Engineering combinatorial bacteria for superior metal and radionuclide bioremediation

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Sources of Chromate [Cr(VI)] and U(VI) contamination

Contaminating Industries

- Metal plating
- Steel making
- Bricks in furnaces
- Dyes and pigments
- Chrome plating
- Leather tanning
- Wood preservation

Nuclear weapons production

Cr(VI) vs. Cr(III)

Cr (VI) (chromate)	Cr(III)
U(VI)	**U(IV)**
Rapid cellular uptake	Not taken up
Carcinogenic	Non-carcinogenic
Soluble (can spread)	Insoluble

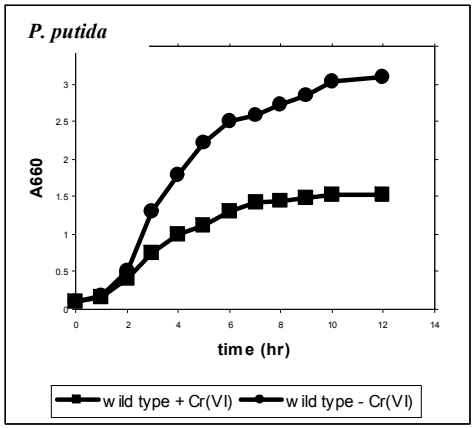
Several bacteria can reduce chromate using soluble enzymes

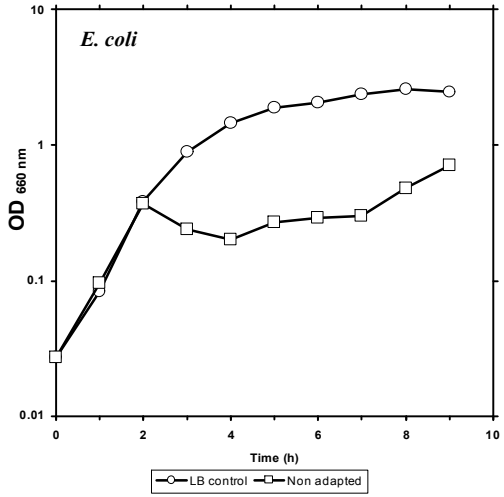
Enterobacter cloacae: anaerobic chromate respirer?

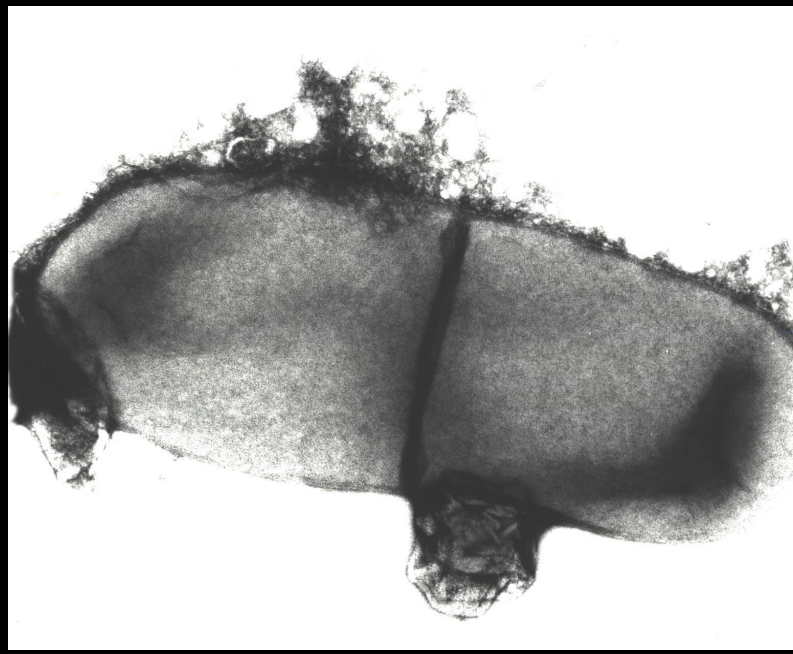
	Activity (nmol/mg protein/h)		
	Grown without chromate	Grown with 0.4 mM chromate	Assayed in presence of 10 mM $CuSO_4$[b.]
P. putida KT2440	8.4	27.6	52.8
P. putida MKI	13.2	42.6	72.0
P. syringae	151.8	N.D.[c.]	N.D.
P. fluorescens	114.6	N.D.	N.D.
B. subtilis	15.6	33.0	40.8
V. harveyi	132.0	264.0	1440.0
E. coli AB117	49.8	32.4	156.0
S. putrefaciens	193.2	N.D.	N.D.
D. radiodurans	186.6	N.D.	N.D.
S. aureus	139.2	N.D.	N.D.
Acinetobacter	133.8	N.D.	N.D.

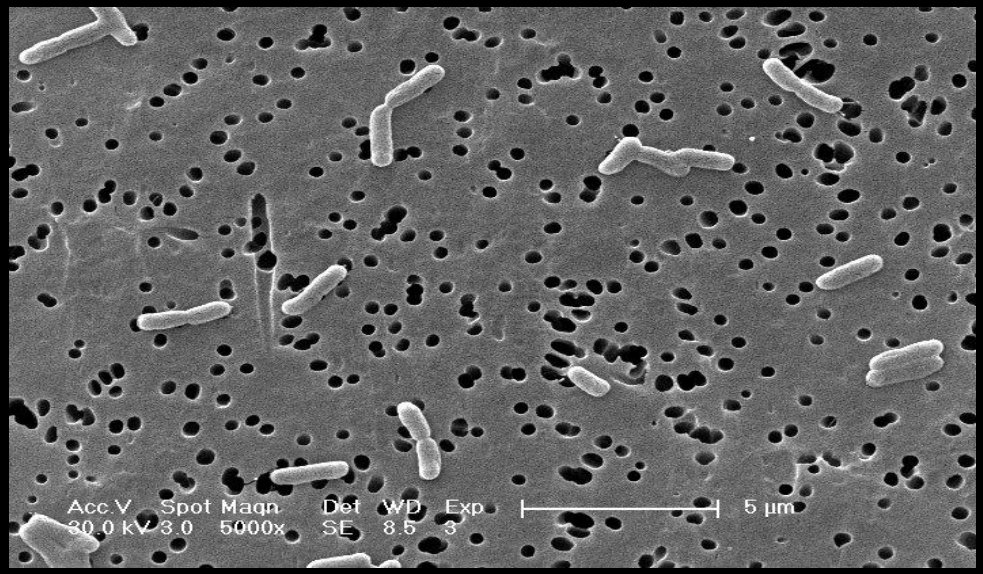
Keyhan, Ackerley, & Matin. 2003.
Batelle Symposium Proc. Paper E06

