Bacterial Genes and Proteins Involved in the Redox Transformations of Metals

Bradley M. Tebo

Overall Laboratory Research Goals

- To understand the molecular mechanisms by which bacteria transform metals.
- To understand the factors controlling the cycling of metals in the environment.
- To understand the effect of toxic metals on microbial communities and their activities.

Mechanisms for the Natural Attenuation of Metal Pollution

- Volatilization
- Sorption
- Precipitation
- **Redox transformations**

The Processes

- Manganese(II) oxidation

  $Mn^{2+} \rightarrow Mn(III,IV)$ oxides (solids)

  – Sequestration of toxic metals (e.g., Pb, Zn, Cd, Cu, Co)

- Chromium(VI) reduction

  $Cr(VI) \rightarrow Cr(III)$ (less soluble)

  – Hexavalent Cr detoxification

Research Objectives

- To identify and characterize the genes and proteins involved in Mn(II) oxidation and Cr(VI) reduction
- To understand the underlying molecular mechanisms of these redox transformations
- To understand how these processes are regulated by environmental cues
- To evaluate the potential of these processes for bioremediation applications

Bacterial Mn(II) Oxidation

Biogenic Mn Oxides

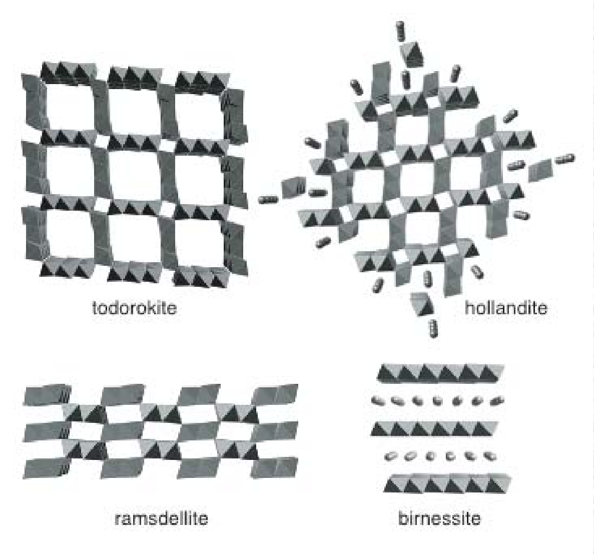
- Reactive!
- Sorb metals
- Oxidize organic compounds

Major players in the biogeochemical cycling of metals and carbon

Manganese Oxide-Coated Creek Sediments

Mn(II)-Oxidizing Bacteria

- Ubiquitous in soils, sediments & natural waters.
- Phylogenetically diverse
- Primary source of reactive Mn oxides found in nature.
- Require O_2



Layer and tunnel structures

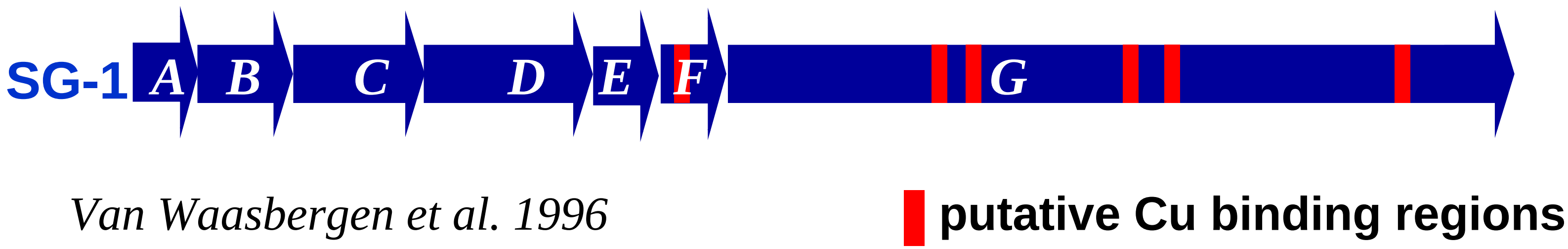
Question

- How do bacteria oxidize Mn (II)?
- Can we exploit Mn(II) oxidation for metal bioremediation?

Marine *Bacillus* sp. strain SG-1

- Spores, not vegetative cells, oxidize Mn(II)
- Mn(II)-oxidizing spores are ubiquitous
- Increase the rate of Mn(II) oxidation by 4-5 orders of magnitude
- Active over a wide range of conditions
  - [Mn(II)] (<nM to >mM)
  - Temperature (2-55°C)
  - pH ($\geq$ 6.5)
  - Osmotic strength

Mnx Genes Involved in Mn(II) Oxidation in *Bacillus* sp. Strain SG-1

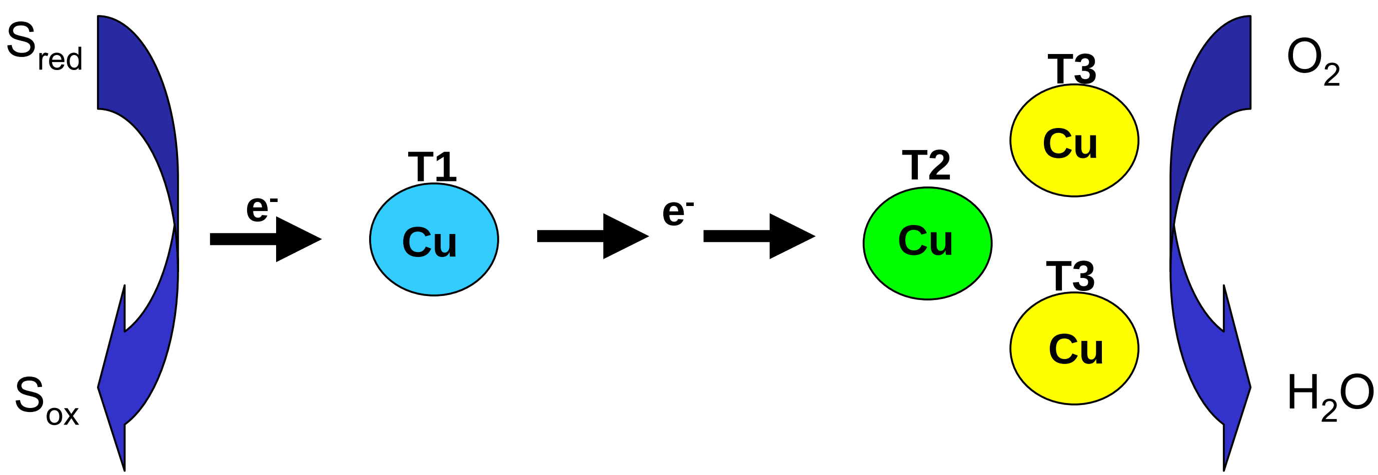


Van Waasbergen et al. 1996

- Transposon mutagenesis identified a region with 7 ORFs that is required for Mn-oxidation activity.
- *mnxG* has copper binding signatures of a multicopper oxidase; 5 Cu binding regions predicted, with a 6th in *mnxF*
- addition of copper enhances Mn(II) oxidation
- Direct link between *mnxG* and active Mn(II)-oxidizing enzyme has never been made.
- Multicopper oxidases involved in Mn oxidation have also been found in *Pseudomonas* and *Leptothrix* spp.

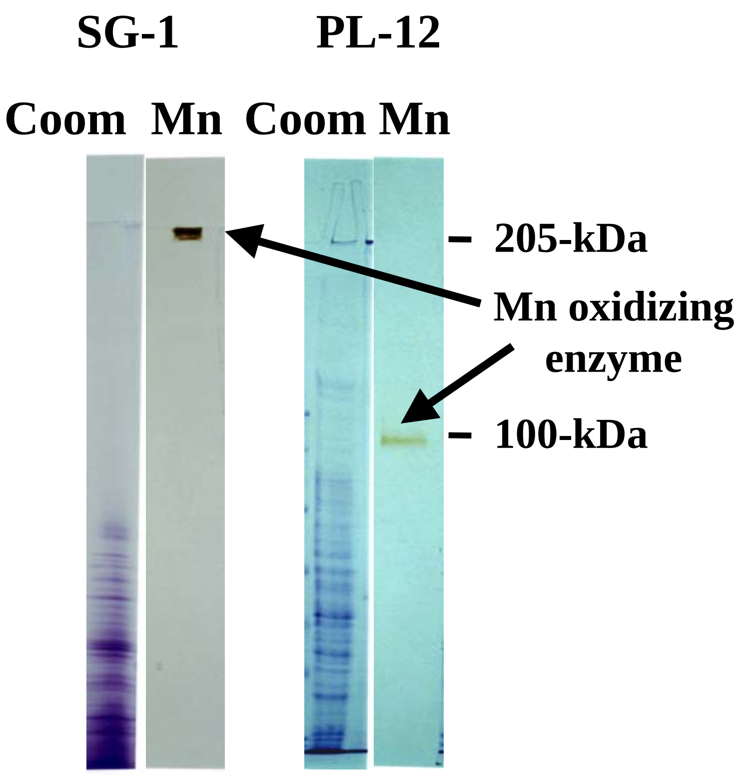
Multicopper oxidases

	A						B						C								D											
		2		3					3		3		1			2		3			3	1	3				1					1
Lac. (fung)	I	H	W	H	G	F	W	Y	H	S	H	L	H	P	F	H	L	H	G	H	H	C	H	I	D	F	H	L	E	A	G	F
Lac. (plant)	I	H	W	H	G	V	W	W	H	A	H	S	H	P	M	H	L	H	G	F	H	C	H	F	E	R	H	T	T	W	G	M
AO (plant)	I	H	W	H	G	I	F	Y	H	G	H	L	H	P	W	H	L	H	G	H	H	C	H	I	E	P	H	L	H	M	G	M
Cerulo (hum)	F	H	S	H	G	I	I	Y	H	S	H	I	H	T	V	H	F	H	G	H	H	C	H	V	T	D	H	I	H	A	G	M
Fet3 (fung)	M	H	F	M	G	L	W	Y	H	S	H	T	H	P	F	H	L	H	G	H	H	C	H	I	E	W	H	L	L	Q	G	L
CopA (P. syr)	I	H	W	H	G	L	W	H	H	S	H	S	H	P	I	H	L	H	G	M	H	C	H	L	L	Y	H	M	E	M	G	M
PcoA (E. coli)	I	H	W	H	G	I	W	Y	H	S	H	S	H	P	I	H	L	H	G	M	H	C	H	L	L	Y	H	M	E	M	G	M
CumA(P. put)	I	H	W	H	G	I	W	Y	H	P	H	V	H	P	I	H	L	H	G	M	H	C	H	V	I	D	H	M	E	T	G	L
MofA (L. dis)	I	H	L	H	G	G	W	Y	H	D	H	T	H	P	V	H	F	H	L	L	H	C	H	I	L	G	H	E	E	N	D	F
MnxG (B. SG1)	M	H	I	H	F	V	F	F	H	D	H	L	H	V	F	H	Y	H	V	H	H	C	H	L	Y	P	H	F	G	I	G	M
MnxG SG-1 #5													H	T	F	H	L	H	G	H												



Many diverse Mn(II)-oxidizing *Bacillus* spores have been identified (Francis & Tebo, 2002)

- An internal region of *mnxG* (900bp) can be PCR-amplified from Mn(II)-oxidizing spores but not from non-oxidizers
- The apparent size of the Mn(II)-oxidizing enzymes varies
- *Bacillus* sp. strain PL-12 has a smaller Mn(II) oxidase
  - more amenable to purification or heterologous expression



Approaches

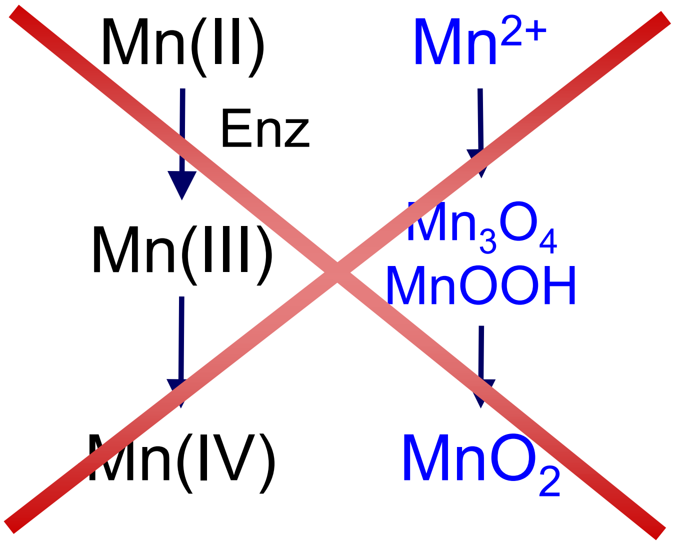
- Direct identification (mass spectrometry of the active protein bands after polyacrylamide gel electrophoresis)
- Standard protein purification and characterization (chromatography, kinetic studies, etc.)
- Cloning and expression

What are the molecular mechanisms of Mn(II) oxidation by bacteria?

