Mechanism of Bacterial Uranium and Technetium Reduction

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PROJECT GOAL

Identify genes and proteins required for bacterial metal respiration

- Reductive dissolution of solid **iron [Fe(III)]**
- Reductive dissolution of solid **manganese [Mn(IV)]**
- Reductive volatilization of solid **elemental sulfur [S(0)]**
- Reductive precipitation of soluble **uranium [U(VI)]**
- Reductive precipitation of soluble **technetium [Tc(VII)]**

Despite the importance of bacterial reductive precipitation of U and Tc as an alternative remediation strategy, the molecular mechanism of U and Tc reduction is poorly understood

Model Metal Reducing Bacteria

Shewanella putrefaciens strain 200
Shewanella oneidensis strain MR-1

Electron Donors:
Organic acids
Amino acids
Sugars
Hydrogen

Electron Acceptors:
Oxygen [O_2]
Nitrogen compounds [NO_3^-, NO_2^-, NO]
Manganese oxides [Mn^{4+}, Mn^{3+}]
Ferric iron [Fe^{3+}]
Sulfur compounds [SO_3^{2-}, $S_2O_3^{2-}$, S(0), DMSO]
Uranium [U^{6+}]
Technetium [Tc^{7+}, Tc^{4+}]
Selenium [Se^{4+}]
Trimethylamine-*N*-oxide [TMAO]
Fumarate
AQDS (electron shuttle)
Others: Arsenate, Chromate, Vanadate, Neptunium(V), Deaminated histidine, Antibiotics

EXPERIMENTAL STRATEGY TO IDENTIFY METAL REDUCTION-SPECIFIC GENES

A. Genetic complementation analysis of metal reduction-specific mutants

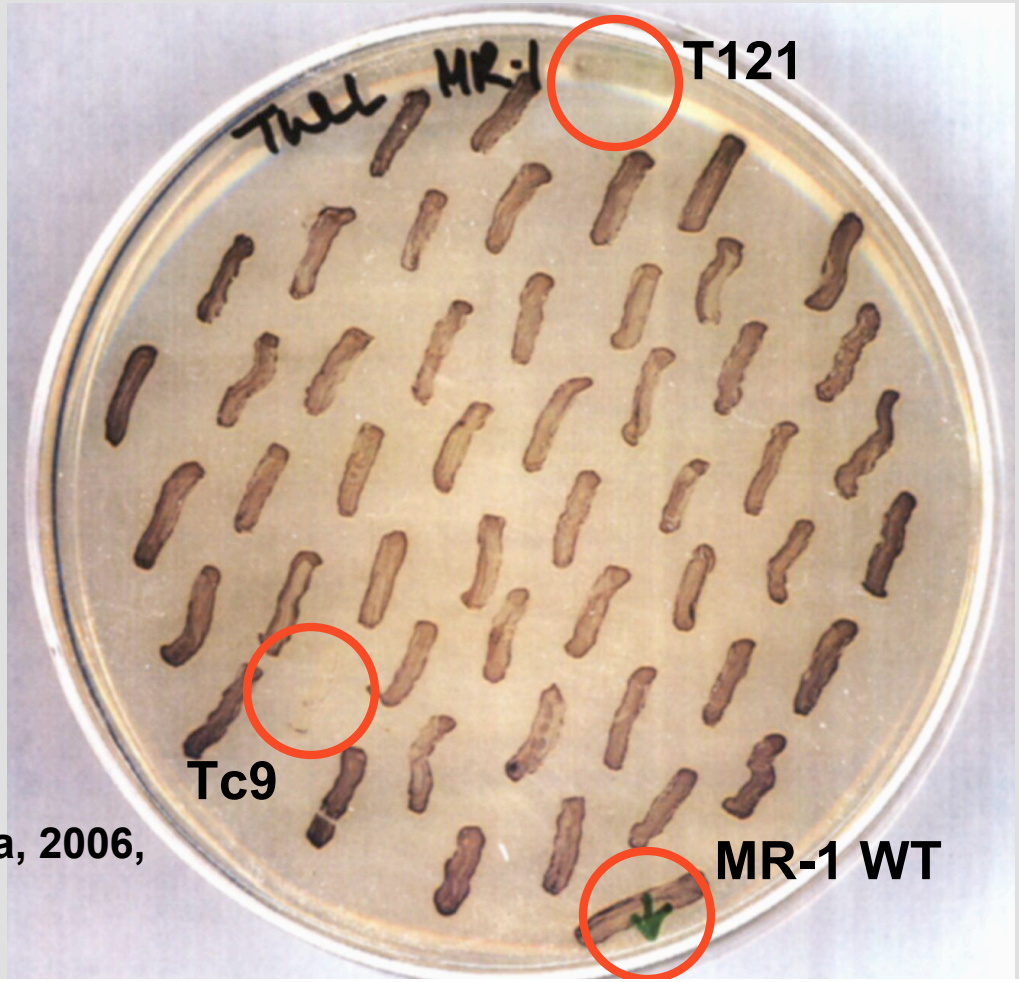
   - random point mutants identified via metal reduction-specific screens

   - mutated genes identified via genetic complementation with random WT DNA fragments

