly connected to the Finnigan electrospray source with 100µm id fused silica.
- For all 1D LC/MS/MS data acquisition, the LCQ was operated in the data dependent mode, where the top four peaks in every full MS scan were subjected to MS/MS analysis.
- To increase dynamic range separate injections were made with a total of 4 or 5 separate m/z ranges scanned.

2-D-LC-MS/MS:

- Two-dimensional LC-MS/MS experiments were performed on an Famos/Switchos/Ultimate 2D HPLC system (LC Packings) coupled to an LCQ-DECA ion trap MS equipped with an Finnigan nanospray source (figure 5).
- Sample and salt (Ammonium Acetate) injections are made with the Famos autosampler on to a LC Packings SCX column (500µm id x 15mm), which sits on Valve A of the Switchos system.
- Peptides that elute from the SCX column are captured on an LC Packings precolumn (300µm id x 5mm, 300Å PepMap) on valve B.
- After desalting on the precolumn the precolumn flow rate was flipped in-line with a nano resolving column VYDAC 218MS5.07515 C18 (75µm id x 15cm, 300Å with 5µm particles).
- After the injection and each subsequent salt bump a RP gradient was run for 2 hours to elute peptides into the mass spectrometer.
- For these experiments 1 or 2 m/z ranges were scanned and MS was operated as described above.

Direct Comparison:

- The goal is to directly compare *fur* and *wt S. oneidensis* samples using
  - Microarray data
  - Proteomic data
- The criteria for up or down regulation is at least 30% sequence coverage and/or 4 or more unique peptides for proteomic data in replicate analysis.
- The criteria for microarray data; is if the mean is above 1 then up regulated if mean is below 1 down regulated.

Figure #4 1-D-LCMS/MS instrumentation

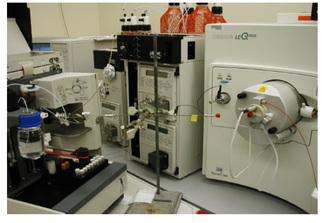
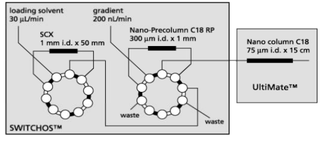


Figure #5 Column switching configuration (figure provided by LC Packings)



Results:

Protein Identification:

Figure #6 Protein Identification numbers for Wt and *fur*

Fur Protein Numbers	
1D-LC-MS/MS 5m/z	481
2D-LC-MS/MS 1m/z	751
2D-LC-MS/MS 2m/z	808
Total	963

WT Protein Numbers	
1D-LC-MS/MS 5m/z	523
2D-LC-MS/MS 1m/z	582
2D-LC-MS/MS 2m/z	669
Total	837

- WT and *fur* samples were analyzed by both 2-D-LC-MS/MS and 1D-LC-MS/MS to test which experiments gave better results.
- 2D-LC-MS/MS experiments yielded the most protein identifications and gave better sequence coverage while using less sample material.
- Future experiments will be carried out by 2D-LC-MS/MS.

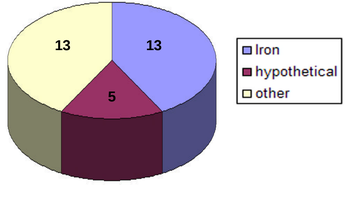
Microarray and Proteome Comparison of WT and Fur:

Figure #7 *S. oneidensis* Fur Vs. WT comparison by proteome and whole genome array data.

