O-066

Investigation of Protein-Protein Interactions in the Metal-Reducing Bacterium *Desulfovibrio vulgaris* Hildenborough

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ESPP Environmental Stress Pathway Project

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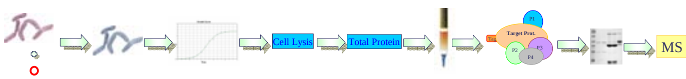
INTRODUCTION

Desulfovibrio vulgaris is a sulfate reducing bacterium of interest due to its potential use in bioremediation as well as its economic impact in the petroleum industry (bio-corrosion of pumping machinery). This sulfate reducing bacterium has been shown to reduce toxic metals (such as chromium and uranium) to insoluble species making them a good model system for understanding molecular machines involved in bioremediation of contaminated soils and ground water.

We have implemented an approach for the isolation of protein complexes from *D. vulgaris* by generating *D. vulgaris* cell lines that produce a "bait" protein of interest fused to an affinity tag (strep tag). Lysate from these cells is passed over an avidin column, and the bait protein with its associated proteins is captured and can be selectively eluted from contaminating proteins. One advantage of this approach is that complexes are formed and captured under native conditions. Nanoscale liquid chromatography-tandem mass spectrometry (nLC/MS/MS) is used identify the captured proteins.

To determine bait proteins of interest, we computationally identified open reading frames that are involved in stress response (including oxygen, heat, pH and salt) based on homology to known stress related genes from other prokaryotic species. We have also selected proteins that are unique to this sulfate reducer ("signature" genes), expected to yield novel complexes related to sulfate/metal reduction. To validate our methods and address the challenge of non-specific binding, we have included some bait proteins whose interacting partners are well characterized in prokaryotic systems and have validated our methods by isolating the protein partners of these targets.

PROTOCOL OUTLINE



A. Tagged-Protein Generation

1. The gene encoding the target protein is amplified with a modified stop codon.
2. The amplified product is introduced in a plasmid containing the tag sequence such that the tag is in the same reading frame as the gene of interest.
3. Wild type *D. vulgaris* is electroporated with the construct from Step 2.
4. Positive strains are isolated followed by extraction of the chromosomal DNA.
5. Chromosomal presence of the tagged gene is confirmed by PCR/Southern analysis.

B. Tagged Protein Isolation and Identification

1. Mutant strains are grown until mid-late log phase.
2. Cells are harvested and lysed using sonication. Protease inhibitors are added to the lysis buffer.
3. Total soluble protein is estimated using the BCA assay.
4. Extracted protein mixture is fed to a *Strep*-Tactin resin affinity column followed by gravity flow separation.
5. Each column is washed with wash buffer to remove non-specific proteins.
6. The tagged protein along with its interacting partners are eluted with a buffer containing a biotin analog (desthiobiotin).
7. Eluted proteins are either denatured and run on an SDS-PAGE Gel or are digested in-solution via trypsin.
8. Bands observed after Coomassie-blue staining are excised and tryptically digested.
9. Digested peptides (in-gel or in-solution) are identified using nLC/MS/MS analysis.

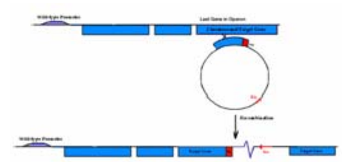
QUALITY CONTROL

- ✓ Sequence verification of PCR-tag constructs
- ✓ Single colony isolation of transformants
- ✓ PCR testing of transformants
- ✓ Southern analysis of transformants

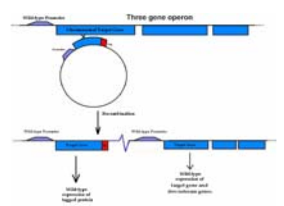
CONSTRUCTION OF TAGGED MUTANTS

Homologous recombination of gene with tagged sequence in circular plasmid with chromosomal copy of the target gene.