B. In-frame deletion of targeted genes identified in genome

Technetium reduction

Tc^{7+} clear, soluble → Tc^{4+} black precipitate



5,000 screened, 6 identified

Tcr mutants tested for anaerobic growth capability in liquid culture

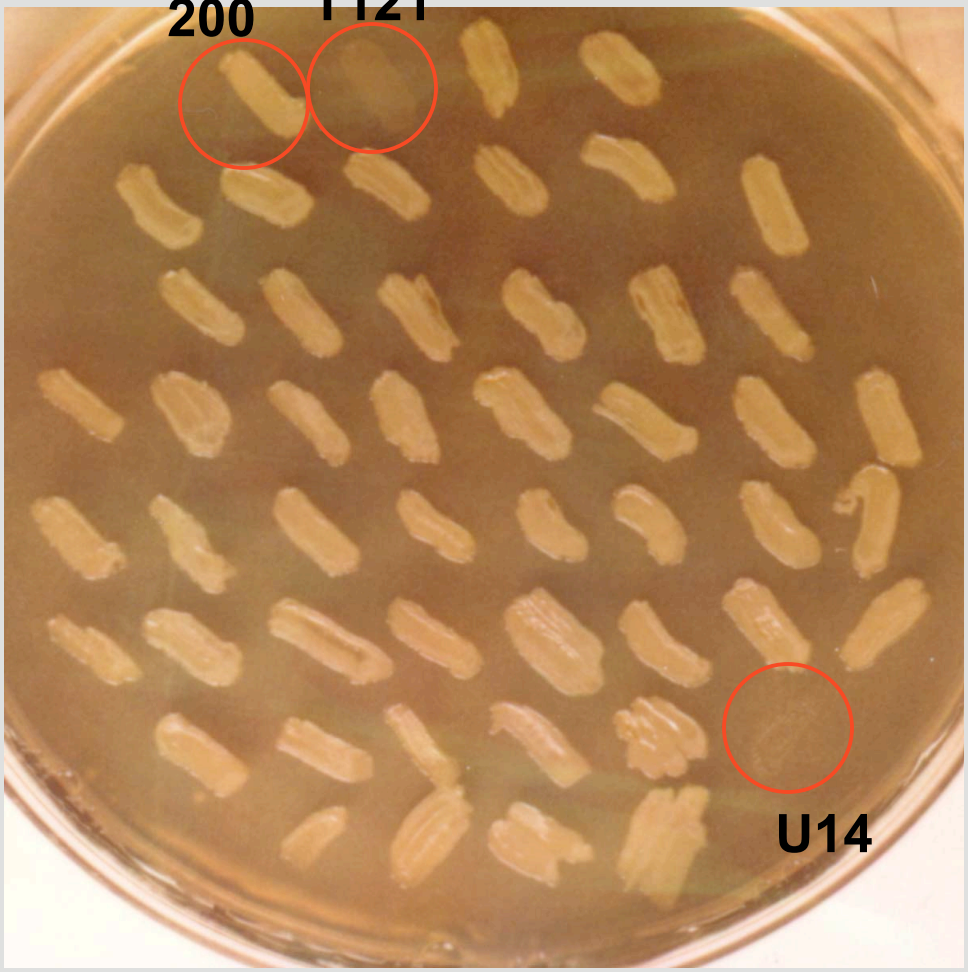
Payne and DiChristina, 2006, *FEMS Microbiol. Lett.*

Uranium reduction

U(VI) ⟶ U(IV)