Up Regulated in FUR	Proteome Data									Array Data	
ORNL Gene #	FUR_5mz % Cov.	FUR_1mz % Cov.	FUR_2mz % Cov.	WT_5mz % Cov.	WT_1mz % Cov.	WT_2mz % Cov.	Fur_pept Tot pept	WT_pept Tot pept	MEAN (Fur/WT)	ORNL Annotation
264	37.9	34.8	26.5	0	0	0	21	0	26.81	ferrichrome-iron receptor
525	30.2	36.5	37	0	0	0	7	0	0.50	alkyl hydroperoxide reductase c22 protein
613	10.4	18.4	18	0	0	0	9	0	0.39	TonB-dependent receptor
775	775	16.8	19.5	4.6	4.6	0	9	1	0.92	formyltransferase/IMP cyclohydrolase
798	58.7	63.1	54	0	0	0	12	0	N/A	probable oxidoreductase
844	36.5	18.5	23.5	16.1	4.7	4.7	21	8	0.41	ornithine decarboxylase, inducible
989	27.1	32.3	40.5	0	6.2	6.2	19	1	26.20	Ferrichrome-iron receptor
1106	69.2	56.9	54.6	7.8	4.2	6.8	33	4	5.61	Fe-regulated outer mem. virulence protein, TonB receptor family
1109	10.7	10.7	25	0	0	0	8	0	8.21	ferric vibriobactin receptor
1320	41.1	42.2	42.2	6.6	20.9	20.9	6	3	0.75	uridine phosphorylase
1462	19.5	10.9	24	10.4	0	0	15	4	2.83	probable outer membrane receptor for iron transport
1594	53.5	41.6	43.8	15.4	9.8	9.8	30	7	25.45	probable tonB-dependent receptor HI0262 precursor
1595	17.2	18.3	18.3	0	0	0	2	0	37.50	conserved hypothetical protein
1596	37.3	42.7	31.4	7	0	0	9	1	59.66	conserved hypothetical protein
2429	25.9	31.7	25.9	11.6	0	0	9	2	N/A	alanine dehydrogenase
3789	44.6	42.5	35.5	0	13	13	11	2	N/A	TonB-dependent receptor, iron-siderophore receptor
3790	35	11.3	15.8	0	0	0	8	0	N/A	Similar to ferric aerobactin receptor
4265	0	11.7	42.3	0	0	0	4	0	1.17	FKBP-type peptidyl-prolyl cis-trans isomerases
4308	2.2	9.9	12.7	2.2	0	0	6	1	0.77	Cation efflux system protein: AcrB/AcrD/AcrF family
4719	37.6	58	58	0	20	5.1	13	2	15.47	conserved hypothetical protein
4960	14.7	12.9	25.8	0	3.1	0	12	1	2.48	heme-hemopexin utilization protein C precursor
5234	13.2	16	14.1	4.2	4.2	4.2	8	1	1.11	UDP-sugar hydrolase
Up Regulated in WT										
125	0	0	0	4.2	12.5	13.6	0	5	0.17	deca-heme type cytochromes
126	0	0	0	7.5	15.7	17.7	0	10	0.19	deca-heme type cytochromes
1552	0	0	0	21.5	8	13.1	0	12	0.94	Hypothetical
2240	0	0	0	3.6	25.8	25.8	0	3	1.92	hypothetical protein
2404	0	8.7	19.7	22.1	37	39.4	2	8	0.94	cytochrome c oxidase, subunit CcoO
3043	4.5	10.5	4.3	25.6	37.1	40.2	4	28	1.32	TonB-dependent (outer membrane) receptor, mostly iron transport
3608	0	0	0	11.8	15.3	17.3	0	8	0.97	cytochrome c maturation protein c
4840	0	0	0	23.3	26.7	25.5	0	16	1.47	putative membrane protein, Membrane protease subunits
75a	0	0	0	6.3	10.6	6	0	7	1.24	probable acylamino-acid-releasing enzyme

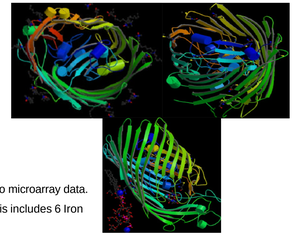
Rules for acceptance: at least a 30% change in sequence coverage and/or 4 or more unique peptides difference
Code:
Black and Bold = high probable change and is involved in metal uptake or utilization
Black and normal font= high probability not directly involved in metal uptake or utilization
Red and normal font= medium probability involved in metal uptake
Microarray: if the mean is above 1 then it is up regulated if mean is below 1 then down regulated

Figure #8 categories of proteins changing in WT and fur



- In WT and fur experiments 31 proteins show significant change.
- 17 proteins agree with microarray data, 10 do not, and 4 there is no microarray data.
- 13 of the 31 proteins are Iron regulation or acquisition proteins. This includes 6 Iron siderophore receptors.