- How does the enzyme catalyze electron transfer and Mn oxide formation?
- Is Mn(III) an intermediate?
  - Solid phase? (e.g., Mn_3O_4, MnOOH)
  - Enzyme bound?

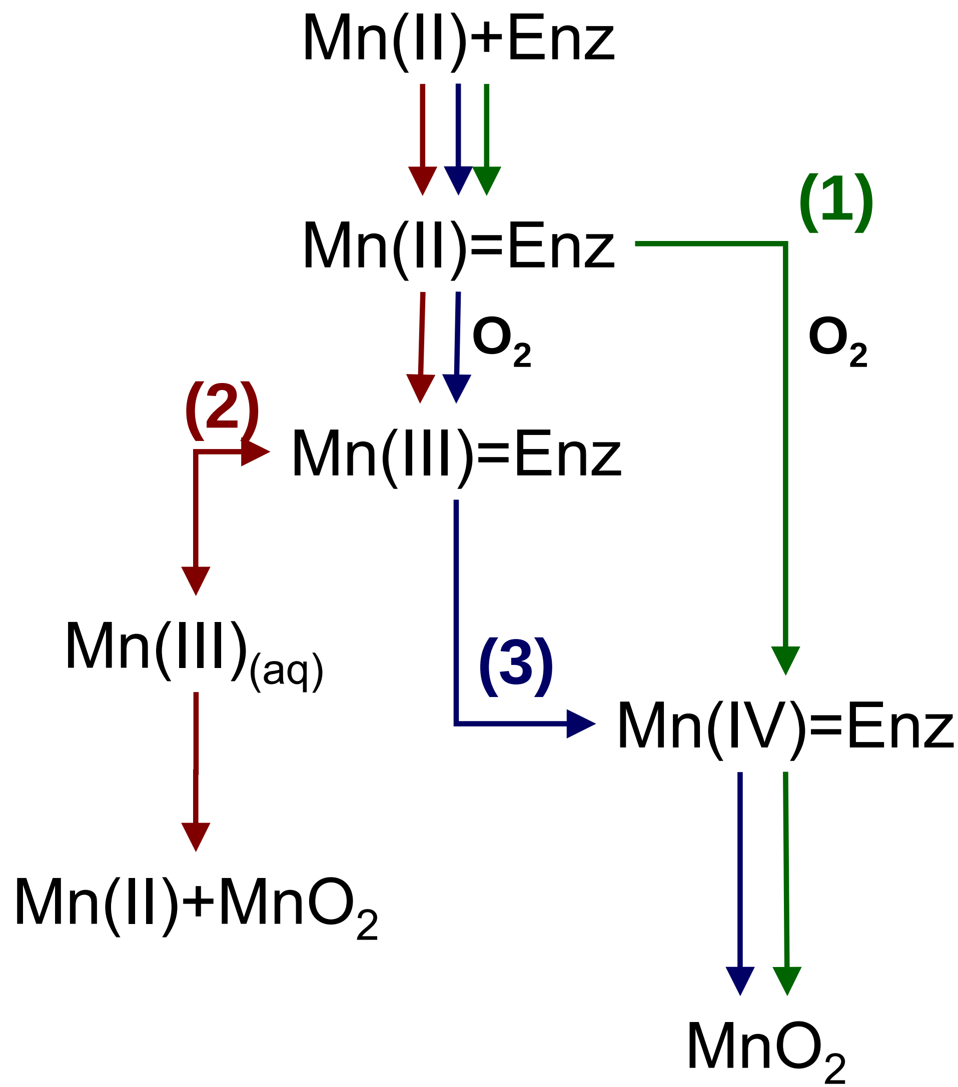
Possible Mechanisms of Bacterial Mn(II) Oxidation

Solid Phase Intermediate

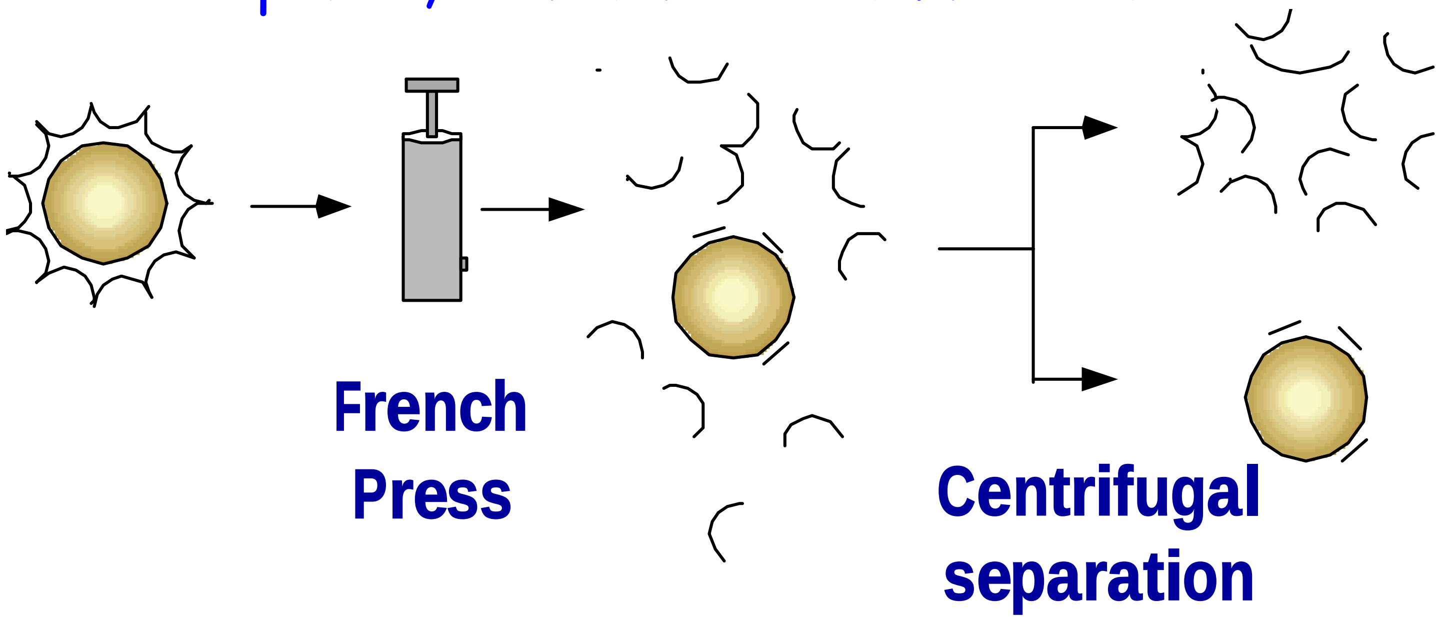


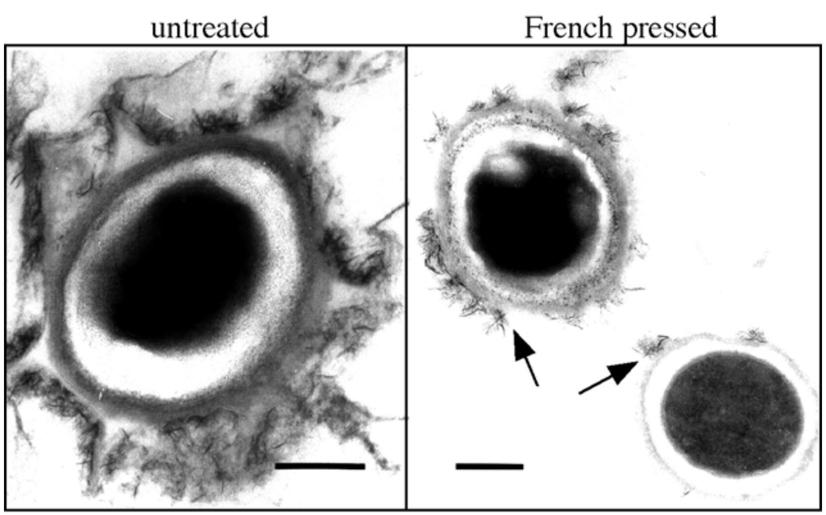
Ruled out by X-ray Absorption Spectroscopy

Enzyme-bound Intermediate



The exosporium, the outermost layer covering the spores, contains the "Mn oxidase"



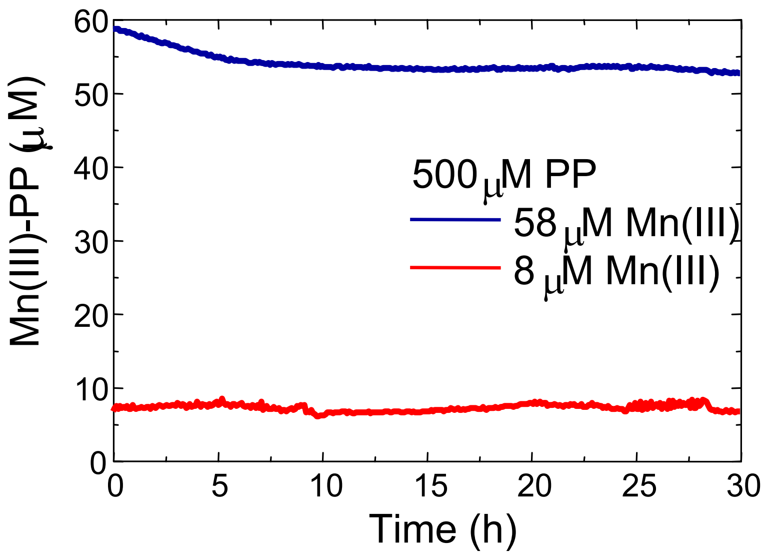


Mn(III) Trapping Experiments

- Exosporium from SG-1 (pH 7.5)
- Monitor UV-Vis absorbance in situ while biogenic oxides are produced
  - Presence and absence of pyrophosphate (PP)
    - Forms a colored complex with Mn(III) at 258 & 480 nm
  - Monitor absorbance at 5 min intervals
  - Correct for Mn oxide particles
- Experimental Parameters
  - Varied initial Mn (10µM, 50µM, 100µM)
  - Inhibitors (KCN, NaN_3)
  - No O_2
  - *mnxG*⁻ mutants