Soluble, Clear

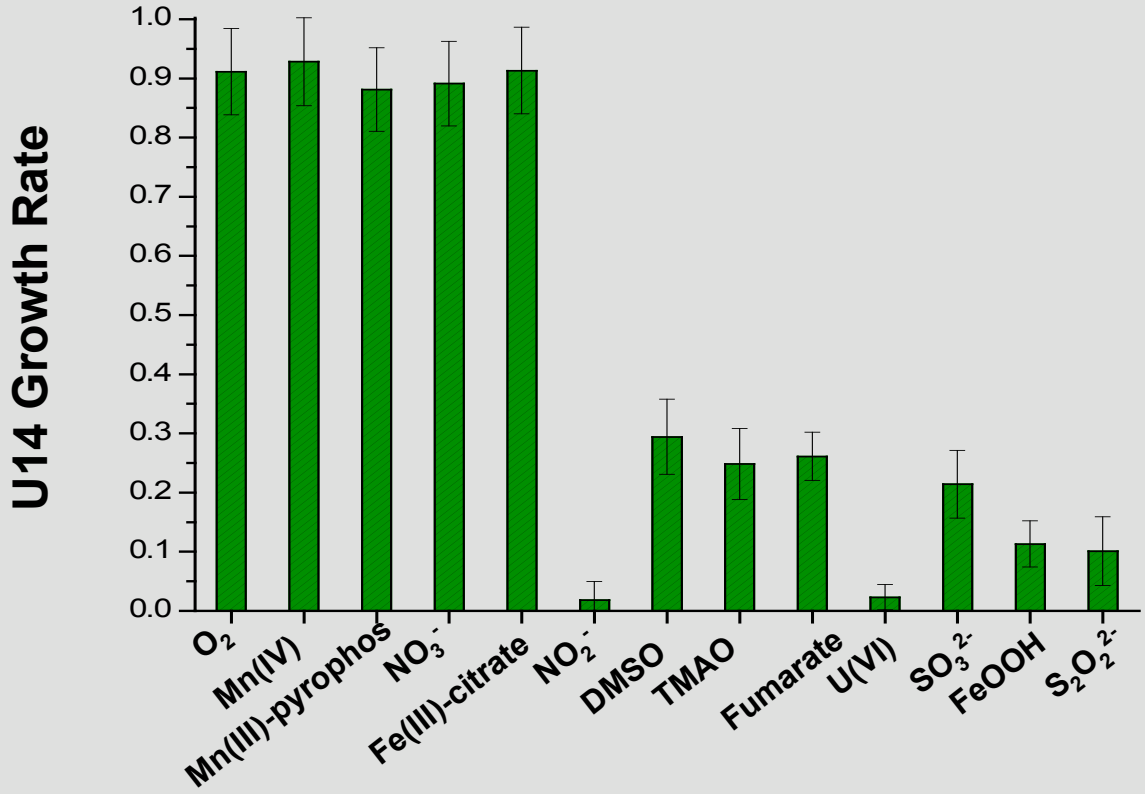
Insoluble, Brown Precipitate



7,000 screened,
4 identified

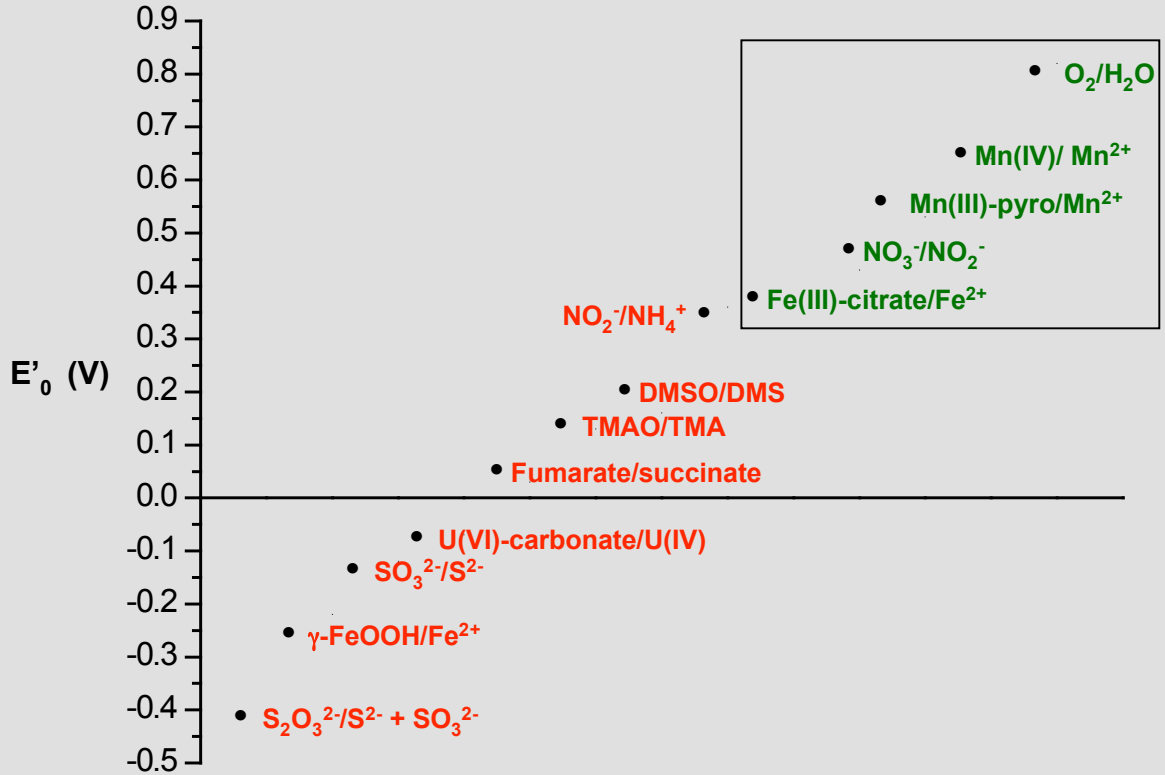
U14 tested for anaerobic growth capability in liquid culture