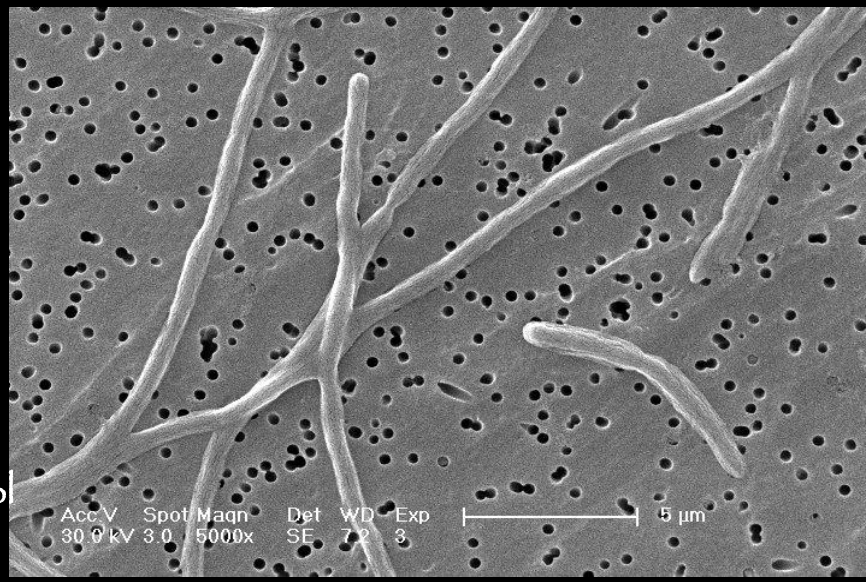
Effect of chromate toxicity







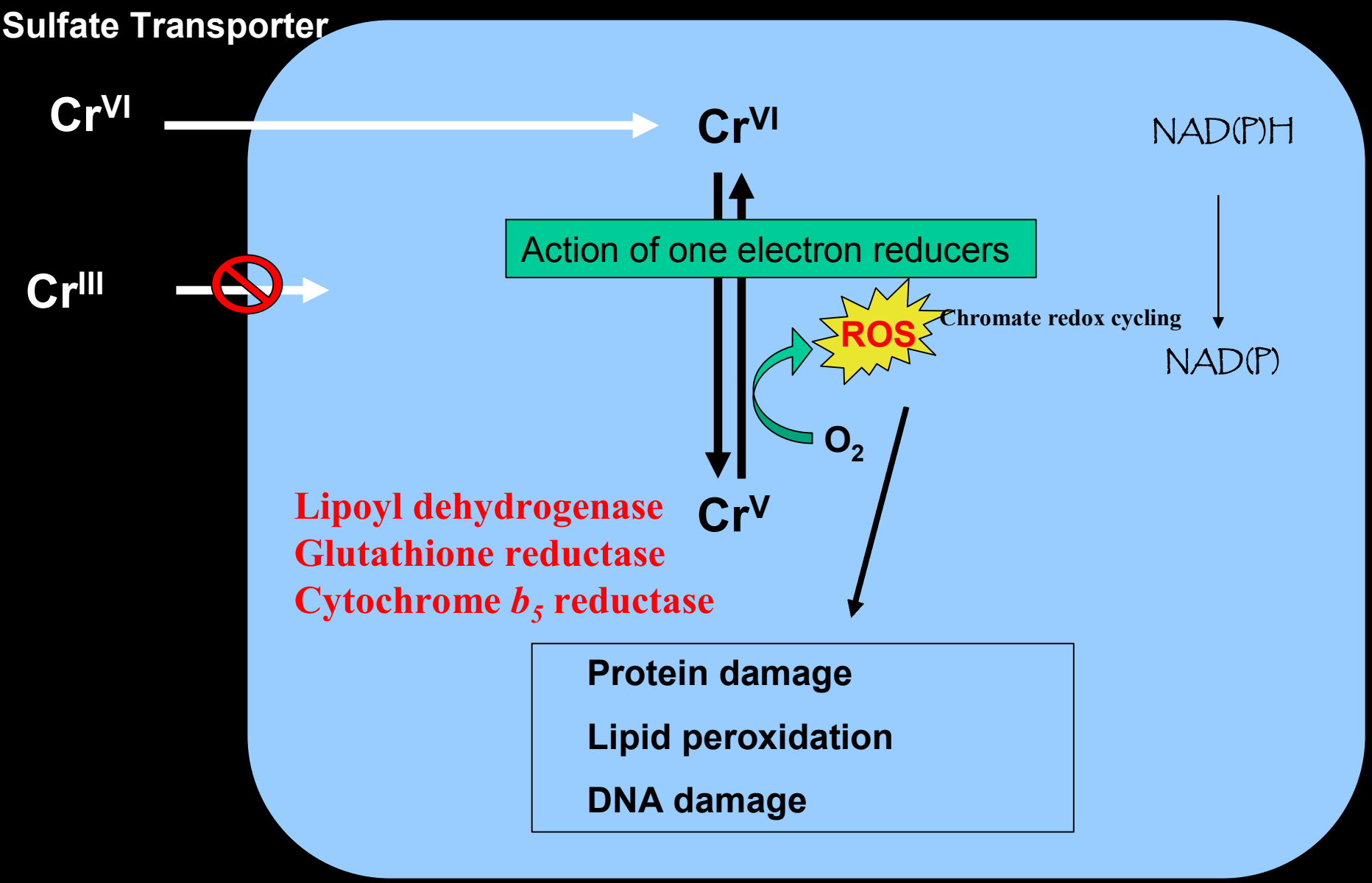




Ackerley, Barak, Lynch, Curtin, and Matin. 2006. J. Bacteriol 188 *In press*

Shi & Dalal, using ESR, showed that many enzymes (e.g., lipoyl dehydrogenase, glutathione reductase, and cytochrome b_5 reductase generate Cr(V) from Cr(VI), indicating one-electron reduction

Cr(V) redox cycling



NAD(P)H ELECTRONS TRANSFERRED TO CHROMATE & H_2O_2 (μM; 1 min)