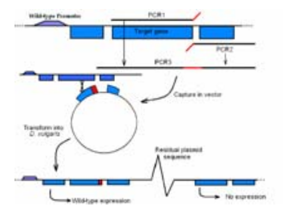
Scheme for last gene in an operon or monocistronic gene



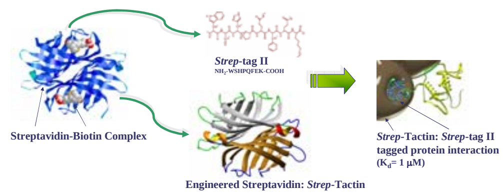
Scheme for first gene in an operon



Scheme for penultimate gene in an operon



THE STREP TAG II SYSTEM



- Based on the well known binding of biotin to streptavidin
- *Strep*-tag II ®: consists of 8 amino acids (WSHPQFEK) and binds to the biotin pocket of streptavidin.
- Engineered Streptavidin: *Strep*-Tactin ® has 100 times higher binding affinity to *Strep*-tag II than Streptavidin

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STRAINS GENERATED

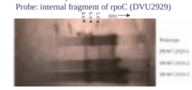
Strain Name	Target Protein	Gene ID	Description
JW907-0811	DnaK	DVU0811	Molecular chaperone
JW907-2928	RpoB	DVU2928	DNA-directed RNA polymerase, beta subunit
JW907-2929	RpoC	DVU2929	DNA-directed RNA polymerase, beta prime subunit
JW907-1577	HslV	DVU1577	ATP-dependent protease
JW907-0503	Pnp	DVU0503	Polyribonucleotide nucleotidyltransferase
JW905-3184	Rub	DVU3184	Rubredoxin
JW905-0846	ApsB	DVU0846	Adenylylsulphate reductase, beta subunit
JW907-1568	Ftn	DVU1568	Ferritin
JW913-1397	Bfr	DVU1397	Bacterioferritin

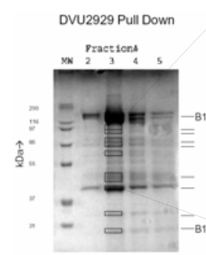
Some interacting partners obtained

MODEL SYSTEM: DNA-directed RNA polymerase complex

We generated the strain JW907-2929 to validate our protocol for protein complex identification in *D. vulgaris*. DVU2929 encodes for the DNA-directed RNA polymerase, RpoC, and occurs in a two gene operon preceded by one of its interacting partners, RpoB (DVU2928). The construct used for transforming the wild type strain was verified by confirming the sequence of the tagged gene and the recombination was confirmed by southern analysis (left). The pull down experiment generated at least 10 distinct bands on an SDS-PAGE gel. The tagged protein (RpoC) as well as the expected interacting partners, RpoA and RpoB were all detected by MS-MS analysis of the typically digested bands.

Southern Analysis.
Probe: internal fragment of rpoC (DVU2929)





MW = Mark12 ladder (Invitrogen, Carlsbad, CA).
SDS PAGE: 10% Tris-Glycine.