U14 displays multiple respiratory deficiencies



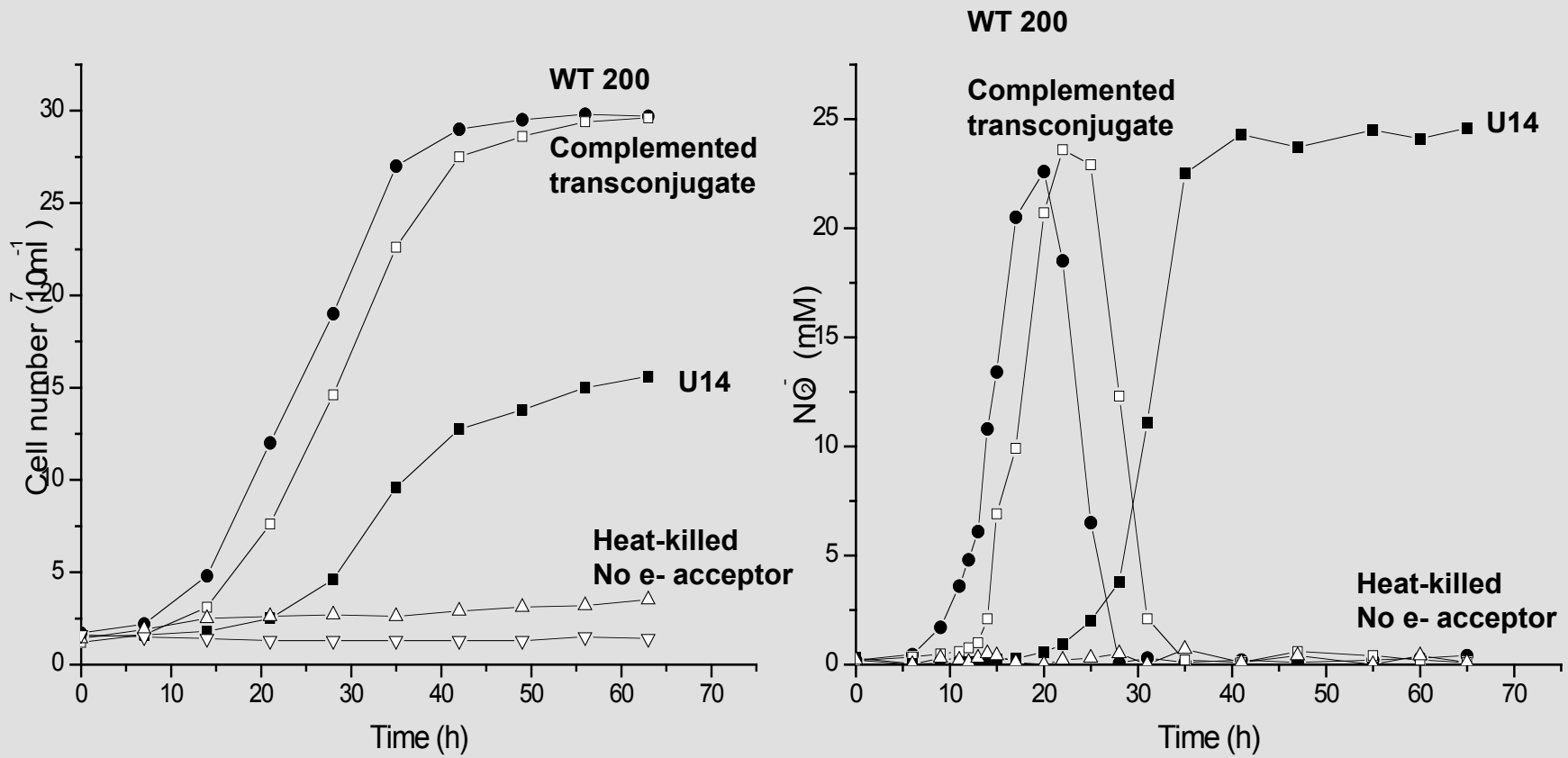
Dale et al., 2007, *J. Bacteriol.*,189:1036-1043

U14 respires on electron acceptors with high (but not low) mid-point redox potential (E'_0)

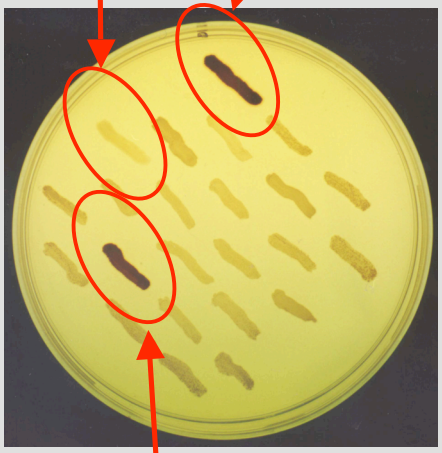


E'_0 cutoff is between NO_3^-/NO_2^- couple (0.43 V).....