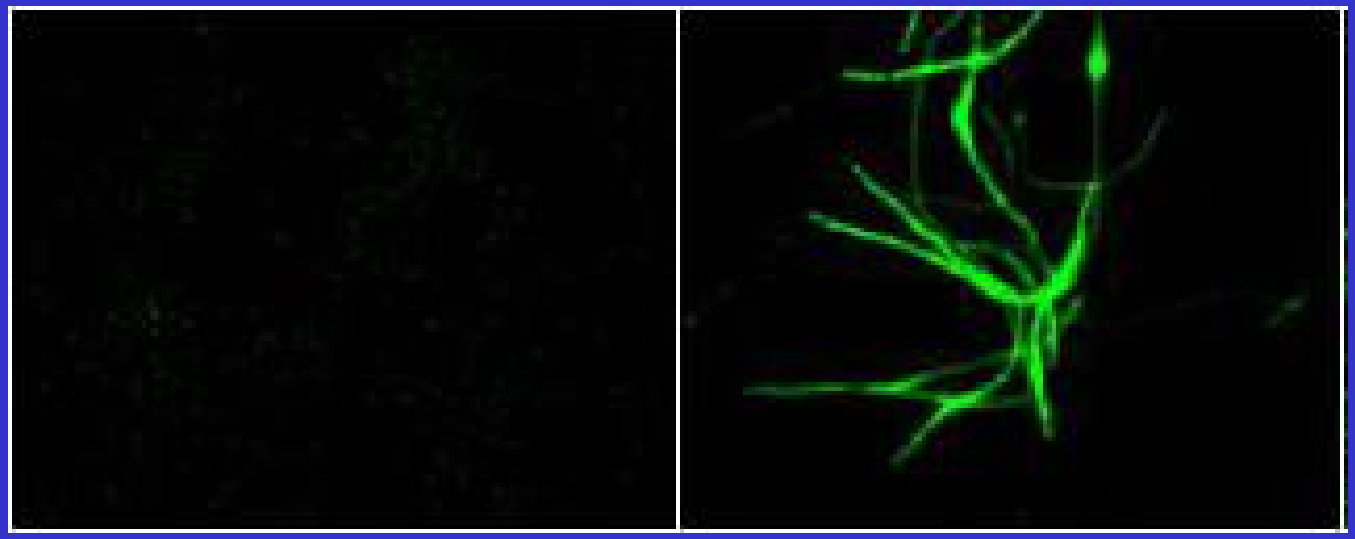
Lipoyl dehydrogenase

Enzyme	NAD(P)H e^- consumed	e^- used in chromate reduction	e^- used in H_2O_2 formation
LpDH	**80**	**21 (26%)**	**59 (74%)**

Single electron reducer

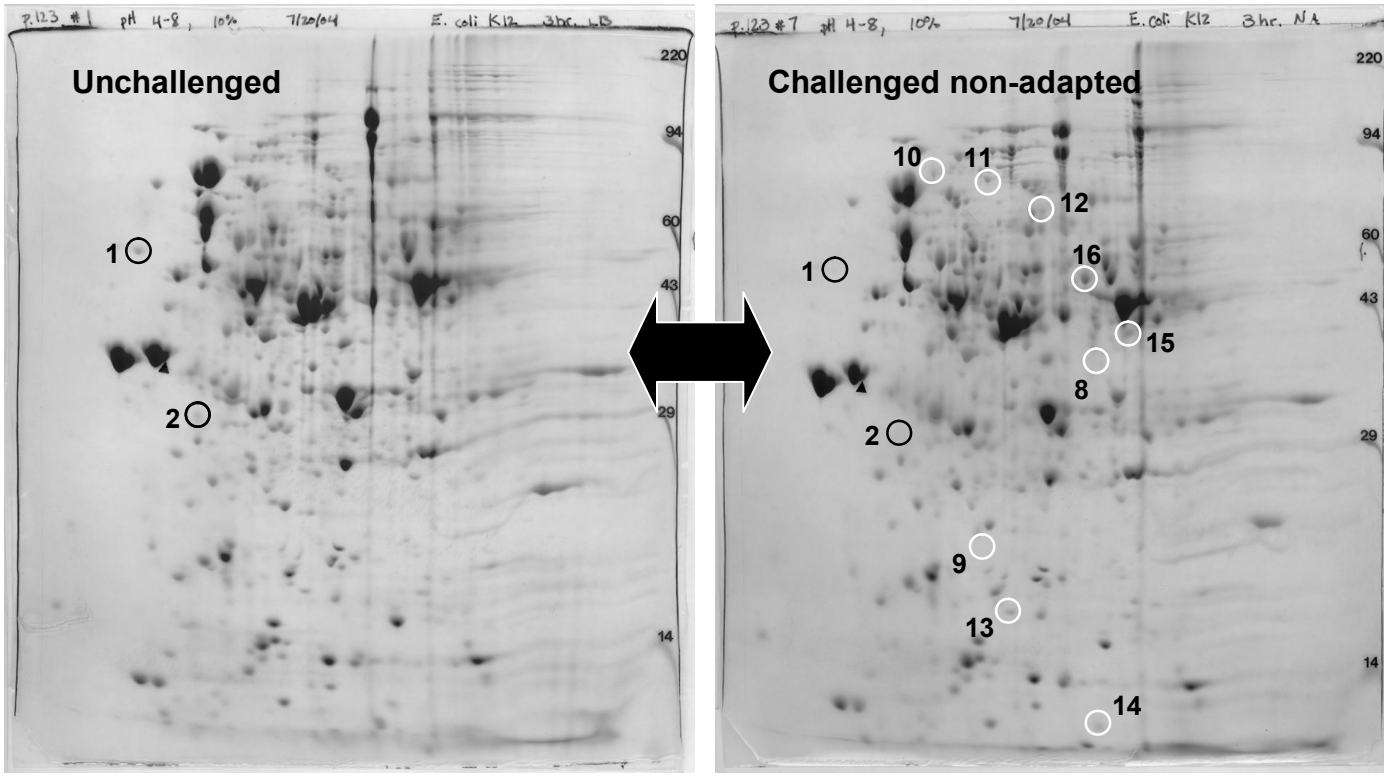
Ackerley, Gonzalez, Park, Blake II, Keyhan, & Matin. 2004. Environmenal Microbiology 6: 851-860

In vivo oxidative stress detected using the ROS-responsive dye H_2DCFDA



Ackerley, Barak, Lynch, Curtin, and Matin. 2006. J. Bacteriol 188 *In press*

Partial snap-shot of proteomics of chromate challenge



Ackerley, Barak, Lynch, Curtin, and Matin. 2006. J. Bacteriol <u>188</u> *In press*

Partial snap-shot of proteins altered upon chromate challenge

Ackerley, Barak, Lynch, Curtin, and Matin. 2006. J. Bacteriol 188 *In press*

spot #	protein	gene	fold-change	known or suspected function
3 h samples				
1	Flagellin	*fliC*	11-fold down	Flagellar subunit
2	ADP-L-glycero-D-manno-heptose-6-epimerase	*hldD*	4.1-fold down	Lipopolysaccharide biosynthesis
5 h samples				
3	Superoxide dismutase (ferric; chain B)	***sodB***	+++	**Decomposition of $O_2^{\cdot -}$**
4	Sulfate adenylyl transferase	***cysN***	6.3-fold up	**Cysteine biosynthesis**
5	Cysteine synthase A	***cysK***	4.1-fold up	Cysteine biosynthesis
1	Flagellin	*fliC*	---	Flagellar subunit
2	ADP-L-glycero-D-manno-heptose-6-epimerase	*hldD*	3.8-fold down	Lipopolysaccharide biosynthesis
6	Cytoplasmic ferritin	***ftnA***	3.8-fold down	**Iron storage**
7	Putative formate acetyl transferase	*yfiD*	3.3-fold down	unknown