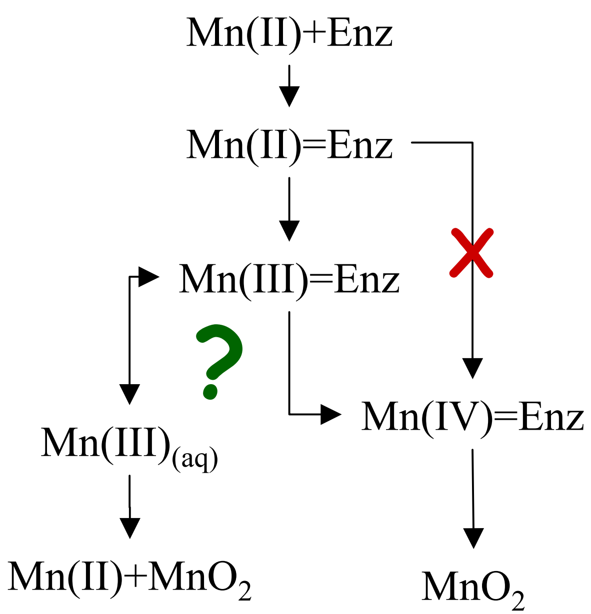
Exo + Mn(II) = δ-MnO_2

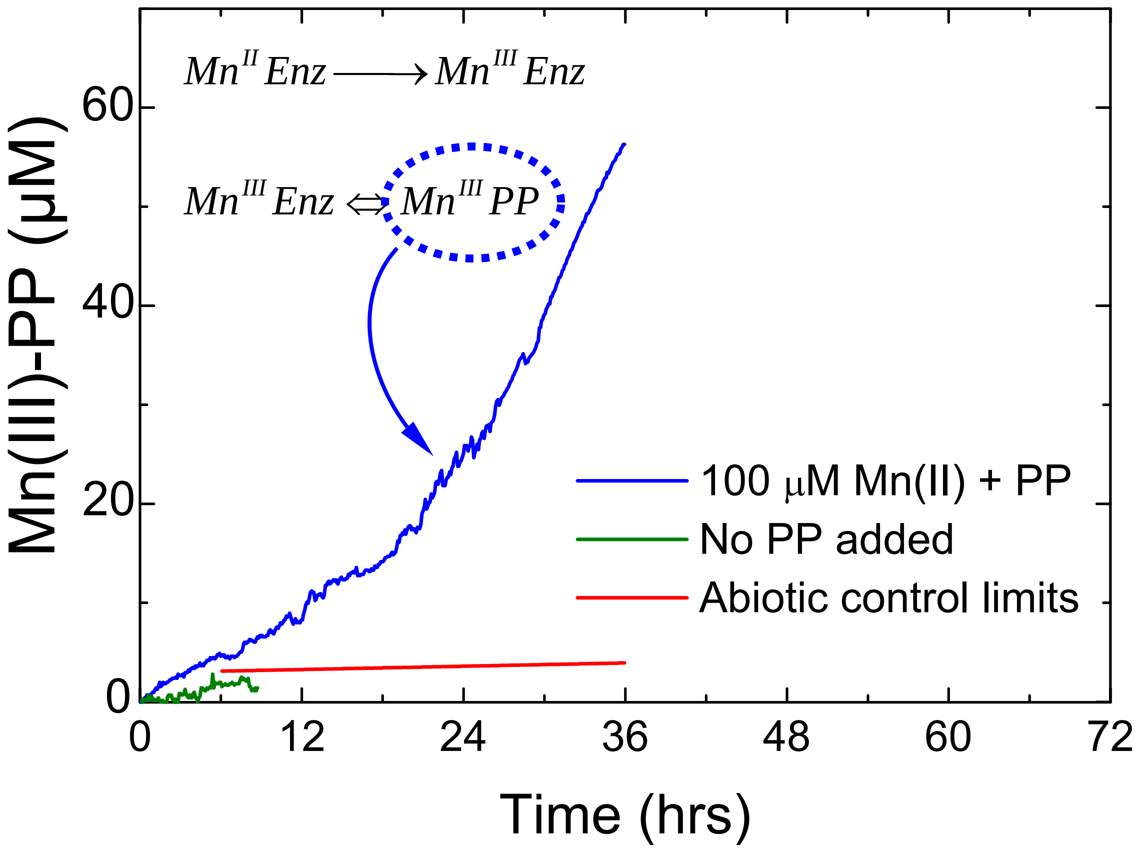
The Mn(III)-PP complex is stable



Mn(III)-PP is produced from Mn(II)

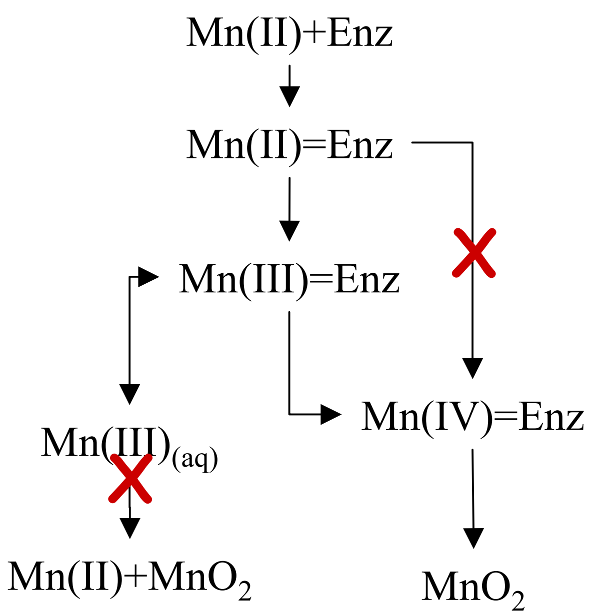
- Production rates occur faster than controls with PP, Mn(II), and synthetic or biogenic oxides

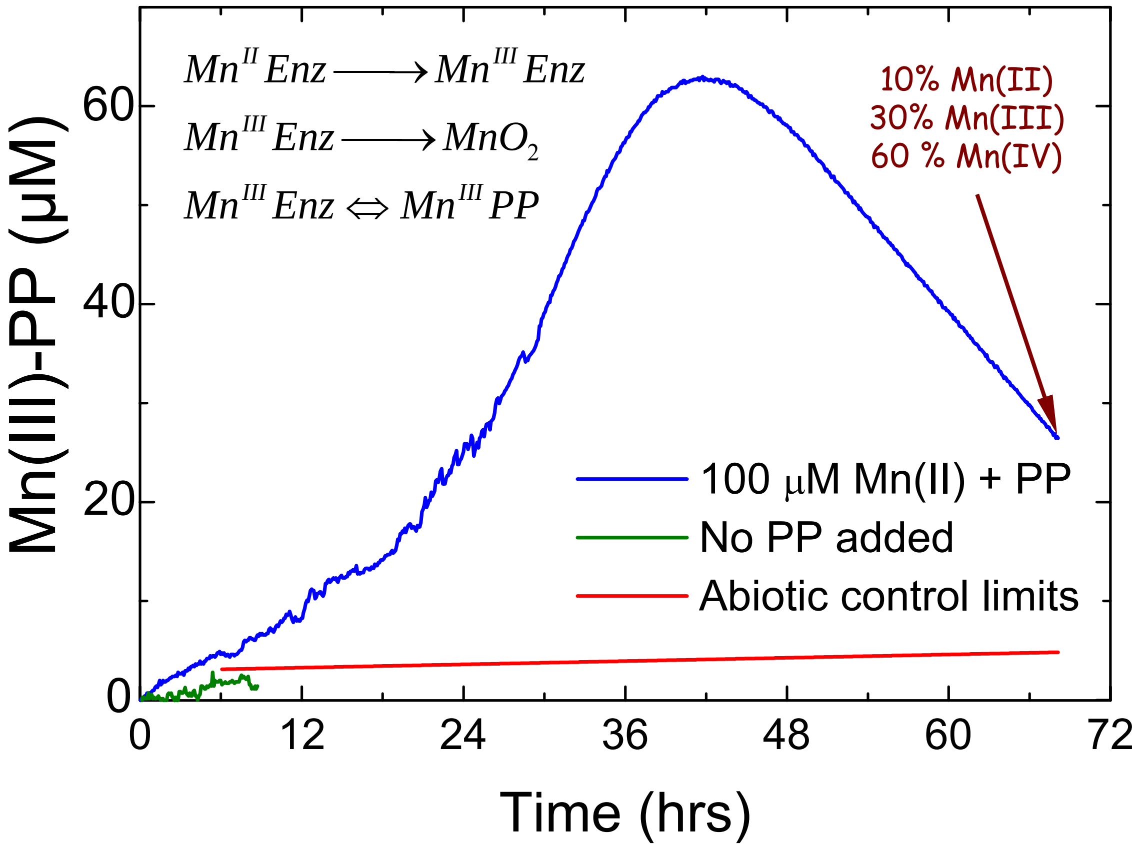




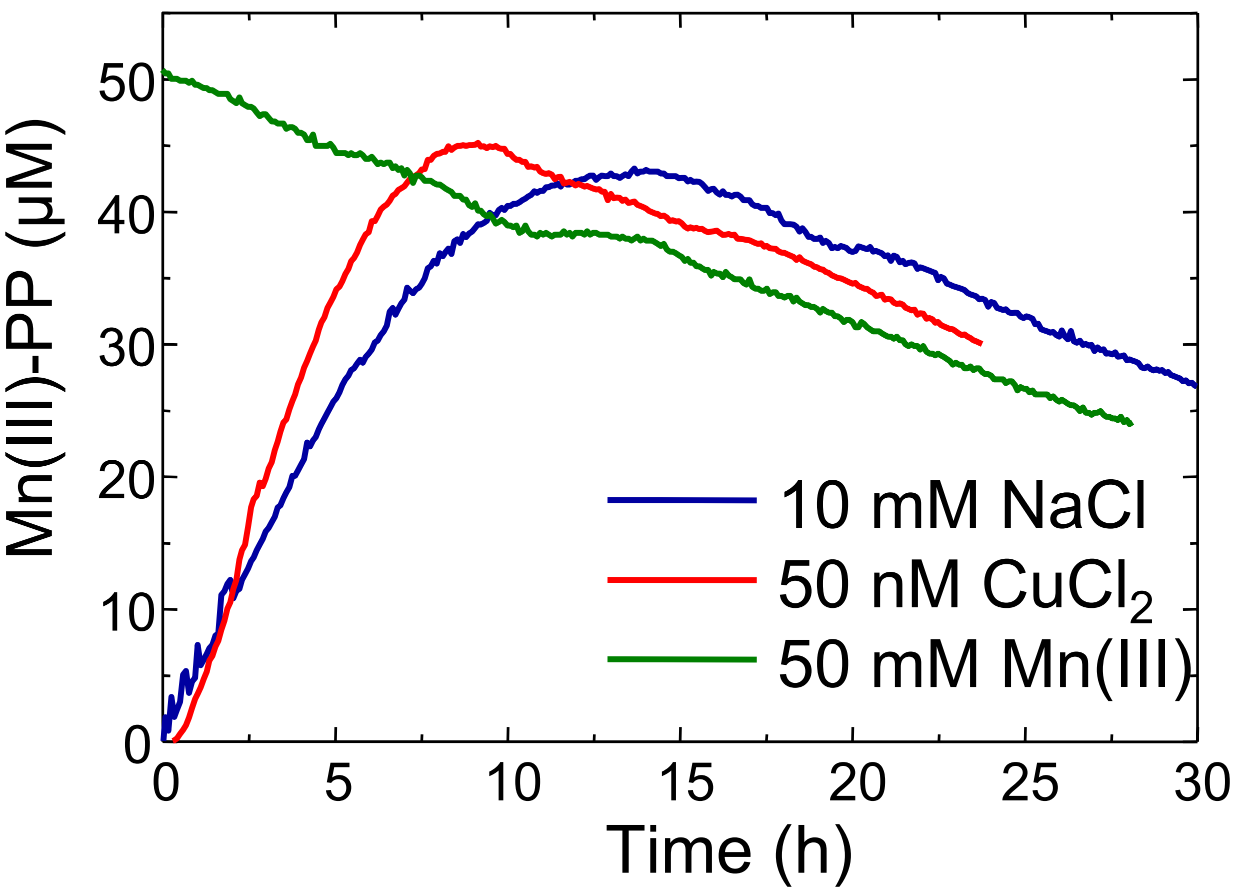
Mn(III)-PP disappears with time!