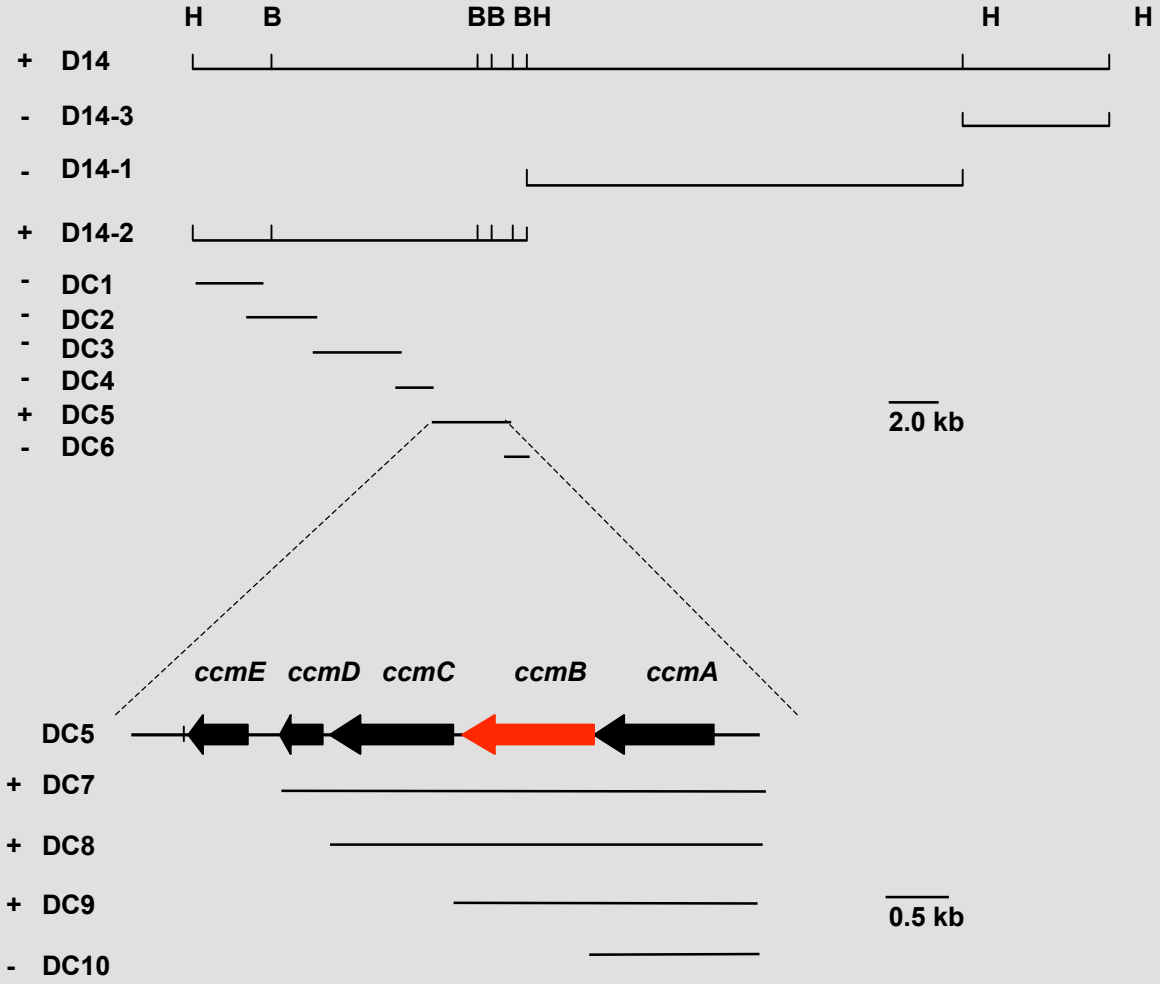
U14 respires NO_3^- but not NO_2^-



Genetic complementation analysis of U14



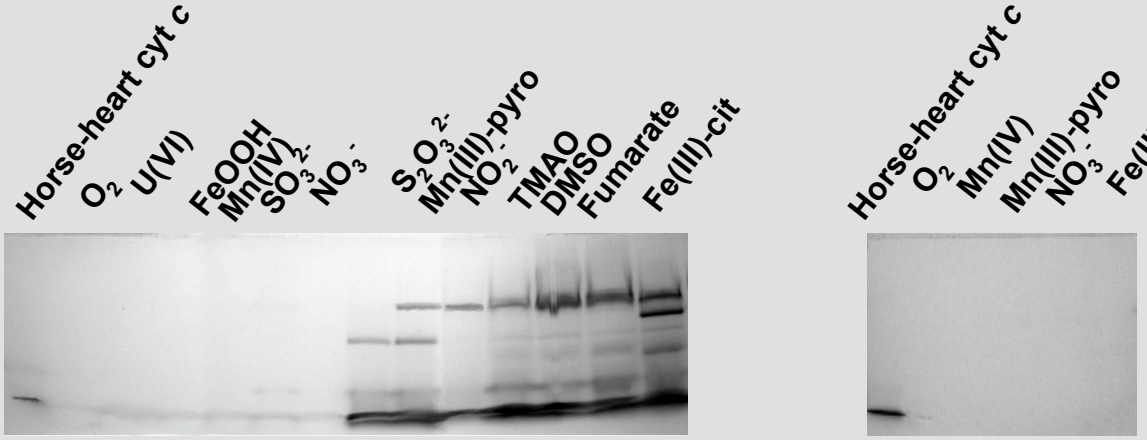
U14 transconjugate with restored U(VI) reduction activity



U14 is mutated in *ccmB*, the permease subunit of the *ccm* cytochrome *c* maturation system

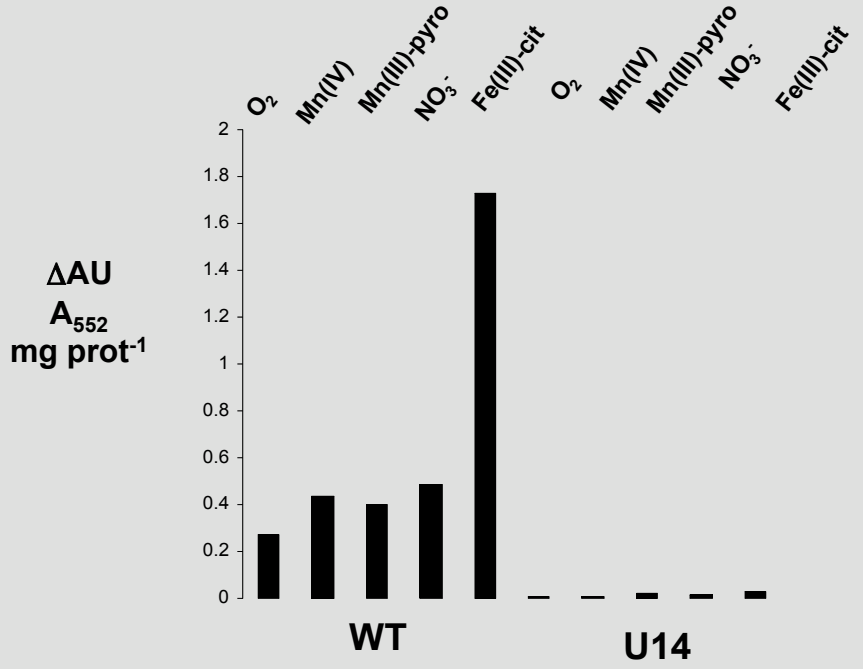
U14 is unable to produce mature cytochrome *c*