Induction also of *sfiA* gene, indicative of activation of the SOS response

Depletion of cellular glutathione and Other free thiols

Ackerley, Barak, Lynch, Curtin, and Matin. 2006. J. Bacteriol 188 *In press*

Chromate toxicity increases in mutants missing anti-oxidant defense genes

	Chromate MIC_{50} (μM)
Wild type	**400**
cysK*	**300**
cysN*	**400**
sodA	450
sodB*	350
katE	**300**
katG	400
gshA	400
gshB	400
yieF	**300**

Ackerley, Barak, Lynch, Curtin, and Matin. 2006. J. Bacteriol 188 *In press*

HYPOTHESIS

Bacteria possess functional homologues of mammalian NQO1 enzyme (DT diaphorase)

NQO1 is an obligatory two-electron reducer of its substrates

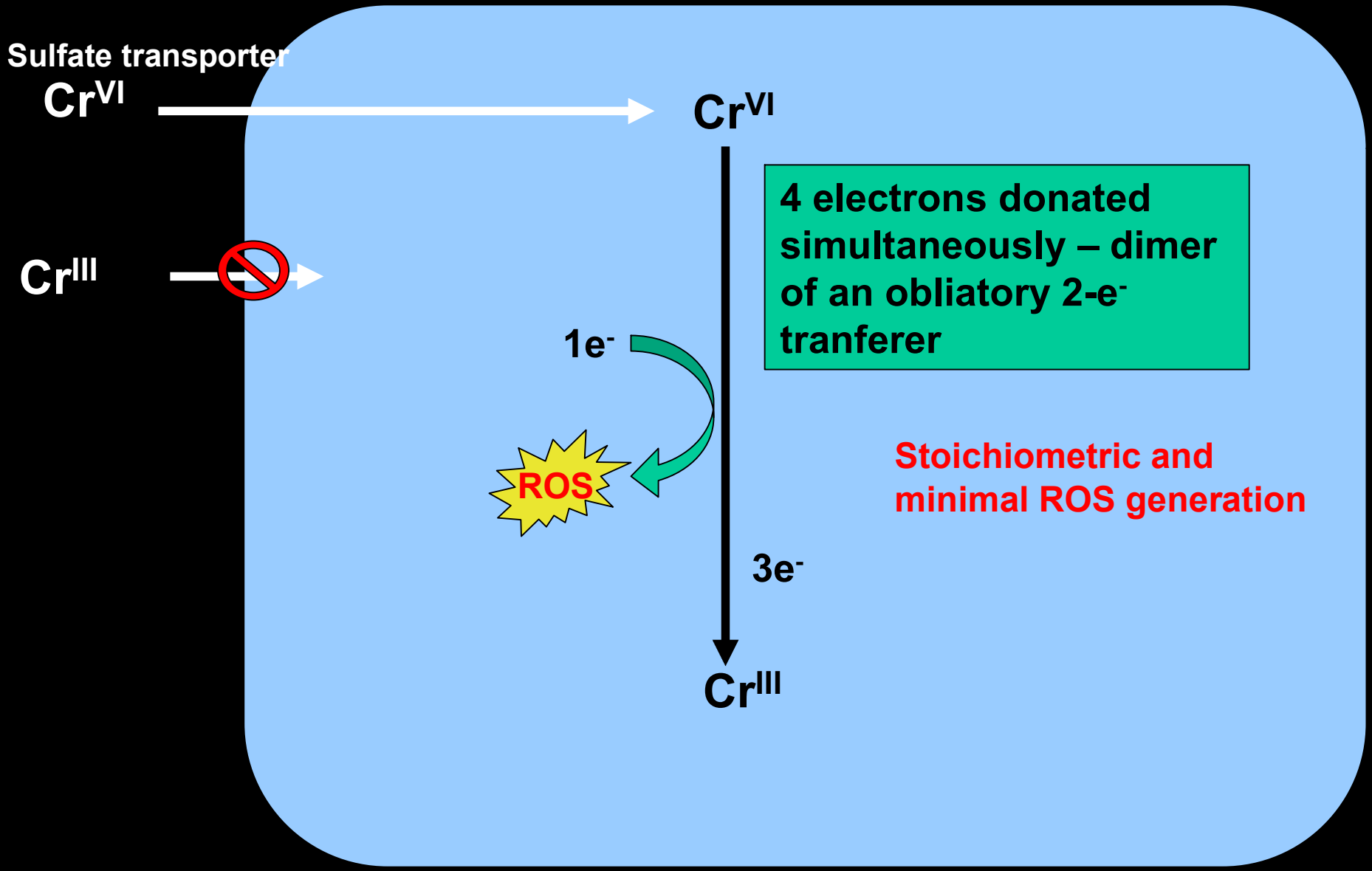
A dimer of a bacterial homologue should be capable of one-step reduction of Cr(VI) to Cr(III), bypassing:

Cr(V) generation

Redox cycling

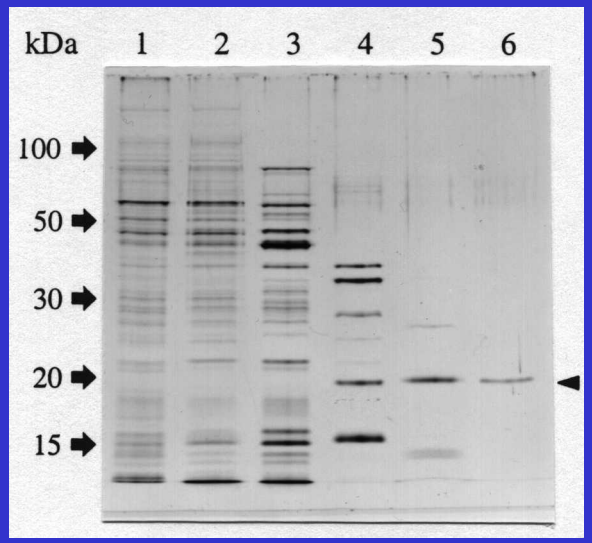
Will generate minimal possible ROS during Cr(VI) reduction

Chromate reduction involving one-step conversion to Cr(III)



1. Classical biochemical methods: *P. putida* chromate reductase purification

(Park, Keyhan, Wielinga, Fendorf, & Matin 2000, AEM 66: 1788-1795)



Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (Unit/mg)	Purification factor (fold)
1. Crude extract	302	8760	7446	0.85	-
2. Ultracentrifugation	407	6198	13016	2.1	2.5
3.Ammonium sulfate (55-70%)	40	880	3168	3.6	4.2
4. DEAE Sepharose CL-6B	3	6	948	158	186
5. Polyphosphate buffer 94	1	0.2	65	325	382
6. Superose 12 HR 10/30	0.75	0.0075	4	533	627

2. Serendipity

3. In-silico approaches

(Park et al., 2002, Remediation and beneficial use of Contaminated sediments, p, 103-111)

4. Gene cloning; homo-logues

5. Pure proteins using molecular techniques

NO ASSIGNED FUNCTION

NAD(P)H electrons transferred to Chromate & H_2O_2 by simultaneous e^- transfer

Enzyme	NAD(P)H e^- consumed	e^- used in chromate reduction	e^- used in H_2O_2 formation
YieF	62	46 (74%)	16 (26%)