- Mn(III)-PP is <u>stable</u> over this time period
- Indicates an enzymatic pathway from Mn(III) → Mn(IV)



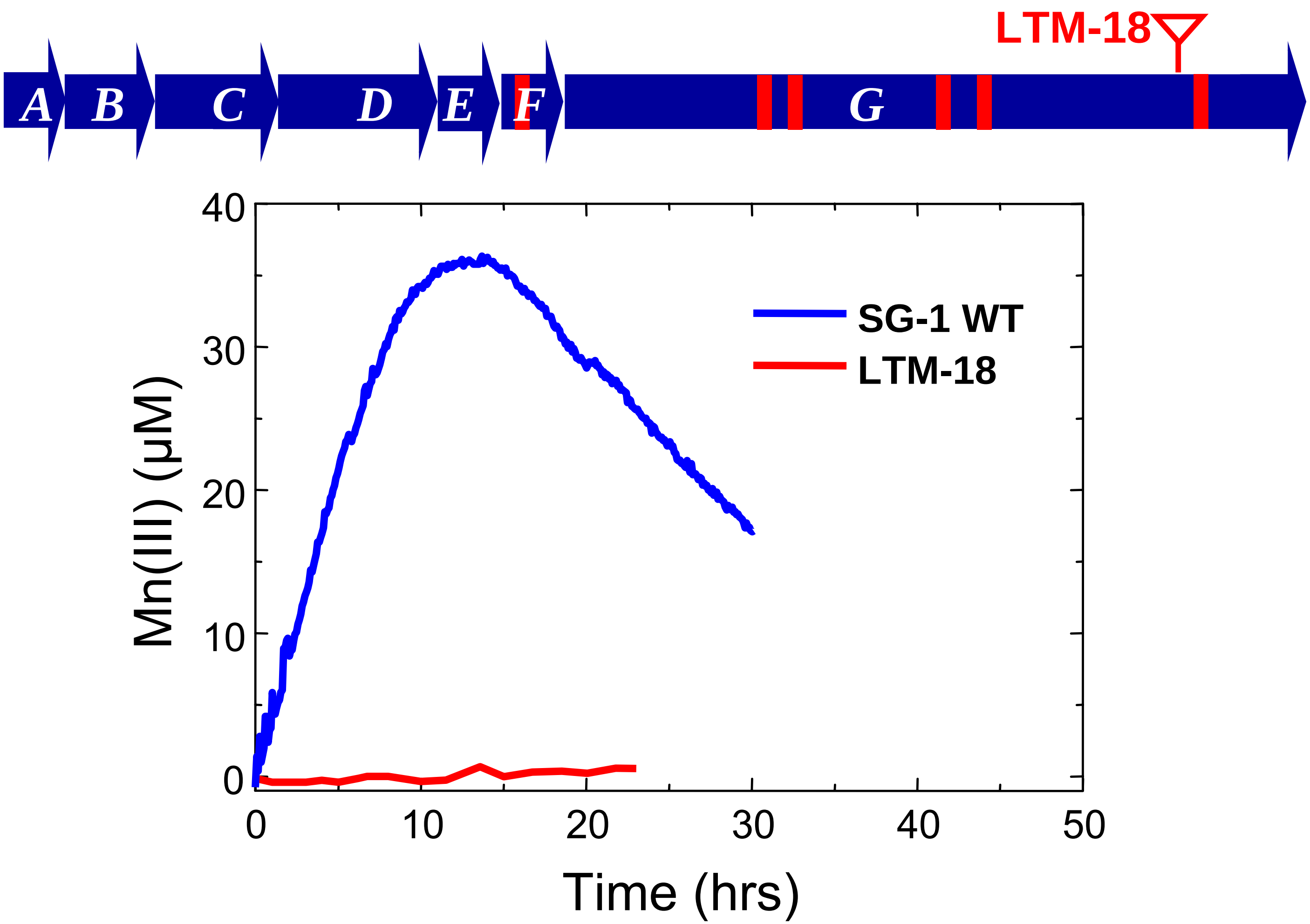


The exosporium oxidizes Mn(III)-PP

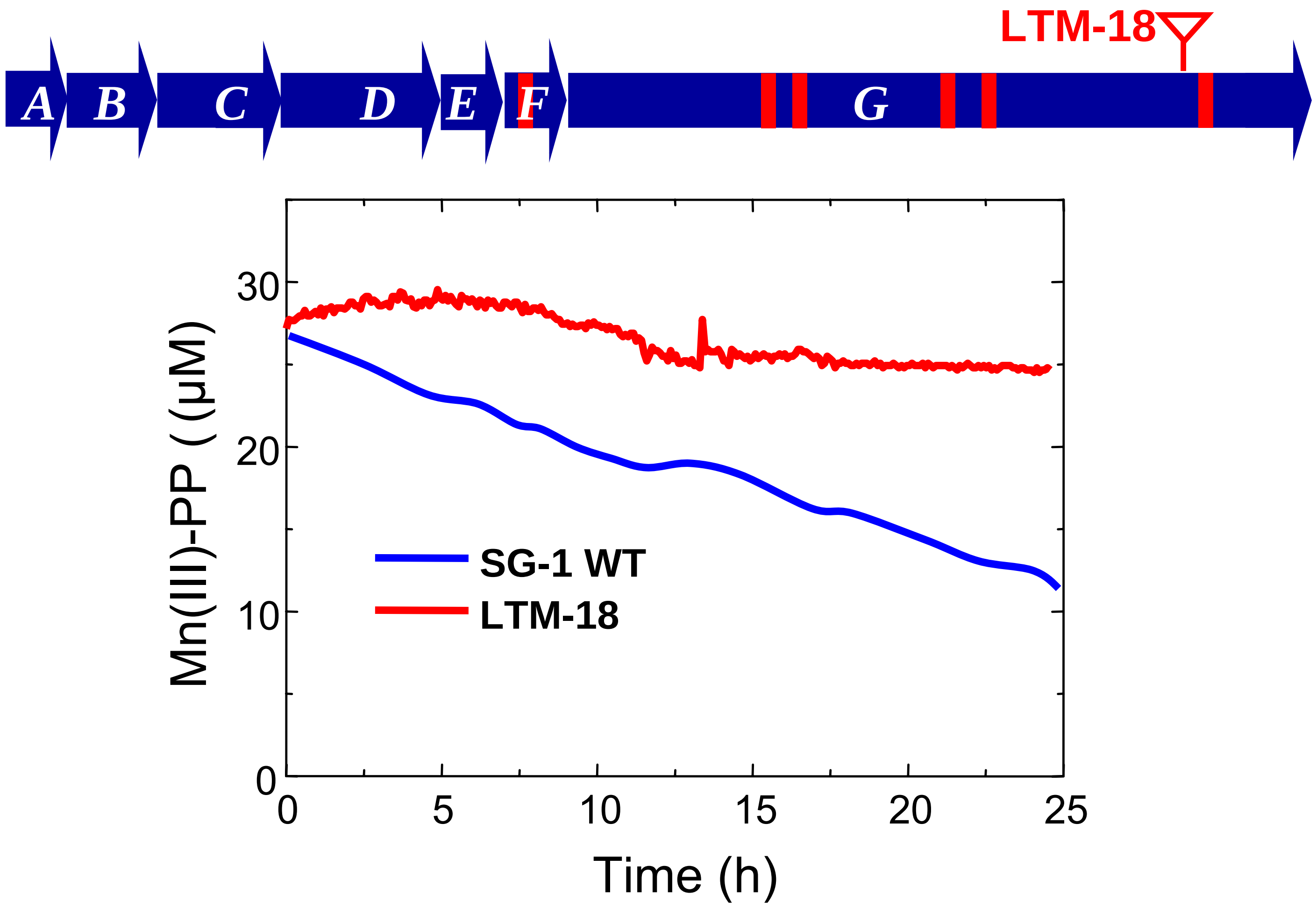


- Rate of Mn(III) decay in Mn(II) incubations is similar to that of Mn(III)+Exo

A *mnxG⁻* mutant is unable to oxidize Mn(II) → Mn(III)



A *mnxG*⁻ mutant is also unable to oxidize Mn(III) → Mn(IV)



Novel Aspects of the Mn(II)-oxidizing Multicopper Oxidase

- Overall: 2 e^{-} oxidation of the substrate: Mn(II) → Mn(III) → Mn(IV)
  - Other MCOs only oxidize their substrate by 1 e^{-}
  - Both steps require MnxG
- Molecular oxygen from O_2 is incorporated into the Mn oxide mineral (^{18}O-labelling studies with whole spores) *[Mandernack, Fogel & Tebo, 1995]*
  - Is the enzyme also an oxygenase?

$$\text{Mn(II)+Enz} \longrightarrow \text{Mn(II)=Enz} \underbrace{\longrightarrow \text{Mn(III)=Enz} \longrightarrow}_{+\text{O=O}} \text{Mn(IV)=Enz}$$

$$\text{Mn(IV)=Enz} \downarrow \text{MnOO}$$

Environmental implications

- Mn(III) is a strong oxidant
  - Mn(III) is involved in lignin (and xenobiotic) degradation by fungi
  - Generation of free radicals
- Cometabolic biotransformations
- Mn oxidation provides several pathways for transformation/sequestration of metal and organic contaminants

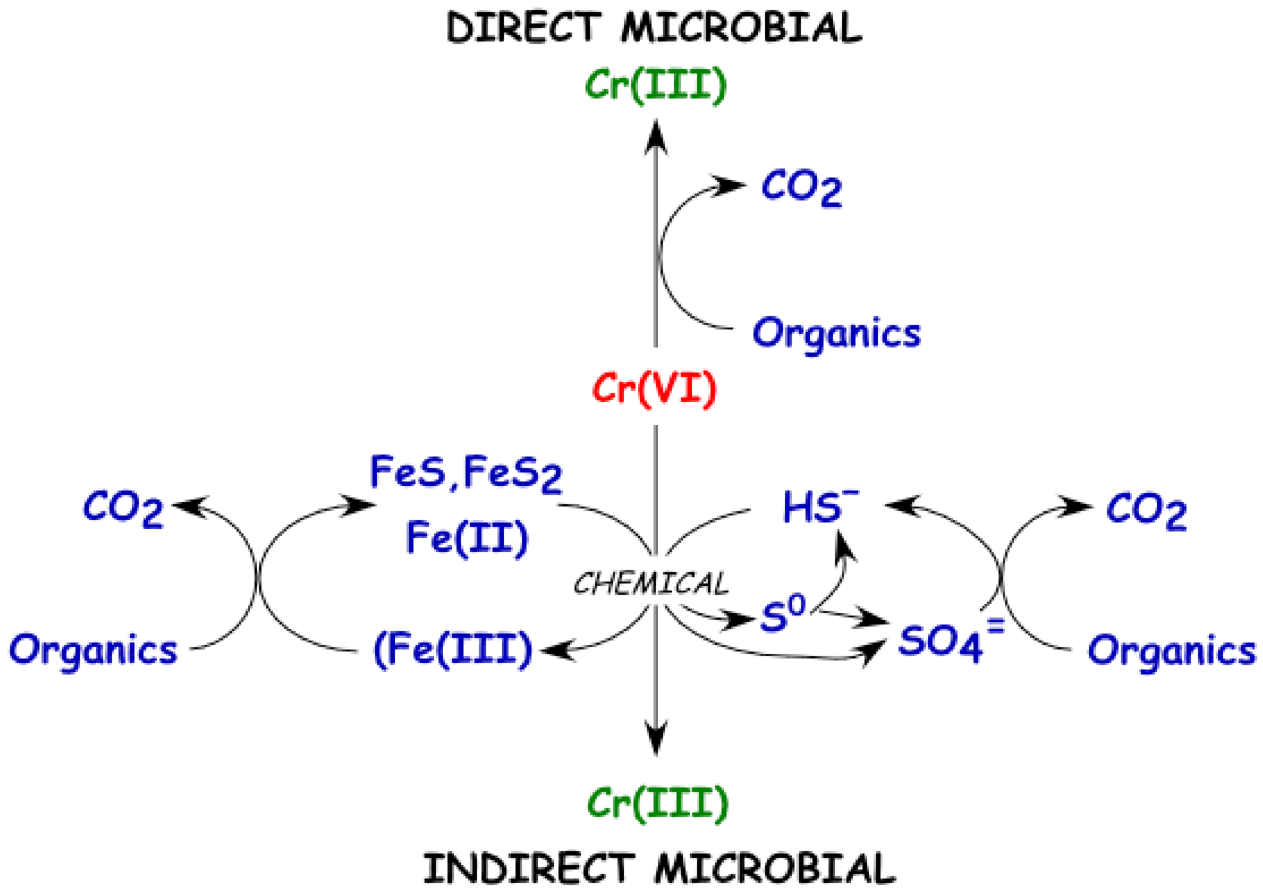
Chromium Chemistry

- Hexavalent Cr ↔ Trivalent Cr
- Cr(VI)
  - Oxyanion (CrO_4^{2-})
  - Soluble, conservative behavior
  - Highly toxic
    - Can be transported across the membrane
    - Cr(VI) is reduced to Cr(III) which binds to proteins and nucleic acids
- Cr(III)
  - $Cr(OH)_3$ or $Cr(OH)^{2+}$
  - Less soluble or particle reactive
  - Relatively nontoxic
    - Not transported across the membrane