SDS-PAGE heme stain of soluble fraction

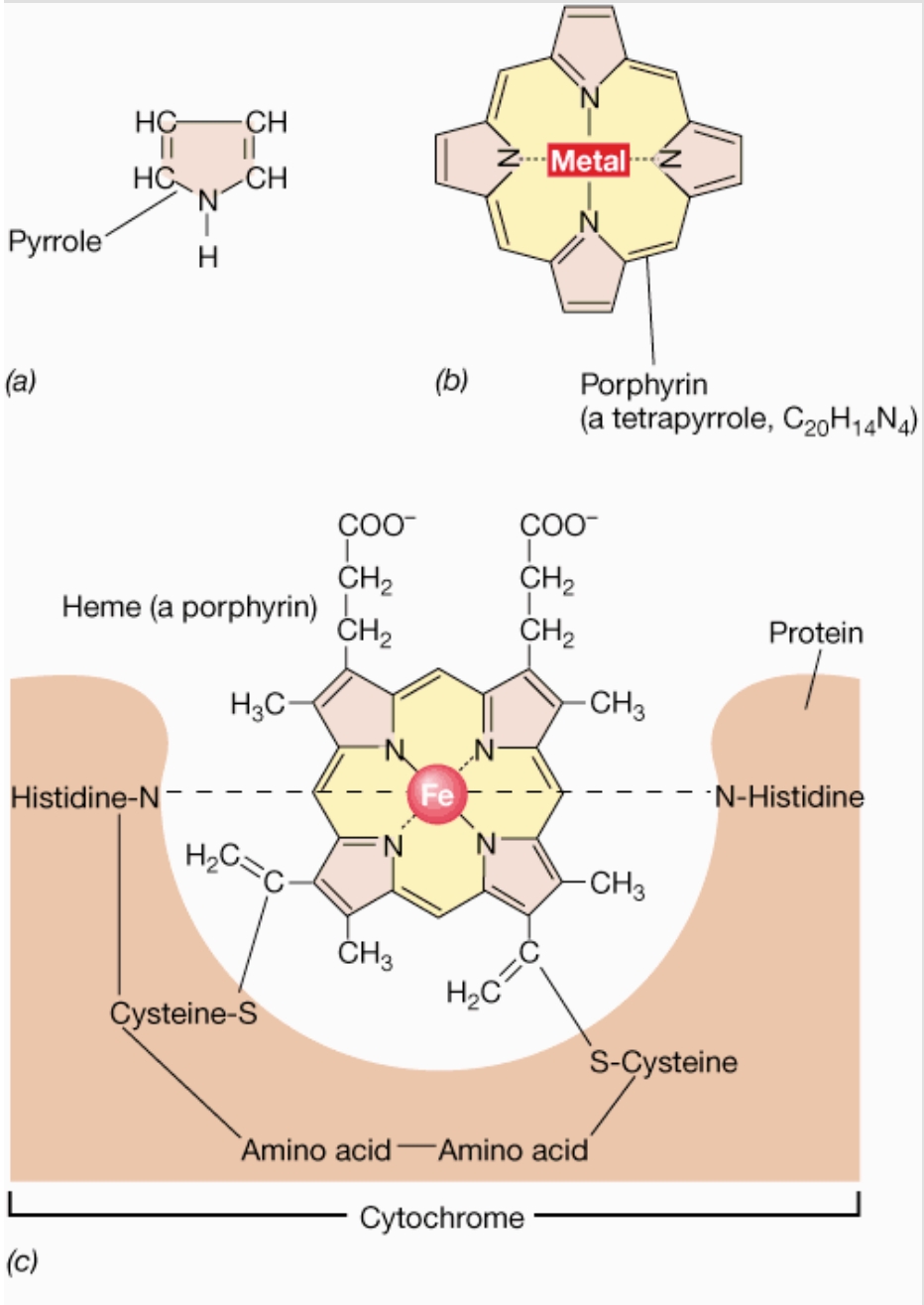


WT **U14**

Cytochrome *c* content of soluble fraction measured by A_{552} in red-minus-ox difference spectra