Simultaneous four electron transfer

Ackerley, Gonzalez, Park, Blake II, Keyhan, & Matin. 2004. Environmenal Microbiology 6: 851-860

Confirmation

Rapid scan spectrophotometry to detect enzyme intermediates ~ Ackerley, Gonzalez, Park, Blake II, Keyhan, & Matin. 2004. AEM 70: 873-882

Electron Spin resonance studies -Ackerley, Gonzalez, Park, Blake II, Keyhan, & Matin. 2004. Environmenal Microbiology 6: 851-860

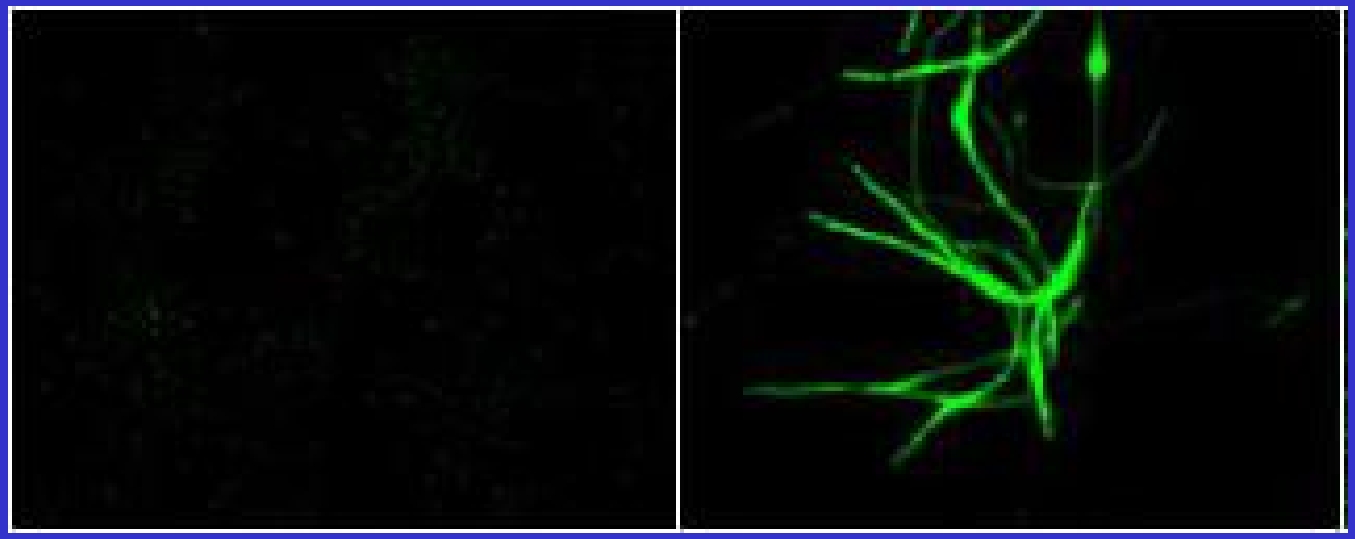
Three "safe" enzyme pure proteins characterized in detail

ChrR of *P. putida*: semitight
NfsA of *E. coli* – semitight
Yief of *E. coli* – tight

YieF is the safest (generates least possible amount of ROS)

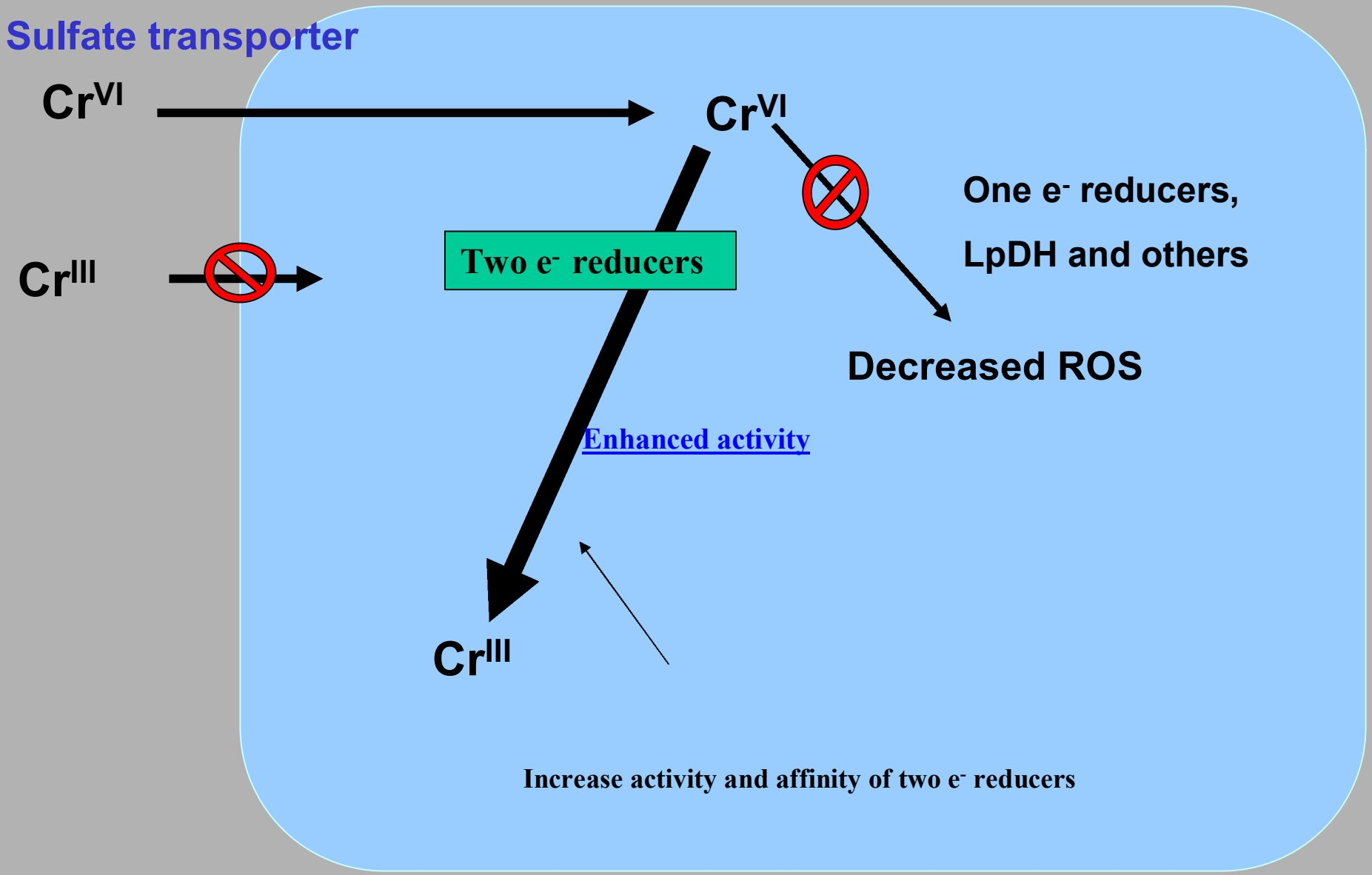
ChrR and NfsA permit some redox cycling but are much safer than one-electron reducers, generating only a fraction of ROS compared to the latter.