Figure #9 Crystal structure of e. coli Iron Siderophore receptors **FecA** (Ferguson et al. Science 20020, **FepA** (Buchanan et al. Nature Structural Biology 1999), and **FhuA** (Locher et al. Cell 1998).



Proteome Data for WT, etrA, and etrA/fur:

Figure #10 S. oneidensis WT, etrA, and etrA/fur proteomic data

Down regulated in ETRA/fur ORNL Gene #	ETRA/fur_1 % Cov.	ETRA/fur_2 % Cov.	ETRA_1 % Cov.	ETRA_2 % Cov.	WT_1 % Cov.	WT_2 % Cov.	ETRA_pept Tot pept	Fur/ETRA_pept Tot pept	WT_pept Tot pept	ORNL Annotation
125	0	0	17.4	6.5	9.8	22	0	7	9	deca-heme type cytochrome
126	0	0	18	13.7	12.4	21.5	0	9	7	deca-heme type cytochrome
1430	0	0	33	33.7	27.2	14.9	0	7	4	Molybdenum-binding periplasmic protein, ModA
2404	0	0	26.4	28.4	26.4	26.4	0	5	5	cytochrome c oxidase, subunit CcoO
2406	0	0	17.7	18.9	17.7	14.3	0	4	4	cytochrome c oxidase, subunit CcoP
3029	0	0	16	6.7	40.2	40.7	0	2	5	superoxide dismutase, Fe
4696	7.3	0	6.2	6.2	15.6	31.6	1	1	7	carbamoyl-phosphate synthase, small subunit
4697	0	0	3.1	3.4	17.2	17.6	0	3	15	carbamoyl-phosphate synthase, large subunit
Up Regulated in ETRA/fur										
196	19.4	16.5	0	0	0	0	4	0	0	methionine aminopeptidase
264	29.1	32.4	0	0	0	0	20	0	0	ferrichrome-iron receptor
613	4	6	0	0	0	0	4	0	0	TonB-dependent receptor
1106	47.7	49.8	0	0	0	0	24	0	0	iron-regulated outer membrane, TonB receptor family
1462	12	23.3	0	0	0	0	13	0	0	probable outer membrane receptor for iron transport
1594	47.6	58	0	0	0	0	26	0	0	probable tonB-dependent receptor HI0262 precursor
1595	24.2	34.4	0	0	0	0	4	0	0	conserved hypothetical protein
1596	75.1	79.5	0	0	0	0	9	0	0	conserved hypothetical protein
2429	21.7	13.7	0	0	0	0	4	0	0	conserved hypothetical protein
3099	12.7	12.7	0	0	0	0	4	0	0	putative siderophore biosynthetic enzyme
3287	38.2	54.6	0	0	0	0	9	0	0	hemin transport system substrate-binding protein
Down regulated in ETRA										
4018	7.4	10.1	0	0	10.5	12.6	5	0	6	putative acidic periplasmic protein
5082	12.4	19.3	0	0	3.3	3.1	8	0	2	phosphomannomutase, putative
UP Regulated in ETRA										
4037	0	0	16.1	14.8	3.6	3	0	5	2	formate dehydrogenase, iron-sulfur subunit
Down Regulated in WT										
3789	26.8	26.8	21.7	23.2	11.1	0	5	5	2	TonB-dependent receptor, iron-siderophore receptor
Up Regulated in WT										
1552	5	5	5	5	14.9	12.6	1	1	6	hypothetical
3029	0	0	16	6.7	40.2	40.7	0	2	6	superoxide dismutase, Fe

- There is a total of 25 proteins showing significant change with at least a 30% change in sequence coverage or 4 or more unique peptides difference
- *etrA/fur* mutant shows the most change with 19 of the 25 proteins showing significant change.
- This was an initial experiment to test conditions for proteomic data and will be repeated
- Microarrays are currently ongoing for these mutants.

Conclusions and Future Plans:

- Repeat analysis of WT and fur by 2D-LC-MS/MSto enhance protein identification.
- Prepare membrane fractions of fur and WT, cut by Proteinase K and CNBr and run by 2D-LC-MS/MS for more extensive coverage of membrane proteins.
- Complete and Compare Microarray data with mutants WT, *etrA*, and *etrA/fur*.

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