Maturation of cytochrome *c*



1. Translate apocytochrome

2. Synthesize 4 pyrroles

3. Cyclize to form porphyrin ring

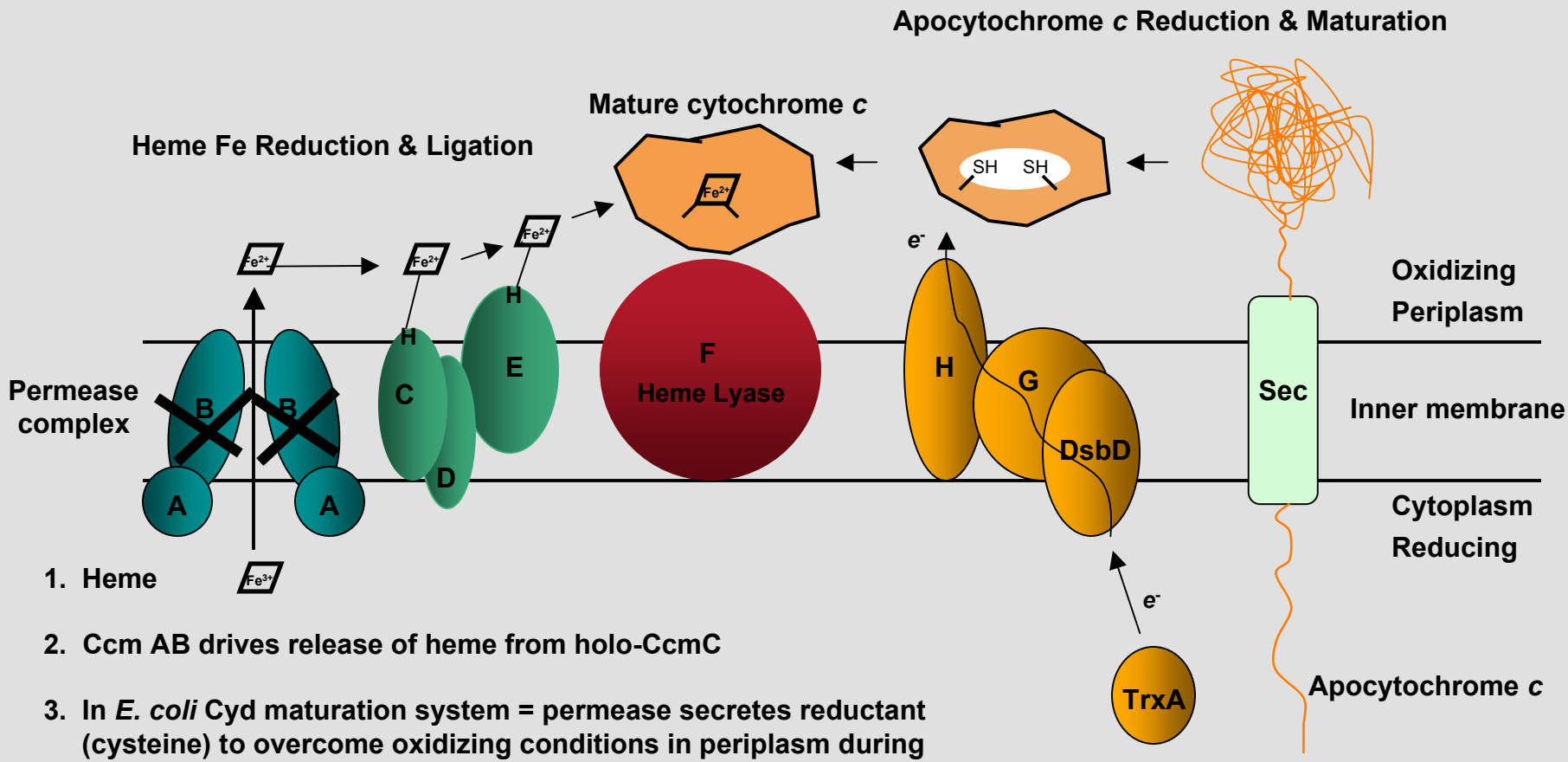
4. Insert Fe metal center to form heme

5. Heme lyase (CcmF) covalently attaches heme vinyl groups to apocytochrome thiols to form a a thioether bridge…..and a mature cyt:

Step 5 requires that the heme Fe and apocytochrome thiols remain in a reduced state, a process carried out by the Ccm system……

Cytochrome *c* maturation in *E. coli* (System I)

S. putrefaciens 200 has a nearly identical ccm system (genome contains two predicted copies of CcmF)



1. Heme
2. Ccm AB drives release of heme from holo-CcmC
3. In *E. coli* Cyd maturation system = permease secretes reductant (cysteine) to overcome oxidizing conditions in periplasm during growth on electron acceptors with high E'_0 (O_2 or NO_3^-)

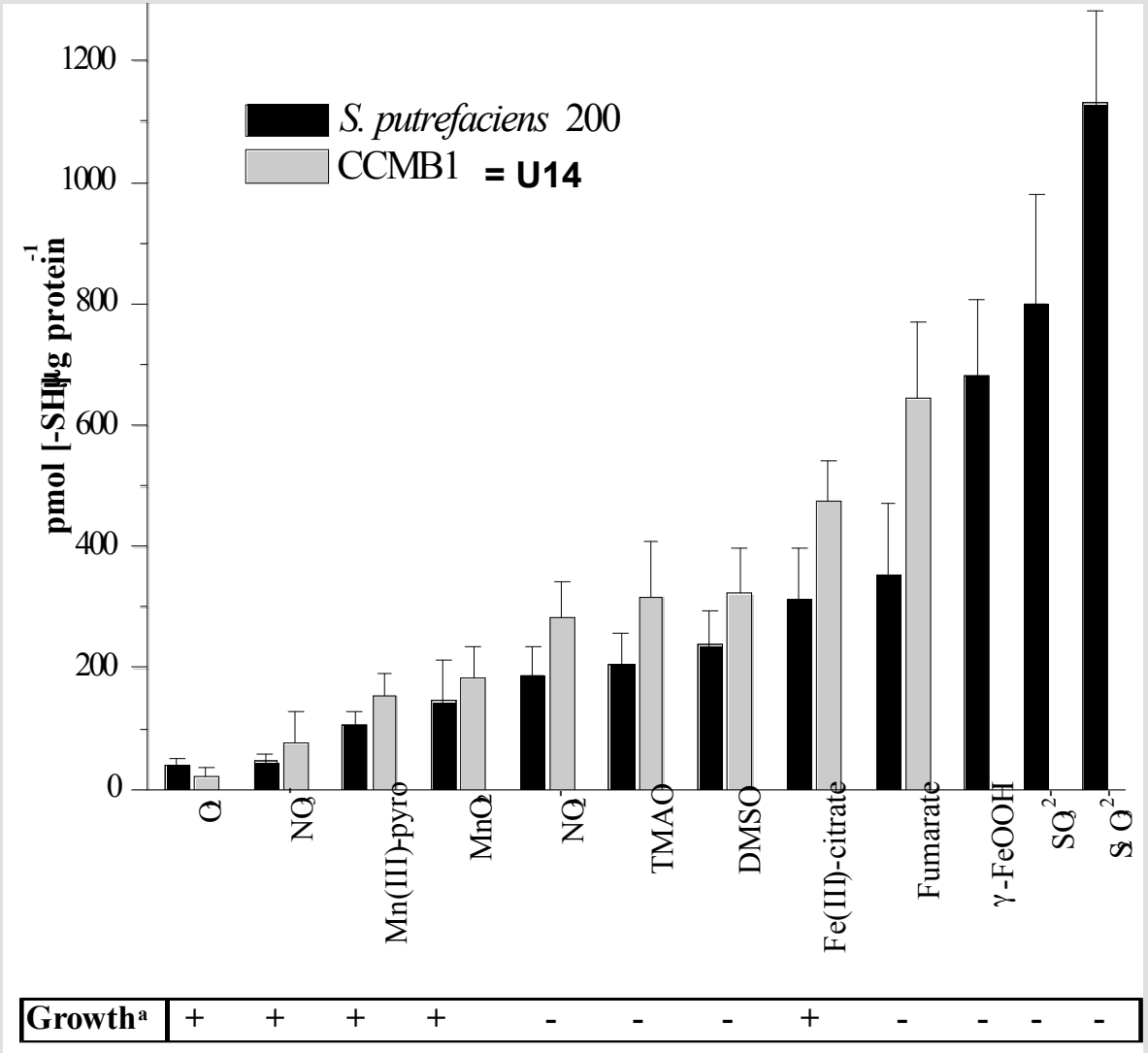
The finding that the *E. coli* Cyd permease secretes a reductant (cysteine) into the periplasm to overcome highly oxidizing conditions during growth on electron acceptors with high E'_0 prompted us to hypothesize that *Shewanella* CcmB secretes an oxidant (cystine?) into the periplasm to overcome highly reducing conditions during growth on electron acceptors with low E'_0