In vivo oxidative stress detected using the ROS-responsive dye H_2 DCFDA

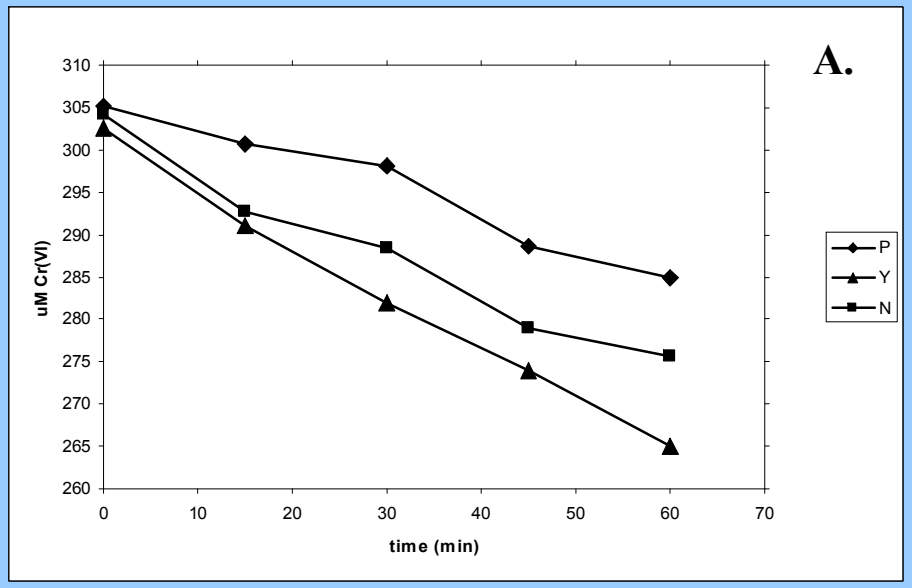


Ackerley, Barak, Lynch, Curtin, and Matin. 2006. J. Bacteriol 188 *In press*

Strategy to detoxify chromate reduction



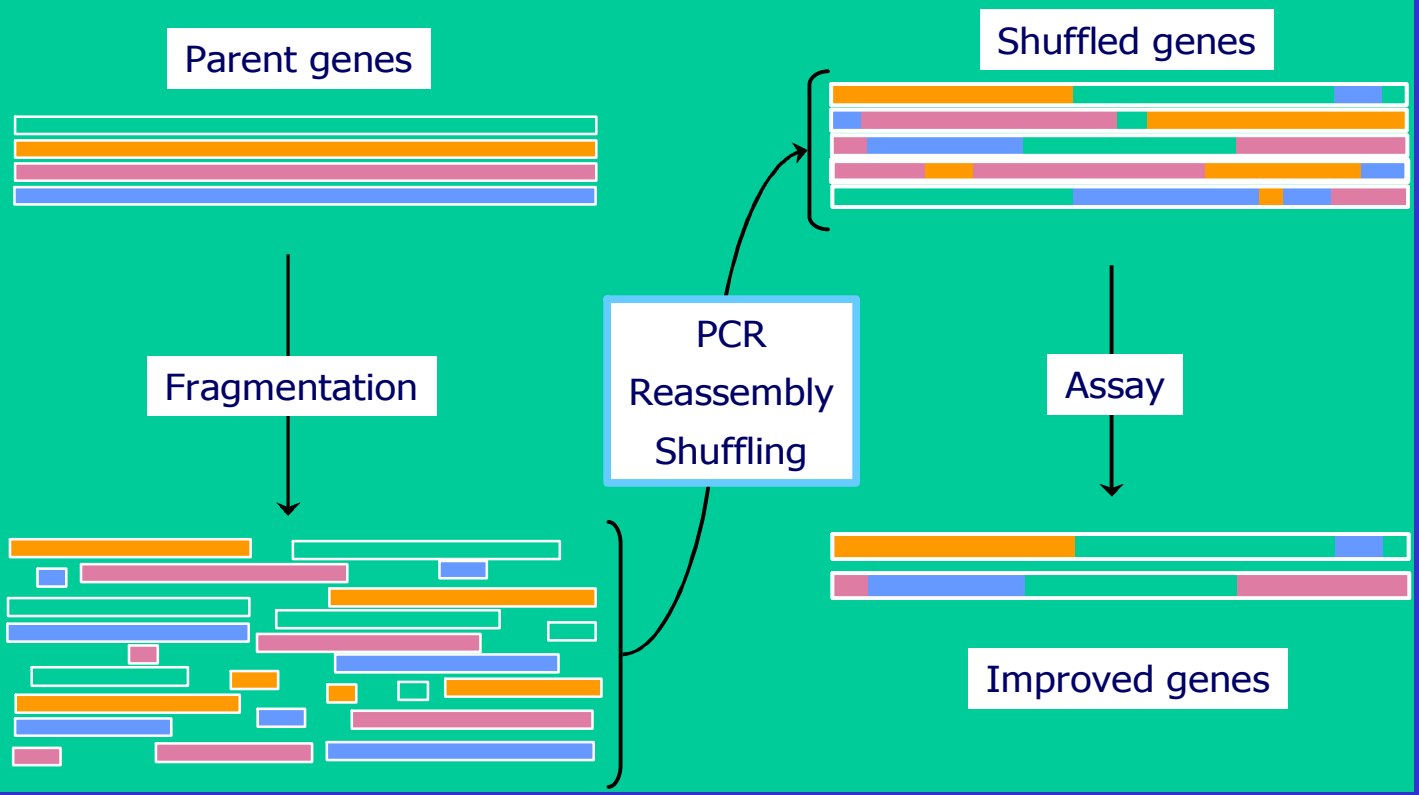
Protection by over expression of two-electron reducers