Uses of Chromium

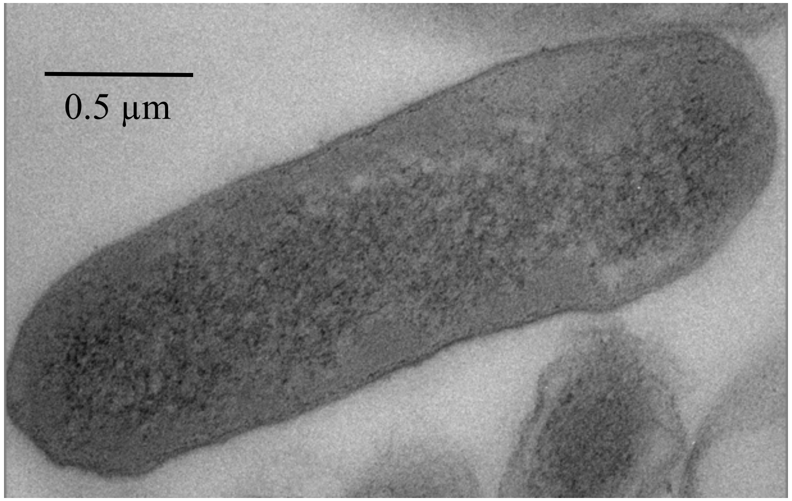
- Metal-finishing industry/alloy construction
- Leather tanning
- Ink, dye, and pigment manufacturing
- Boat paints
- Wood preservative

Mechanisms of Cr(VI) Reduction



Shewanella species

- *S. oneidensis* MR-1 and *Shewanella* sp. MR-4
- Great metabolic versatility- can use >12 electron acceptors: including O_2, NO_3^-, NO_2^-, fumarate, DMSO, TMAO, Fe(III), Mn(IV), U(VI), Cr(VI), Co(III)
- Genome sequence complete; microarrays available
- Important for immobilization of chromium as Cr(III) in contaminated aquifers



Shewanella sp. MR-4

Studies of the Mechanisms of Metal Reduction

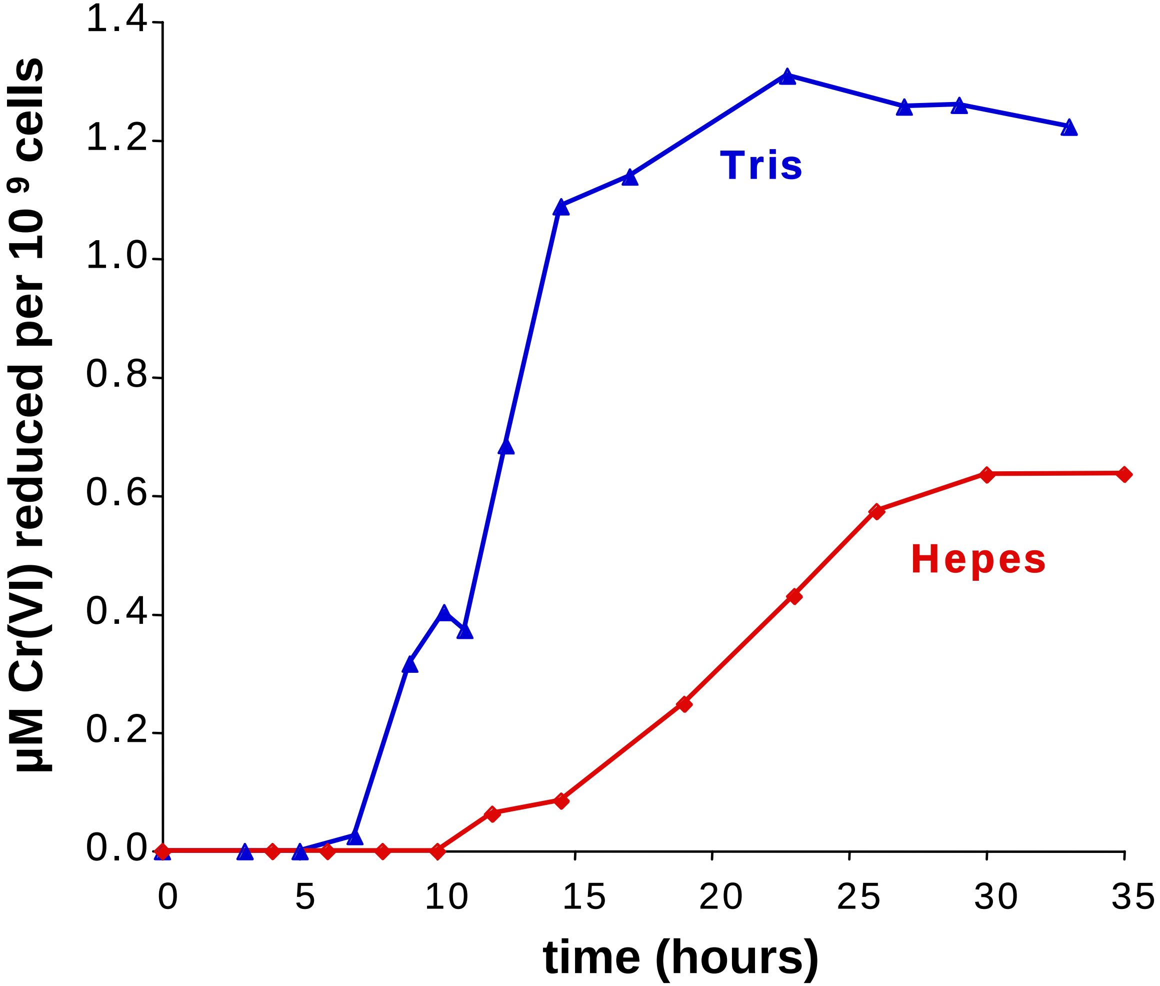
Cells grown on different electron acceptors ±Cr(VI)

↓

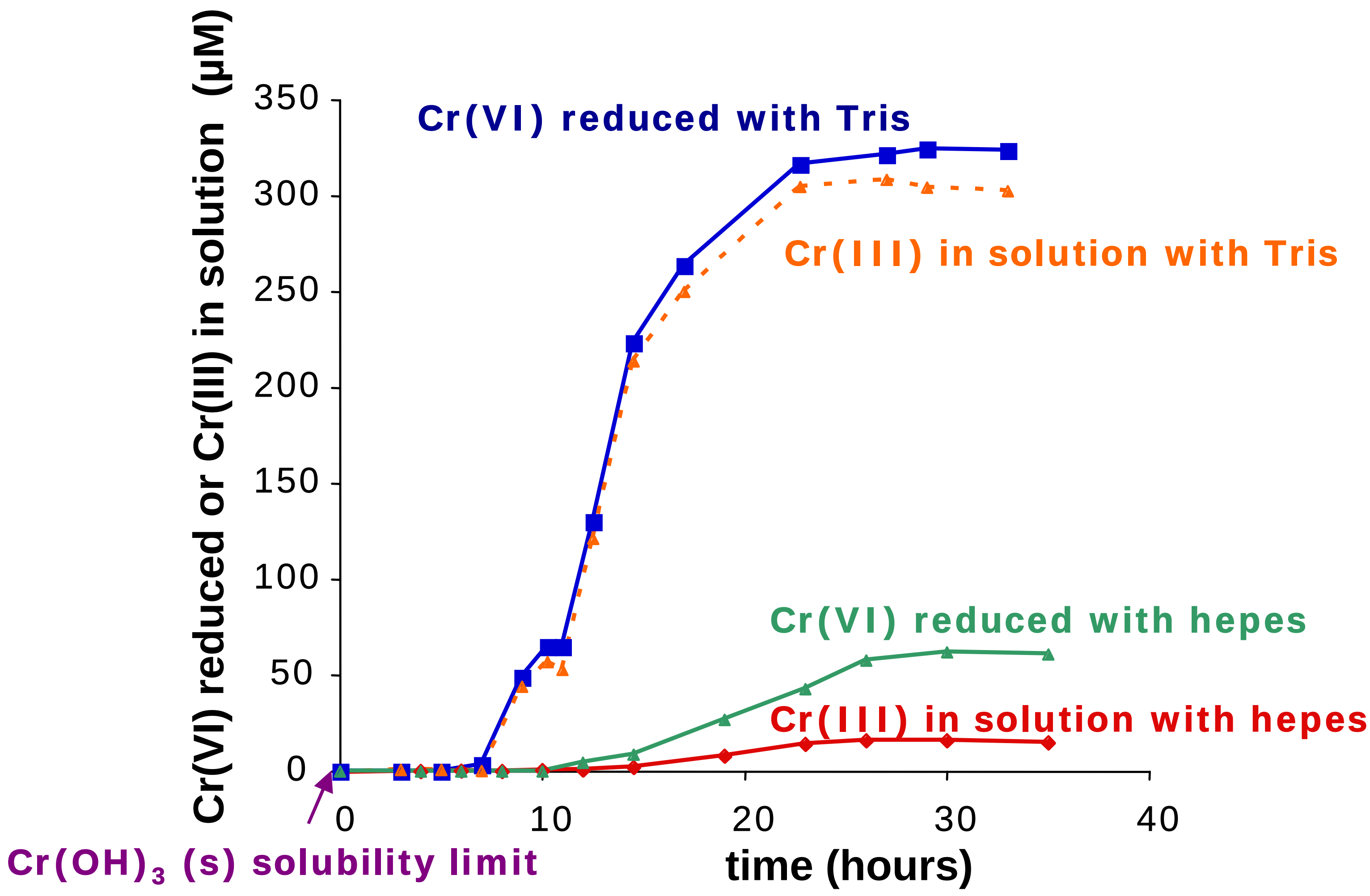
Harvest cells

- Fixation → Transmission Electron Microscopy (TEM) & Electron Energy Loss Spectroscopy (EELS) → Localization of metal reduction
- RNA → Microarrays → Identify genes turned on in the presence but not absence of Cr(VI)
- Protein → 1-D & 2-D Gel Electrophoresis FPLC Protein Purification → Identify proteins expressed (or repressed) when grown in the presence and characterization of Cr(VI) reductases

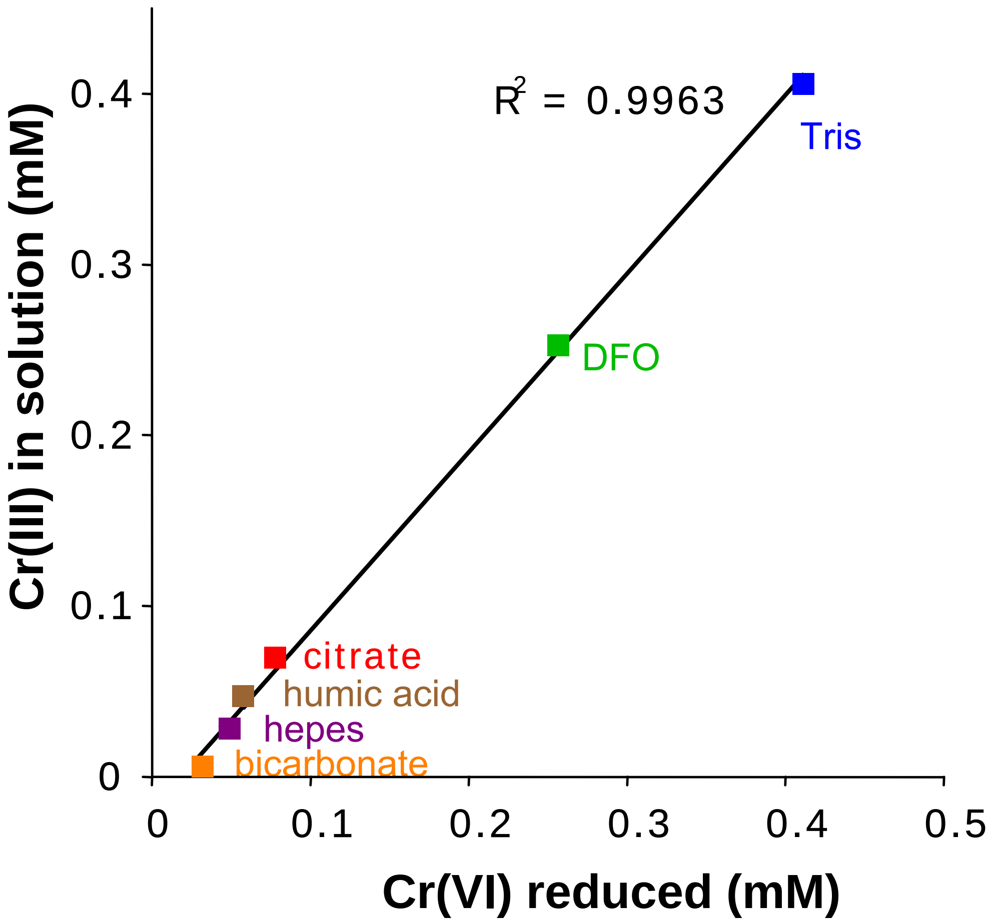
Aerobic Cr(VI) Reduction: Tris increases amount of Cr(VI) reduced