sample	hit #	hit score	# sig pep	accession	annotation	MW (Da)
2929B10	1	1276	24	DVU2928	DNA-directed RNA polymerase, beta subunit (rpoB)	153574
	2	868	16	DVU2929	DNA-directed RNA polymerase, beta prime subunit (rpoC)	155628
	3	139	2	DVU3025	pyruvate-ferredoxin oxidoreductase (poR)	132690
	4	107	3	DVU1834	pyruvate carboxylase, putative	137523
2929B9	1	440	7	DVU2929	DNA-directed RNA polymerase, beta prime subunit (rpoC)	155628
	2	130	2	DVU2928	DNA-directed RNA polymerase, beta subunit (rpoB)	153574
	3	82	1	DVU1834	pyruvate carboxylase, putative	137523
	4	25	1	DVU1300	translation elongation factor G (fusA-1)	76532
2929B8	1	263	5	DVU3033	iron-sulfur cluster-binding protein	80915
	2	142	2	DVU1834	pyruvate carboxylase, putative	137523
	3	86	2	DVU3029	phosphate acetyltransferase (pta)	77295
	4	48	1	DVU1788	RNA polymerase sigma-70 factor (rpoD)	67766
2929B7	1	220	4	DVU0847	adenylyl-sulphate reductase, alpha subunit (aprA)	75491
	2	168	3	DVU3150	ribosomal protein S1 (rpsA)	64698
	3	69	1	DVU1976	chaperonin, 60 kDa (groEL)	58524
	4	61	1	DVU2929	DNA-directed RNA polymerase, beta prime subunit (rpoC)	155628
2929B6	1	204	4	DVU1834	pyruvate carboxylase, putative	137523
	2	99	2	DVU1976	chaperonin, 60 kDa (groEL)	58524
	3	75	1	DVU0510	N utilization substance protein A (nusA)	47870
	4	62	1	DVU0777	ATP synthase, F1 alpha subunit (atpA)	54718
	5	59	2	DVU2543	hybrid cluster protein	58448
	6	48	1	DVU0465	anthranilate synthase, component I	57944
	7	38	1	DVU1179	aldehyde:ferredoxin oxidoreductase, tungsten-containing (aor)	61607
	8	19	1	DVU1043	GMP synthase (guaA)	57265
2929B5	1	173	5	DVU0607	adenosylhomocysteinase (ahcY)	53433
	2	133	2	DVU2109	MTH1175-like domain family protein	50621
	3	115	2	DVU1468	peptidase/PDZ domain protein	51577
	4	100	2	DVU1044	inosine-5`-monophosphate dehydrogenase (guaB)	52584
2929B4	1	162	2	DVU1834	pyruvate carboxylase, putative	137523
	2	153	2	DVU0799	conserved hypothetical protein	50496
	3	138	3	DVU3027	glycolate oxidase, subunit GlcD (glcD)	49545
	4	86	2	DVU2920	translation elongation factor Tu (tuf)	43664
	5	79	2	DVU1295	sulfate adenylyltransferase (sat)	48065
	6	71	1	DVU2364	aminotransferase, classes I and II	43247
	7	57	1	DVU1329	DNA-directed RNA polymerase, alpha subunit (rpoA)	39012
	8	48	1	DVU2109	MTH1175-like domain family protein	50621
	9	37	1	DVU0002	DNA polymerase III, beta subunit (dnaN)	43075
	10	31	1	DVU0797	conserved hypothetical protein	51294
2929B3	1	575	12	DVU1329	DNA-directed RNA polymerase, alpha subunit (rpoA)	39012
	2	179	3	DVU3048	aspartate-semialdehyde dehydrogenase (asd)	38631
	3	43	1	DVU0403	dissimilatory sulfite reductase beta subunit (dvsB)	43232
	4	31	1	DVU0789	rod shape-determining protein MreB (mreB-1)	37447
	7	22	1	DVU1422	OmpA family protein	38500
2929B2	1	154	3	DVU2104	iron-sulfur cluster-binding/ATPase domain protein	32248
	2	130	3	DVU1329	DNA-directed RNA polymerase, alpha subunit (rpoA)	39012
	3	108	2	DVU2103	iron-sulfur cluster-binding/ATPase domain protein	31127
	4	71	1	DVU2076	chemotaxis protein methyltransferase (cheR-2)	34139
	5	68	1	DVU1306	ribosomal protein L2 (rplB)	30300
	6	58	1	DVU0874	ribosomal protein S2 (rpsB)	28627
2929B1	1	106	3	DVU2925	ribosomal protein L1 (rplA)	24877
	2	95	2	DVU1309	ribosomal protein S3 (rpsC)	24456
	3	88	1	DVU1303	ribosomal protein L3 (rplC)	22451
	4	39	1	DVU2224	hypothetical protein	24476
	5	38	1	DVU1328	ribosomal protein S4 (rpsD)	24175

PROTEIN-PROTEIN INTERACTIONS OBSERVED

Putative Interacting Proteins Associated with Tagged Targets - DVU2928, DVU0811, DVU1577 and DVU0503.