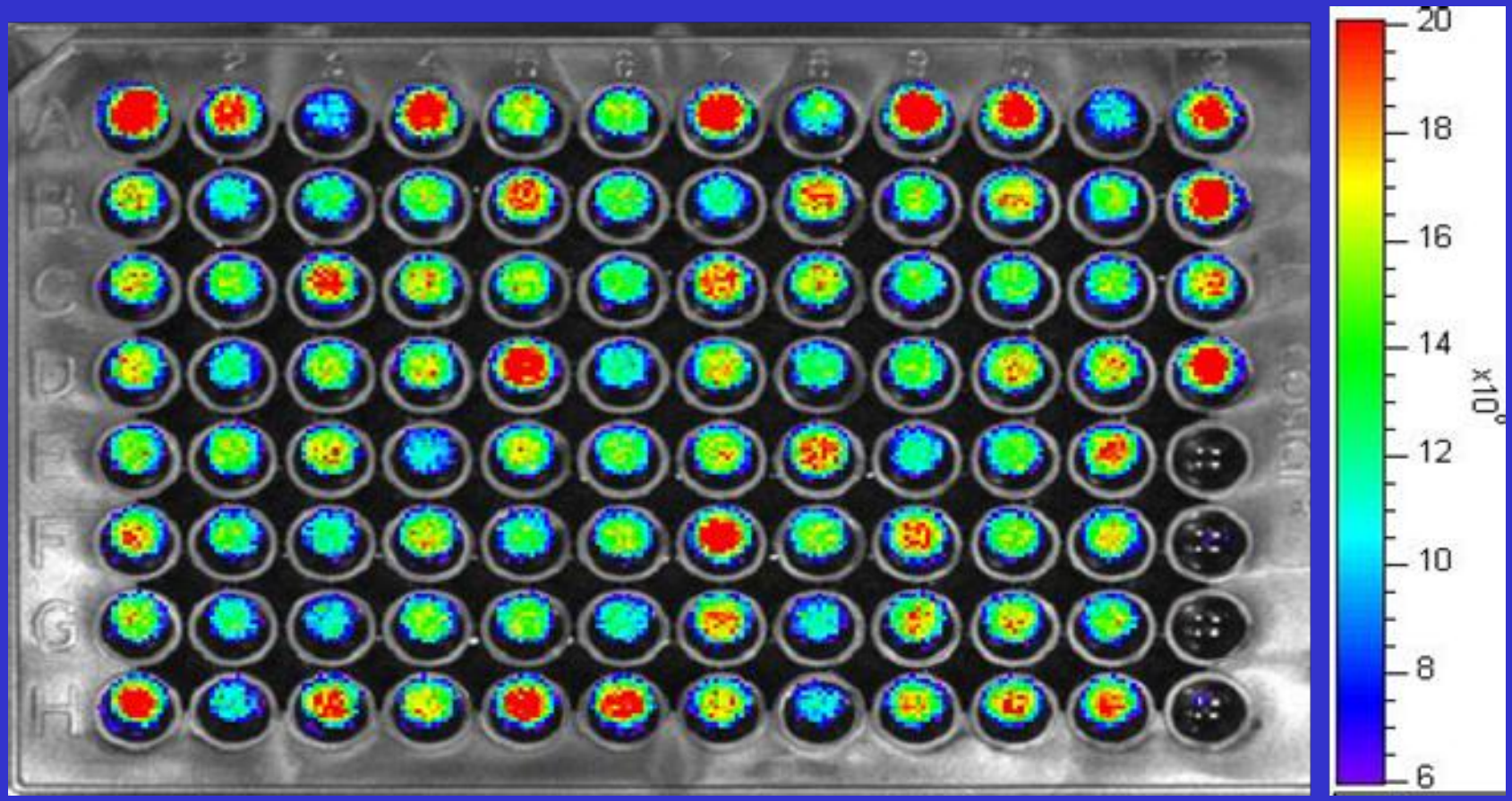


More chromate reduced per unit biomass

Ackerley, Gonzalez, Park, Blake II, Keyhan, & Matin. 2004. Environmenal Microbiology 6: 851-860

Schematic: Standard Shuffling




A
B
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6
x10

Cr(VI) reduction kinetics of YieF and the improved derivative Y6

Strain	V_{max} (nmol Cr(VI) mg protein $^{-1}$ min $^{-1}$)	K_m (μM)	K_{cat}* (S^{-1})	K_{cat}/K_m
YieF	295 ± 27	376 ± 14	30 ± 2	$4.5X10^4 \pm 3 X10^3$
Y6	8,812 ± 611 (30-fold)	41 ± 5	521 ± 18	$1.3X10^7 \pm 3 X10^5$

XANES Analysis showed quantative conversion to Cr(III)
Direct demonstration that Y6 also does not generate redox Cyclinng

Barak, Dodge, Francis a, nd Matin. In preparation

Physiological role

Broad substrate range: Quinones, ferricyanide, DCPIP, Mo (IV), and V(V). Ackerley, Gonzalez, Park, Blake II, Keyhan, & Matin. 2004. AEM 70: 873-882

General role is to protect against electrophiles like the ones mentioned above, with predilection for one-electron reduction and redox cycling - Gonzalez, Ackerley, Lynch & Matin. 2005. Journal of Biological Chemistry 280: 22590-595

Recent results show that U(VI) reduction also subjects the bacterial cell to oxidative stress

YieF & Other "safe" enzymes could reduce also U(VI) – activity detected only when overproduced

U(VI) reduction kinetics of YieF and the improved derivative Y6

Strain	V_{max} (nmol U(VI) mg protein $^{-1}$ min $^{-1}$)	K_m (μM)	K_{cat}* (S^{-1})	K_{cat}/K_m
YieF	194 ± 17	373 ± 49	20 ± 11	$1.6X10^4 \pm 1.7X10^3$
Y6	2,511 ± 421 (12-fold)	335 ± 40	167 ± 39	$5X10^5 \pm 2X10^4$