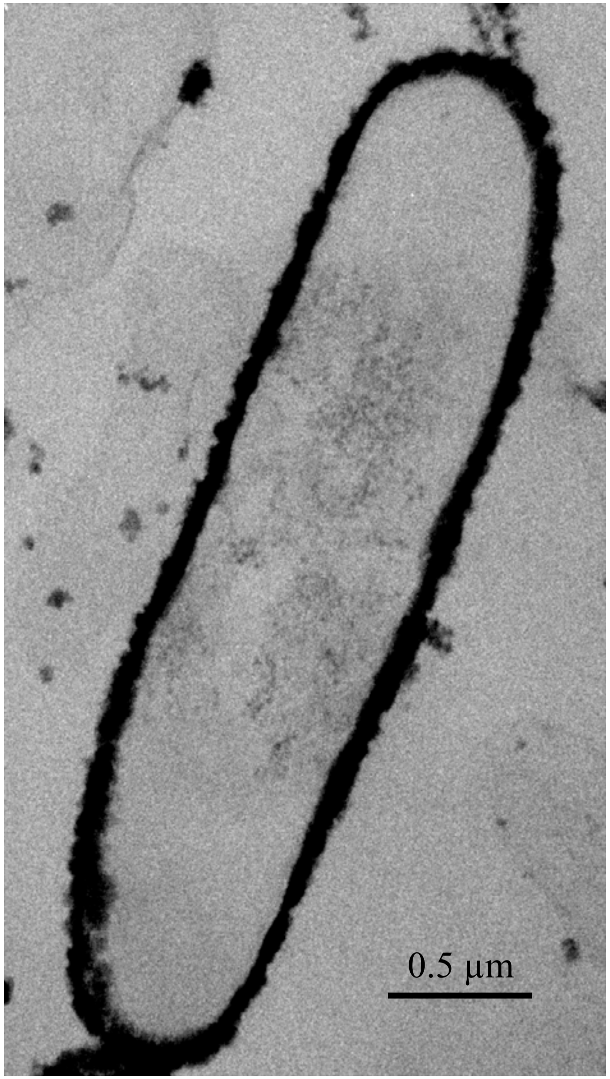
Reduced Cr(III) is soluble in Tris media