Score	# peptides	DVU#	Description	MW (Da)
Target: DVU2928- DNA-directed RNA polymerase, beta subunit (rpoB)				
439	6	DVU2920	translation elongation factor Tu (tuf)	43664
269	6	DVU1834	pyruvate carboxylase, putative	137523
257	6	DVU3025	pyruvate-ferredoxin oxidoreductase (poR)	132690
227	5	DVU2928	DNA-directed RNA polymerase, beta subunit (rpoB)	153574
222	4	DVU1295	sulfate adenylyltransferase (sat)	48065
179	3	DVU2224	hypothetical protein	24476
173	5	DVU1329	DNA-directed RNA polymerase, alpha subunit (rpoA)	39012
151	2	DVU3029	phosphate acetyltransferase (pta)	77295
144	2	DVU2929	DNA-directed RNA polymerase, beta prime subunit (rpoC)	155628
143	2	DVU1013	type I secretion outer membrane protein, TolC family	50106
142	2	DVU3033	iron-sulfur cluster-binding protein	80915
141	2	DVU0799	conserved hypothetical protein	50496
118	2	DVU1976	chaperonin, 60 kDa (groEL)	58524
117	2	DVU3027	glycolate oxidase, subunit GlcD (glcD)	49545
101	2	DVU2109	MTH1175-like domain family protein	50621
Target: DVU0811-dnaK protein (dnaK)				
1789	31	DVU0811	dnaK protein (dnaK)	68787
266	6	DVU1976	chaperonin, 60 kDa (groEL)	58524
247	5	DVU2109	MTH1175-like domain family protein	50621
246	5	DVU0812	heat shock protein GrpE (grpE)	21065
225	6	DVU1834	pyruvate carboxylase, putative 1	137523
174	3	DVU2920	translation elongation factor Tu (tuf)	43664
171	4	DVU0847	adenylyl-sulphate reductase, alpha subunit (aprA)	75491
98	2	DVU2108	MTH1175-like domain family protein	12562
94	2	DVU0846	adenylylsulphate reductase, beta subunit (aprB)	19140
93	2	DVU0775	ATP synthase, F1 beta subunit (atpD)	50824
88	2	DVU0777	ATP synthase, F1 alpha subunit (atpA)	54718
Target: DVU1577-ATP-dependent protease (hslV)				
468	9	DVU2349	carbohydrate phosphorylase family protein	97695
170	3	DVU1834	pyruvate carboxylase, putative	137523
Target: DVU0503-polyribonucleotide nucleotidyltransferase (pnp)				
1514	57	DVU0503	polyribonucleotide nucleotidyltransferase (pnp)	82433
816	18	DVU3150	ribosomal protein S1 (rpsA)	64698
542	11	DVU1834	pyruvate carboxylase, putative	137523
423	6	DVU2920	translation elongation factor Tu (tuf)	43664
271	5	DVU0503	polyribonucleotide nucleotidyltransferase (pnp)	82433
247	4	DVU1328	ribosomal protein S4 (rpsD)	24175
230	6	DVU1306	ribosomal protein L2 (rplB)	30300
219	6	DVU1295	sulfate adenylyltransferase (sat)	48065
211	5	DVU1329	DNA-directed RNA polymerase, alpha subunit (rpoA)	39012
191	2	DVU3150	ribosomal protein S1 (rpsA)	64698
182	3	DVU2929	DNA-directed RNA polymerase, beta prime subunit (rpoC)	155628
168	3	DVU0607	adenosylhomocysteinase (ahcY)	53433
129	2	DVU2109	MTH1175-like domain family protein	50621
120	2	DVU0503	polyribonucleotide nucleotidyltransferase (pnp)	82433
102	2	DVU0874	ribosomal protein S2 (rpsB)	28627
82	2	DVU1044	inosine-5`-monophosphate dehydrogenase (guaB)	52584

We repeated the approach described above for four other targets – DVU2928, DVU0811, DVU1577 and DVU0503. Typically, 7-10 bands were observed for each of the pull down experiments except for DVU1577. The Table on the left lists significant protein hits that were identified from at least two peptides with MOWSE scores of 48 or higher. In all cases except for DVU1577 we were able to identify the tagged protein. The Table below lists proteins that were identified irrespective of the target protein chosen for the pull down experiment. These proteins might be non-specifically interacting with the affinity column. Whether these are observed during pull down experiments with other targets remains to be seen.

Non-specifically Associated Proteins with Tagged Targets DVU2928, DVU2929, DVU0811, DVU1577 and DVU0503.

Gene ID	Description	Average # peptides	Average Score	MW (Da)
DVU1044	inosine-5`-monophosphate dehydrogenase (guaB)	2	92	52584
DVU1295	sulfate adenylyltransferase (sat)	4	173	48065
DVU1834	pyruvate carboxylase, putative	6	289	137523
DVU2109	MTH1175-like domain family protein	2	127	50621
DVU2920	translation elongation factor Tu (tuf)	3	180	43664
DVU3025	pyruvate-ferredoxin oxidoreductase (poR)	3	129	132690

We are currently in the process of tagging the various interacting partners observed for the aforementioned targets. Such cross-tagging experiments help confirm weak interactions as well as identify false positives. Expanding this analysis to the entire proteome of *D. vulgaris* will help us map the 'protein interactome' of this sulfate reducing bacterium.

ACKNOWLEDGEMENT

ESPP is part of the Virtual Institute for Microbial Stress and Survival supported by the U. S. Department of Energy, Office of Science, Office of Biological and Environmental Research, Genomics Program:GTL through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S. Department of Energy.