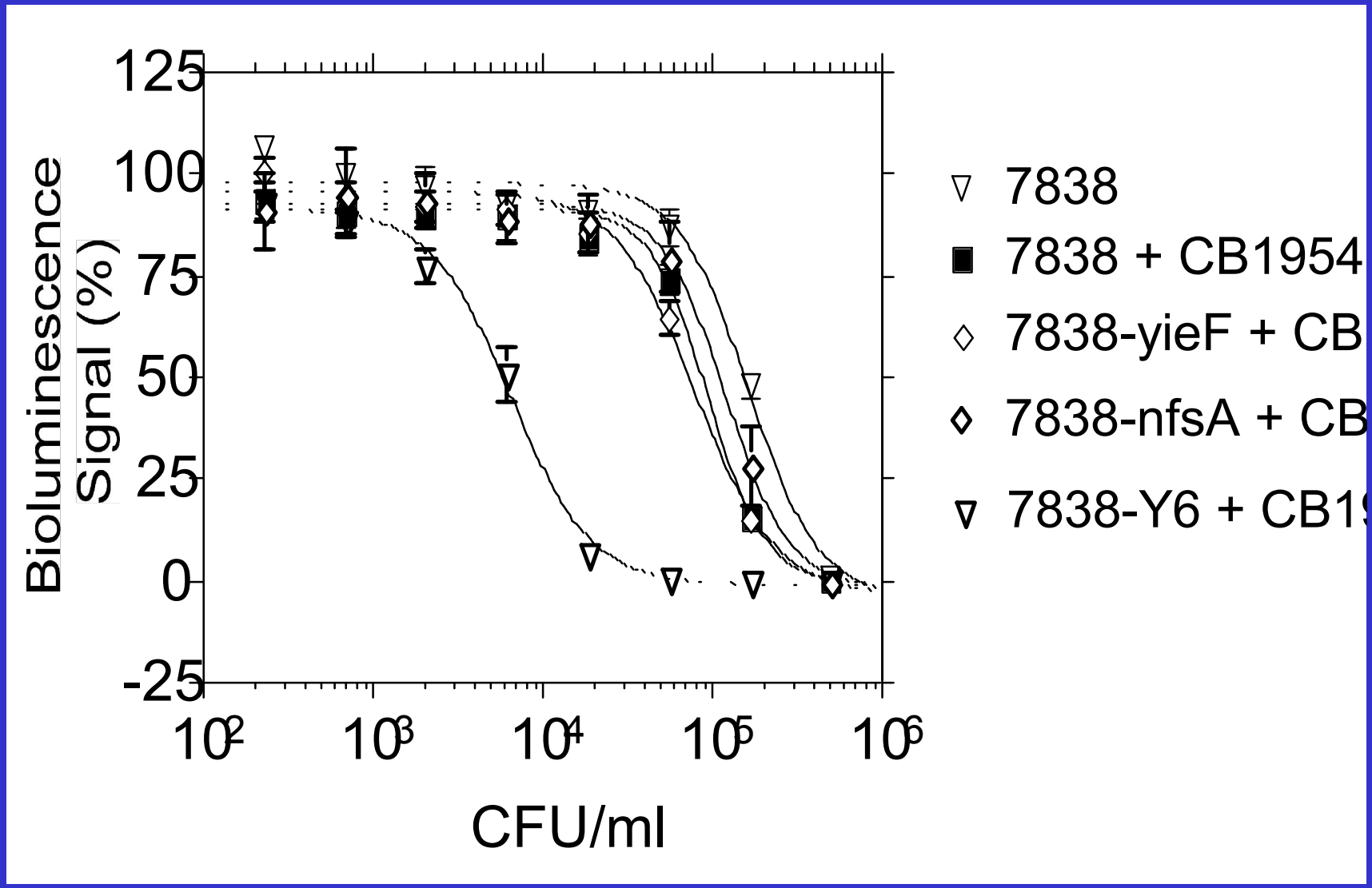
Analysis showed quantitative conversion to U(IV)
Direct demonstration that Y6 also does not generate redox cycling

Barak, Dodge, Francis a, nd Matin. In preparation

Surprise application in cancer prodrug chemotherapy

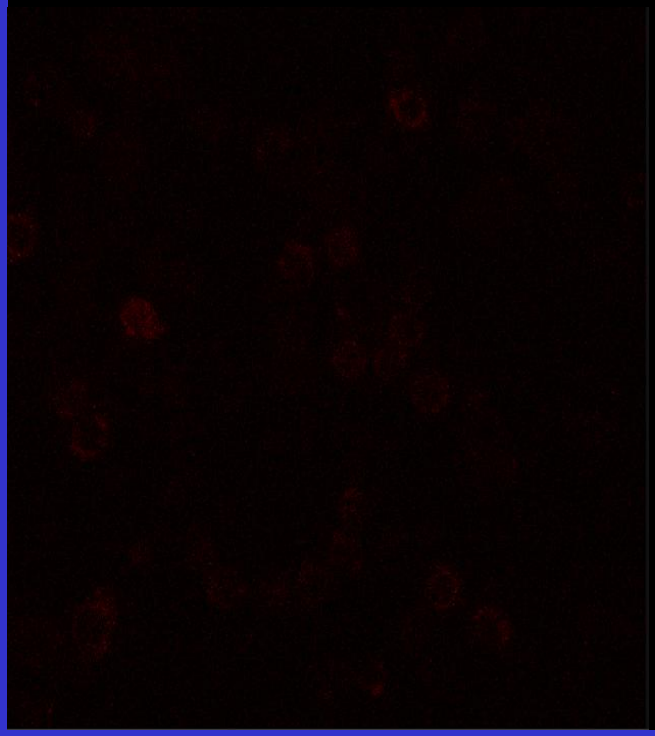
Reductive prodrugs (e.g., CB 1954 and CNOB [discovered by us] become toxic only upon reduction

Y6 and other evolved enzymes are highly effective in this respect also -Barak, Thorne, Contag, and Matin. 2006 Molecular Cancer Therapeutics.

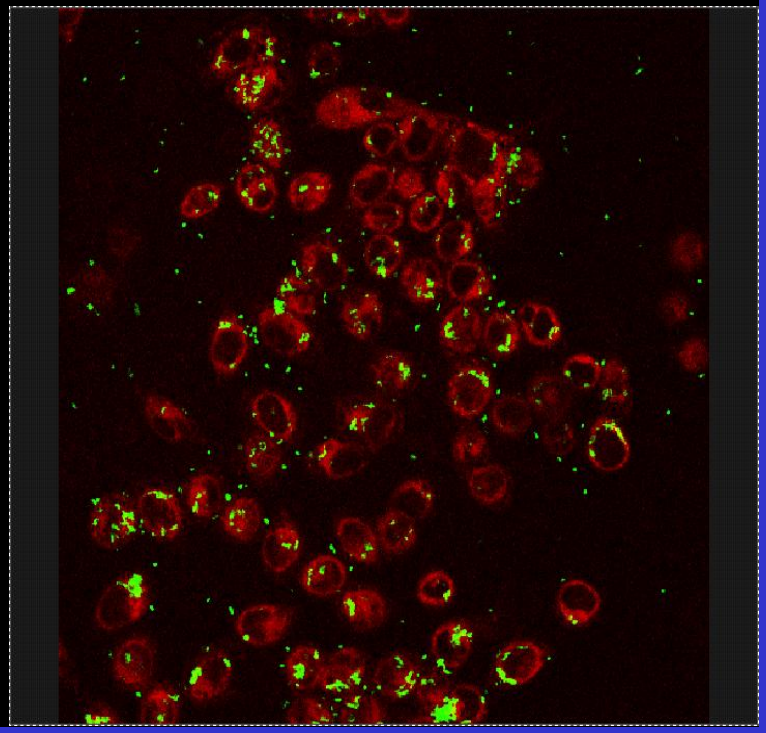


Barak, Thorne, Contag, and Matin. 2006 Molecular Cancer Therapeutics.
Stanford Patent S067

HeLa's +CNOB



HeLa's +CNOB+SL 7838::Y6



Only one change, tyrosine 128 to asparagine, responsible for all three improvements

Application of a stochastic model predicted other beneficial changes; one enzyme has >830-fold improvement

Barak, , Nov & Matin,, In preparation

Work in progress

- Screening of shuffled libraries for other useful reductions, eg., Pu
- Manipulate outer membrane porins to increase *P. putida outer membrane permeability*
- Improve cytoplasmic for chromate transport
- Combine activity with nitrate reduction
- Batch and column studies of efficacy, survival and competitive ability in the presence of FRC material

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