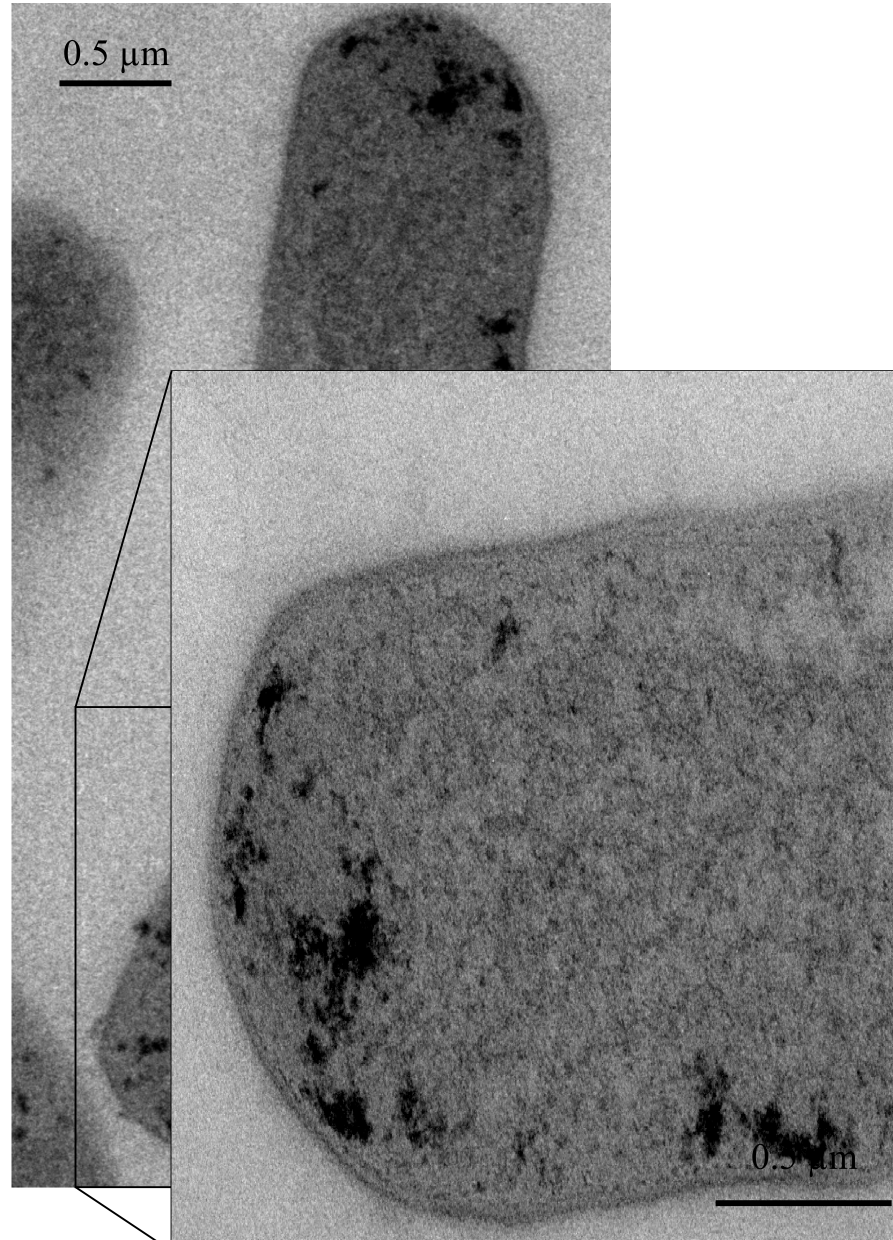


Complexing agents enhance Cr(VI) reduction by MR-4

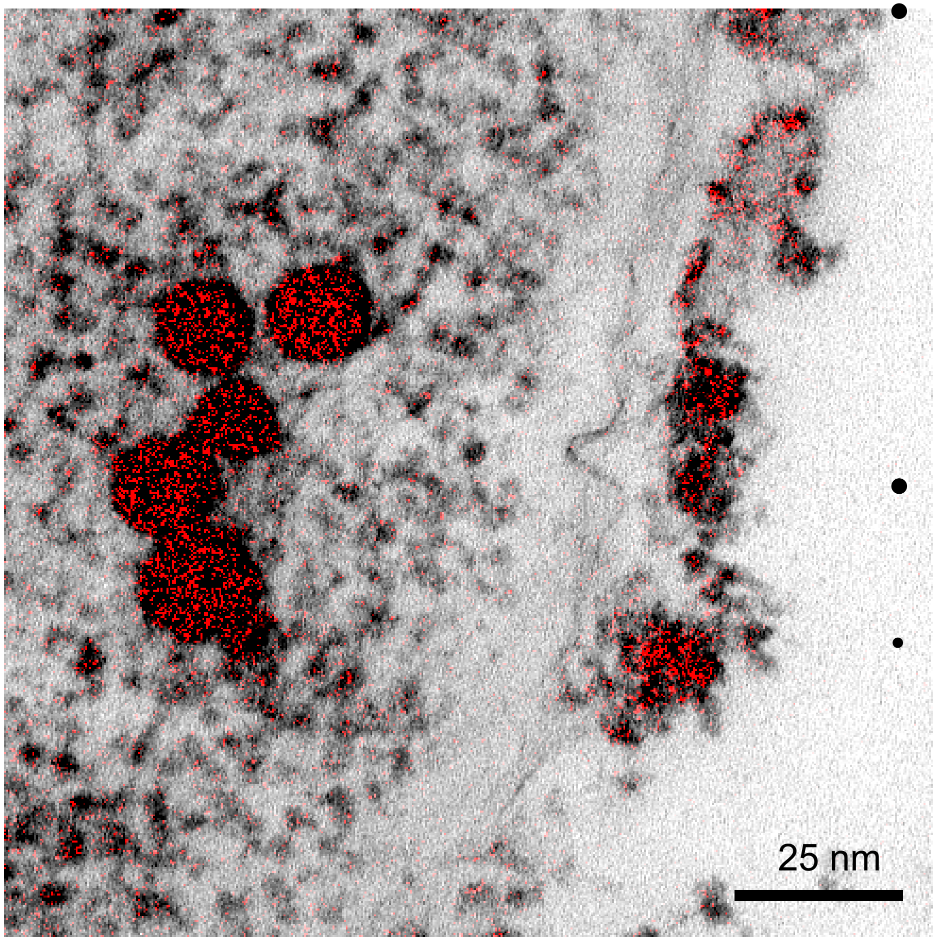


Precipitate formation in and around cells in Hepes



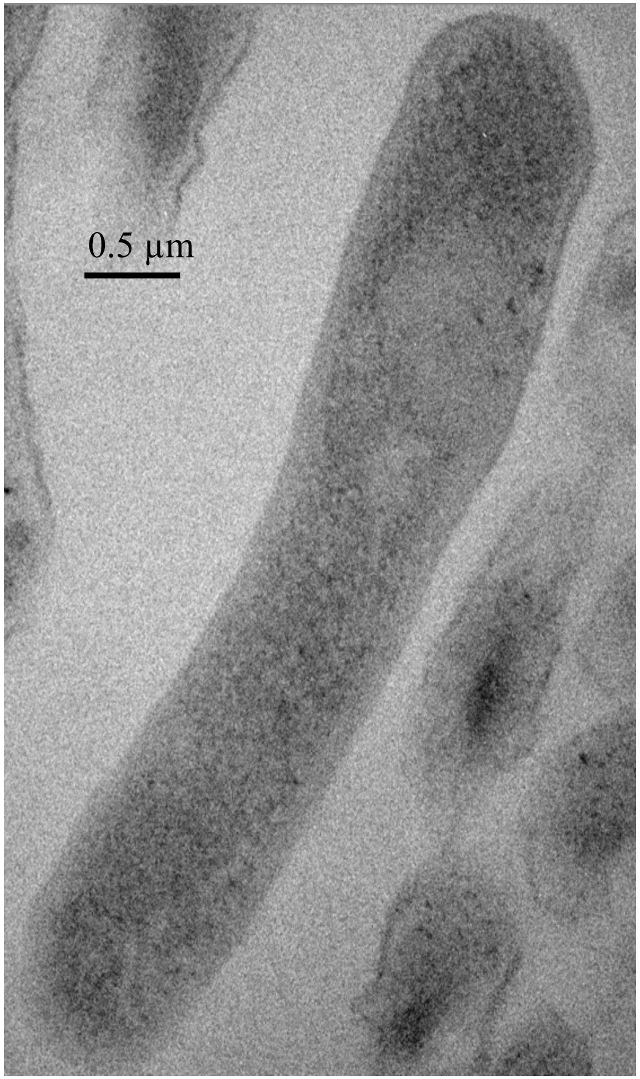


Reduced Cr accumulates intracellularly

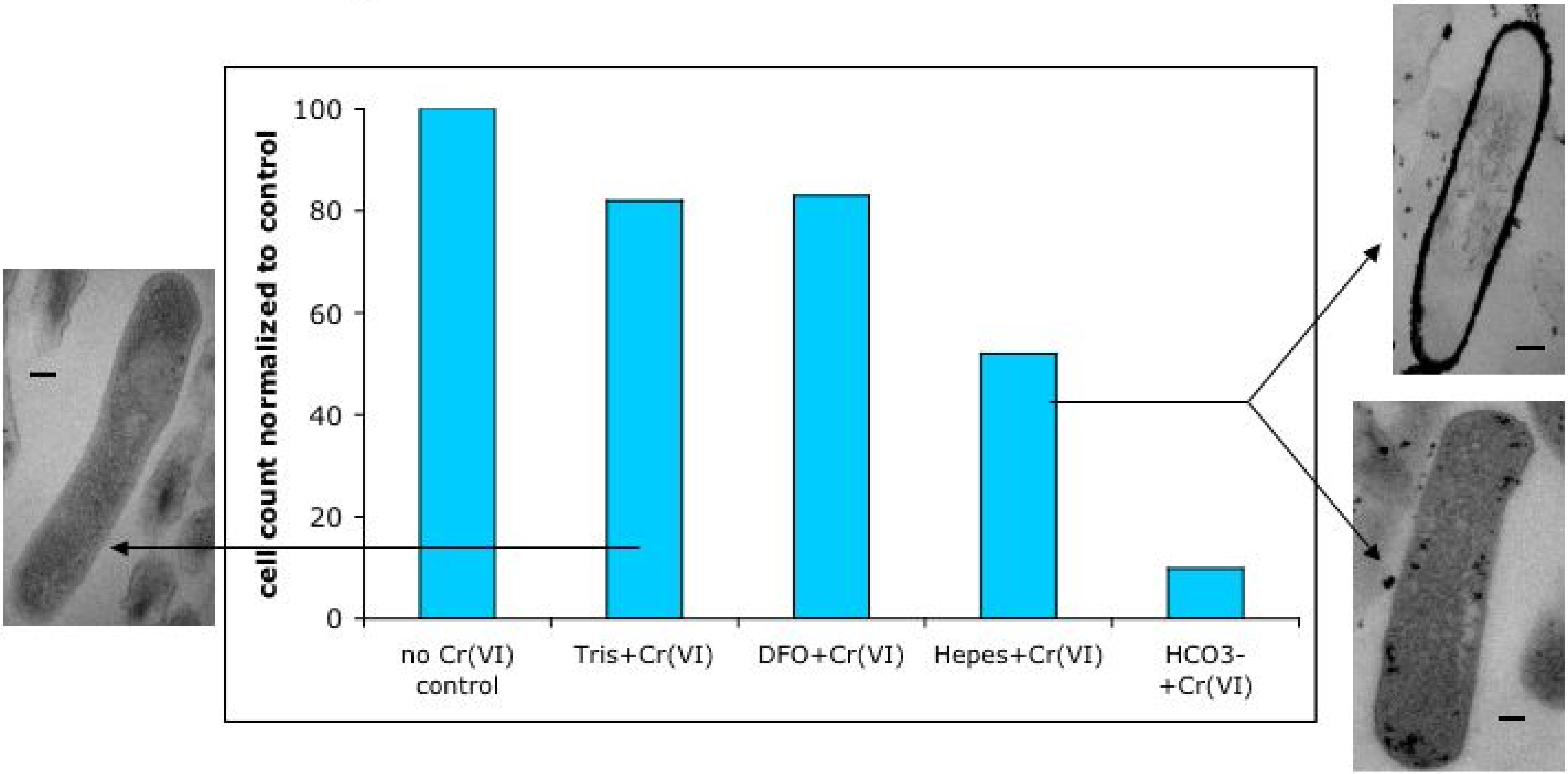


- Energy Electron Loss Spectroscopy (EELS) was used to create an elemental map of Cr (red) overlaid on a zero energy loss filtered image of an MR-1 cell stained with Pb.
- Cr precipitates both intracellular and extracellularly
- *Collaboration with Mark Ellisman and Mason Mackey*

No Cr precipitate in Tris



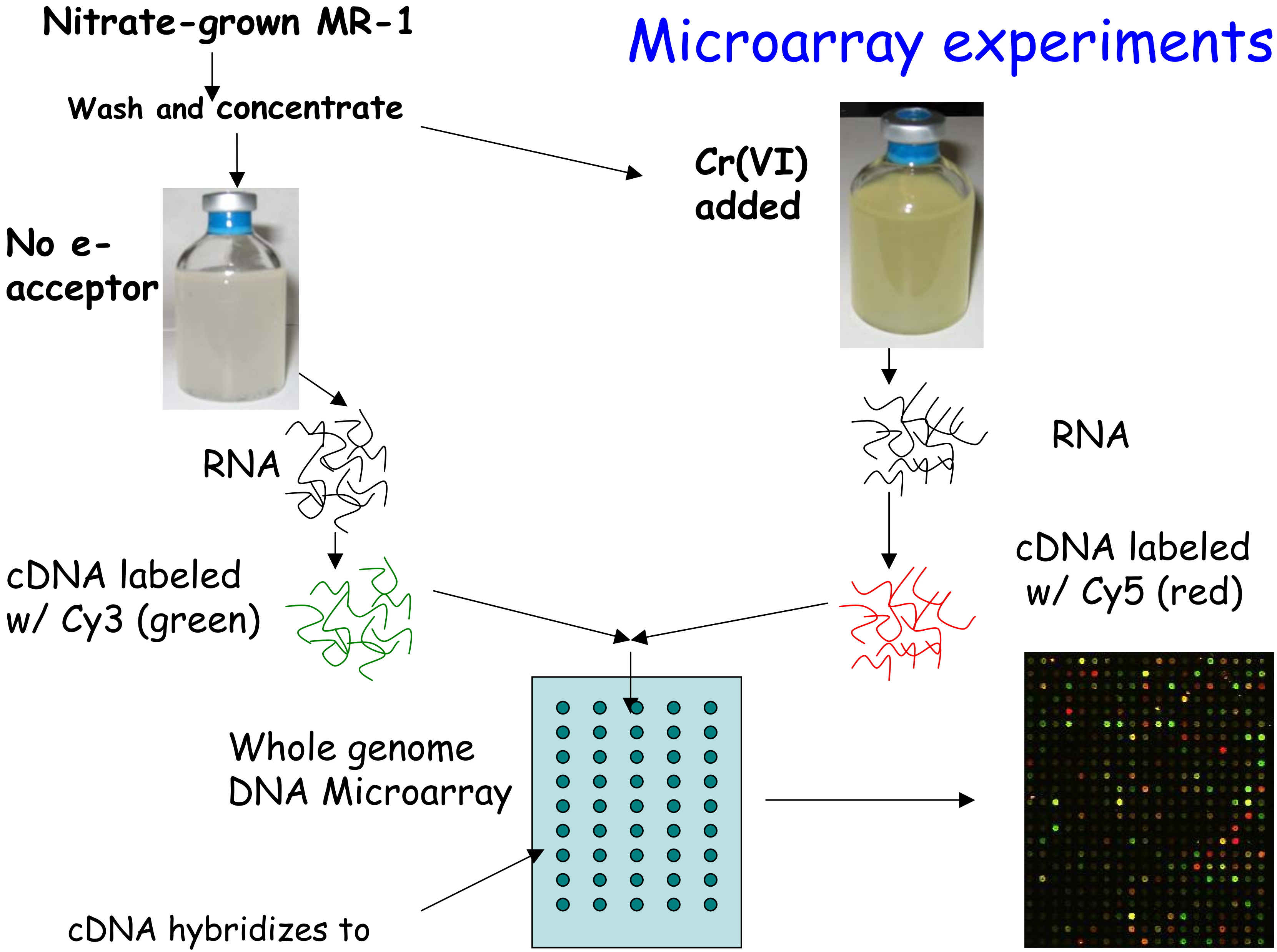
Precipitate formation/lack of Cr(III) complexation reduces cell viability



- Cr(VI) reduction can be enhanced by the addition of complexing agents

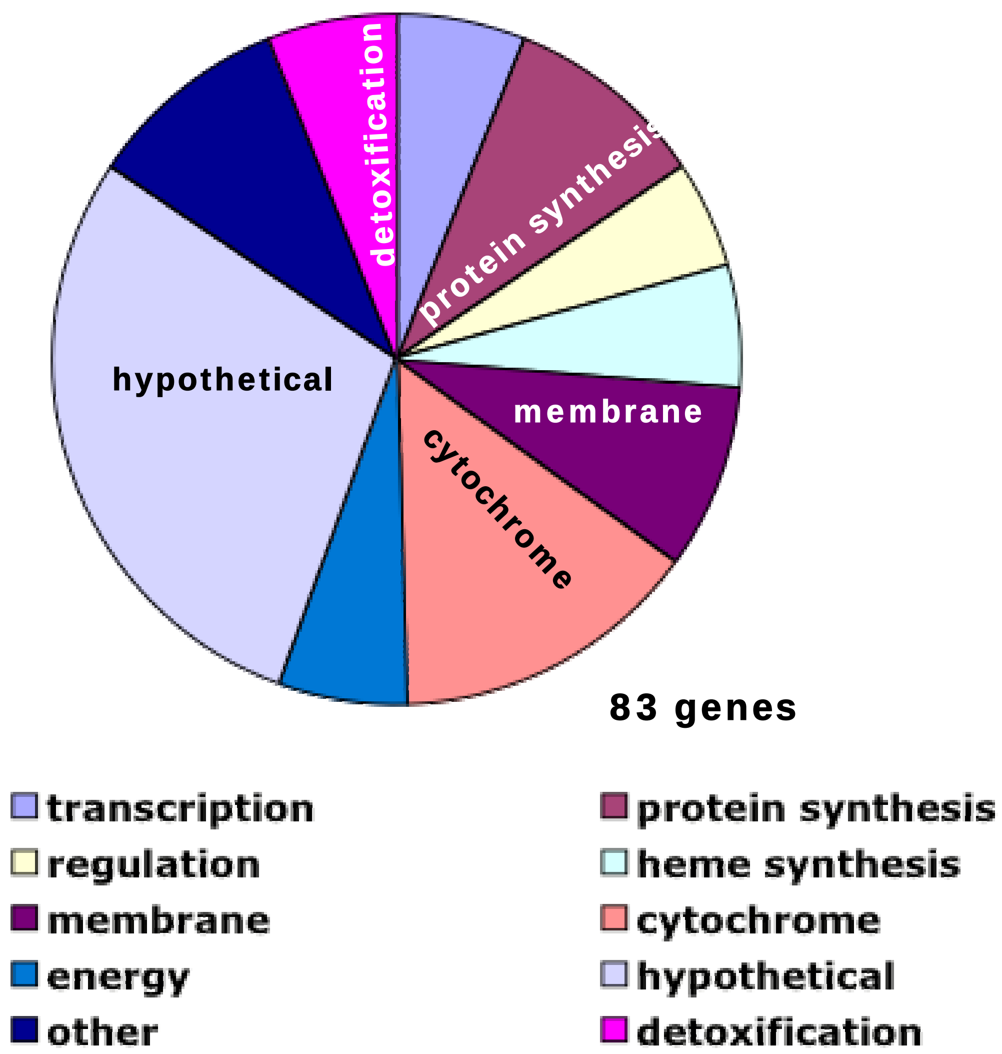
Scale bars = 0.5μM

Microarray experiments



- green, red, yellow spots
- normalization
- cutoff= 3-fold upregulation

Distribution of upregulated genes (≥ 3 fold)

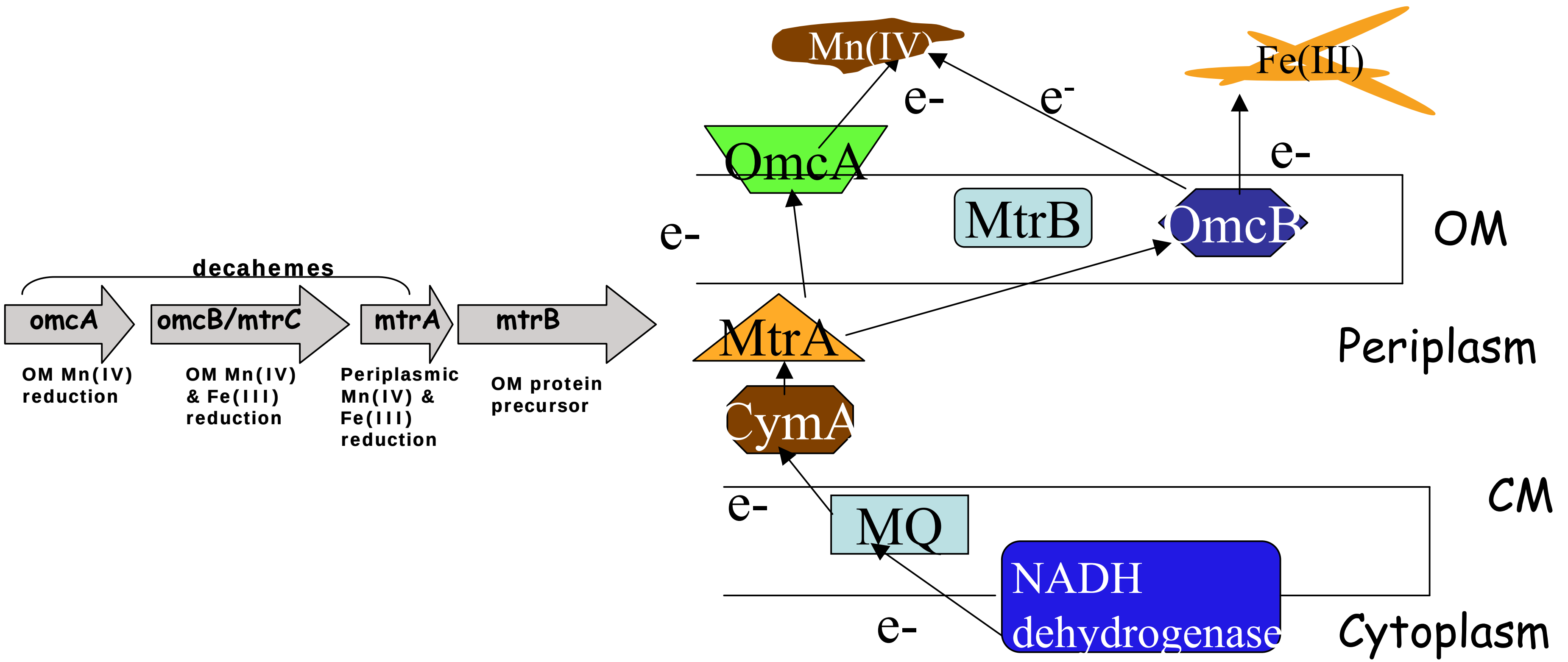


Genes upregulated in response to Cr(VI)

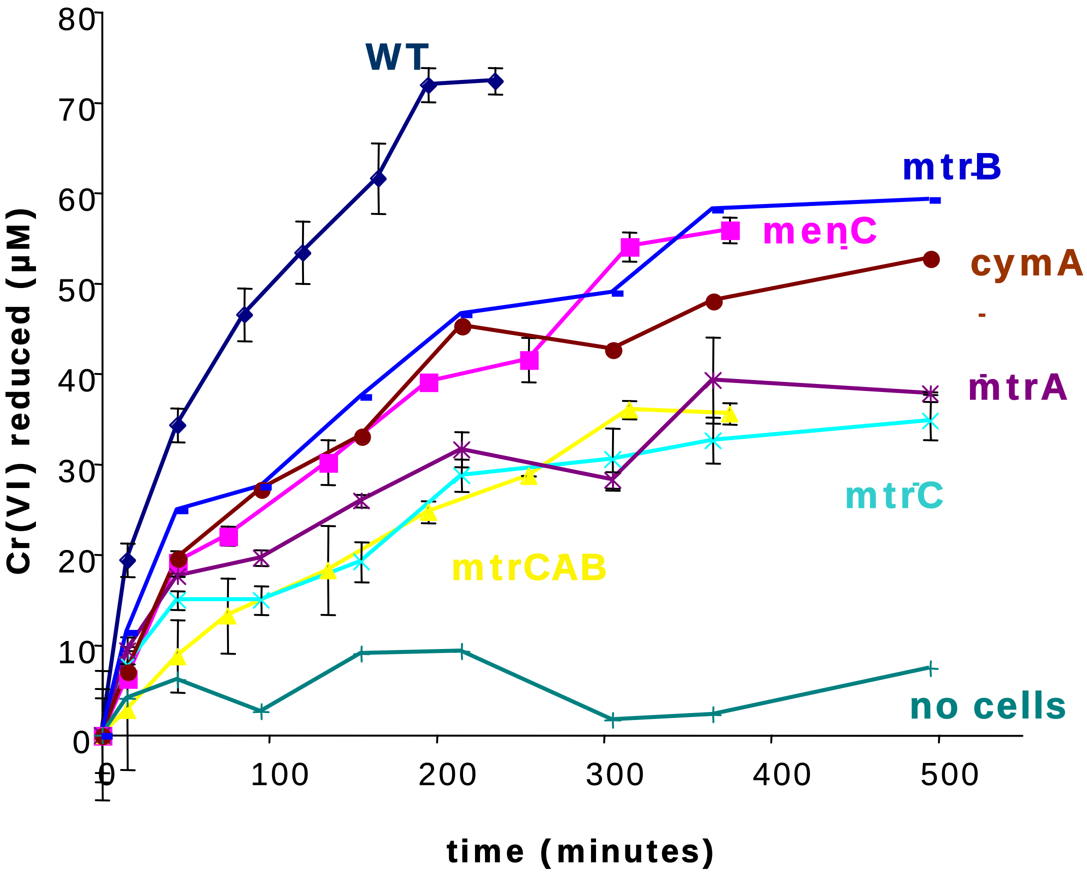
SO#	Annotation	Microarray results	Confirmation by RT RT-PCR
970	fumarate reductase flavoprotein subunit	4.0	√
1274	hypothetical protein	3.7	√
1427	decaheme cytochrome c		
1428	outer membrane protein	2.0	
1429	anaerobic dimethyl sulfoxide reductase (dmsA)	2.3	√
1430	anaerobic dimethyl sulfoxide reductase (dmsB)	4.1	
1431	hypothetical protein		
1776	outer membrane protein precursor MtrB (mtrB)	5.5	√
1777	decaheme cytochrome c MtrA (mtrA)	4.5	√
1778	decaheme cytochrome c (omcB) or (mtrC)	2.6	x
1779	decaheme cytochrome c (omcA)	2.7	x
4483	cytochrome b, putative	8.8	√
4484	cytochrome c-type protein Shp	16.0	√
4485	diheme cytochrome c	11.4	√

Genes known to be involved in Fe(III) and Mn(IV) reduction

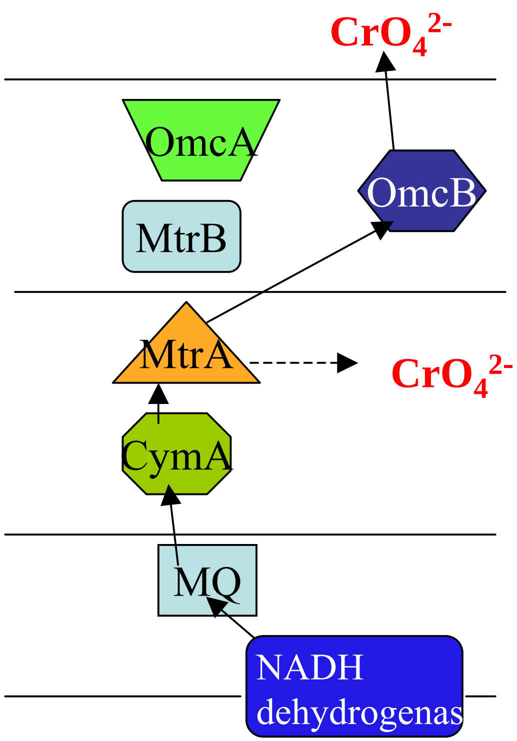
SO#	Annotation	Microarray results	Confirmation by RT RT-PCR
1776	outer membrane protein precursor MtrB (mtrB)	5.5	√
1777	decaheme cytochrome c MtrA (mtrA)	4.5	√
1778	decaheme cytochrome c (omcB) or (mtrC)	2.6	x
1779	decaheme cytochrome c (omcA)	2.7	x



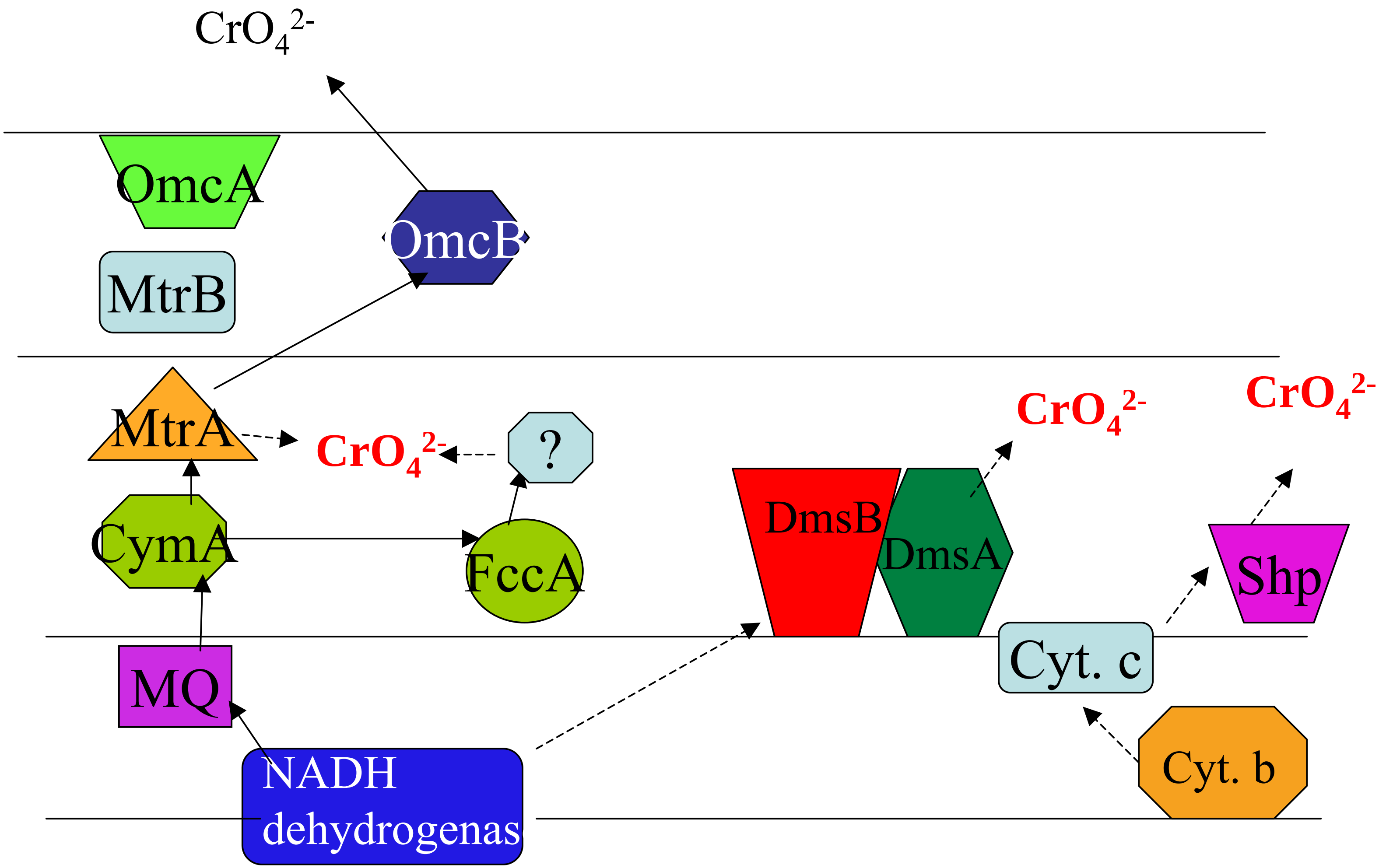
Cr(VI) reduction by MR-1 mutants



gene	array	RT RT-PCR
	Cr	Cr
mtrB	5.5	¦
mtrA	4.5	¦
mtrC	2.6	x



Several potential pathways for Cr(VI) reduction



Implications

- If the genes up-regulated during Cr(VI) reduction are specific to Cr(VI), we should be able to use these genes as biomarkers for Cr(VI) bioavailability.
- Genes and proteins responsible for Cr(VI) reduction can have applications for bioremediation.

Acknowledgments

- Lab Group
  - Greg Dick (graduate student)
  - Rizlan Bencheikh-Latmani (postdoc)
  - Anna Obraztsova (Research Specialist)
- Collaborators
  - Craig Criddle (Stanford; Michigan SBRP)
  - Jizhong Zhou (Oak Ridge Nat'l Lab)
  - Mark Ellisman & Mason Mackey (UCSD SBRP- NCMIR)
  - Betsy Komives & Ross Hoffman (UCSD SBRP-Mass Spec)
  - John Bargar & Sam Webb (Stanford Synchrotron Radiation Laboratory)
- Funding – NIEHS Superfund Basic Research Program