- **Led us to pose two questions:**

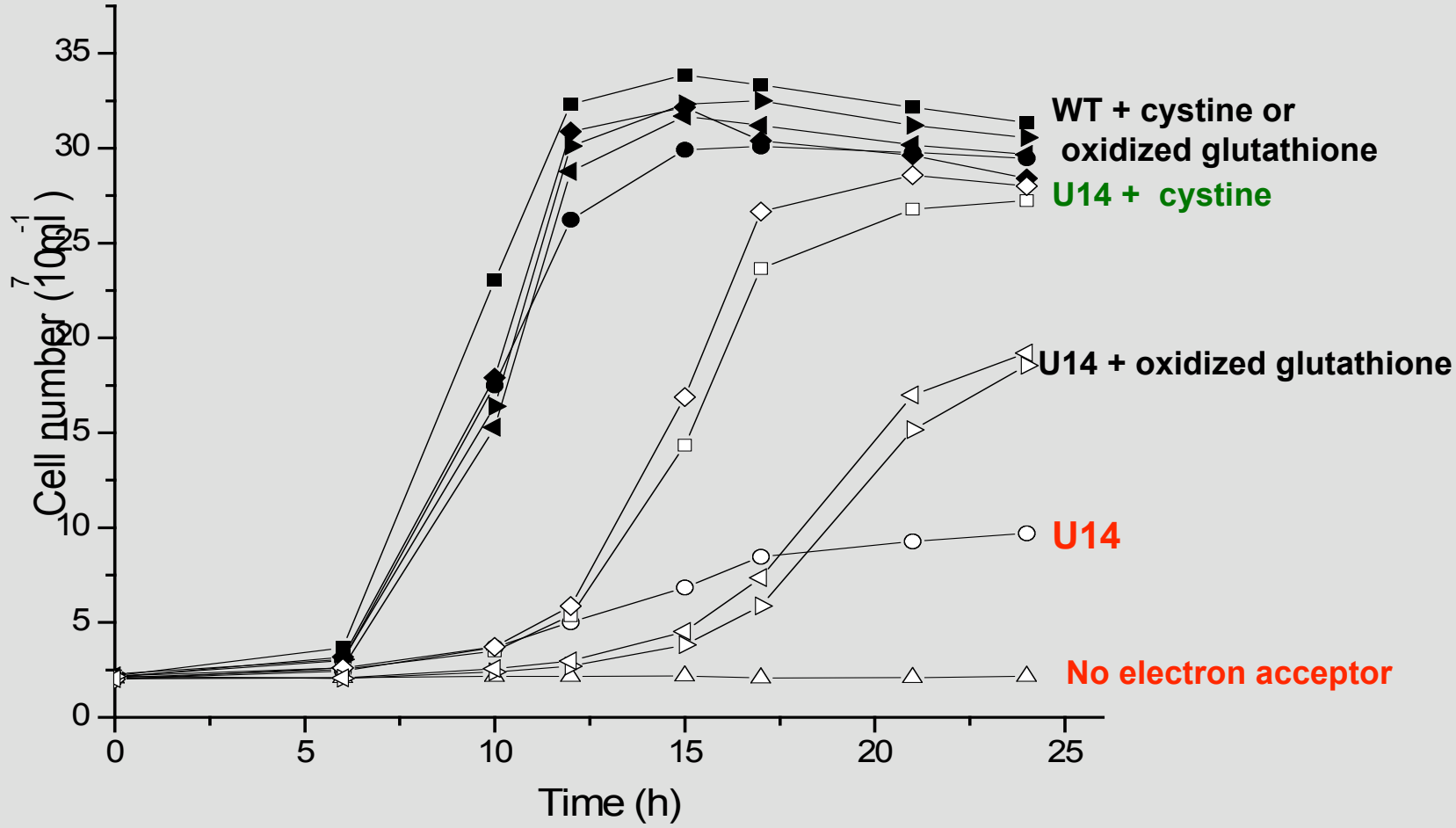
1. **Is the U14 periplasm overly reduced?**

2. **Can the U14 respiratory deficiencies be rescued (chemically complemented) by addition of exogenous cystine?**

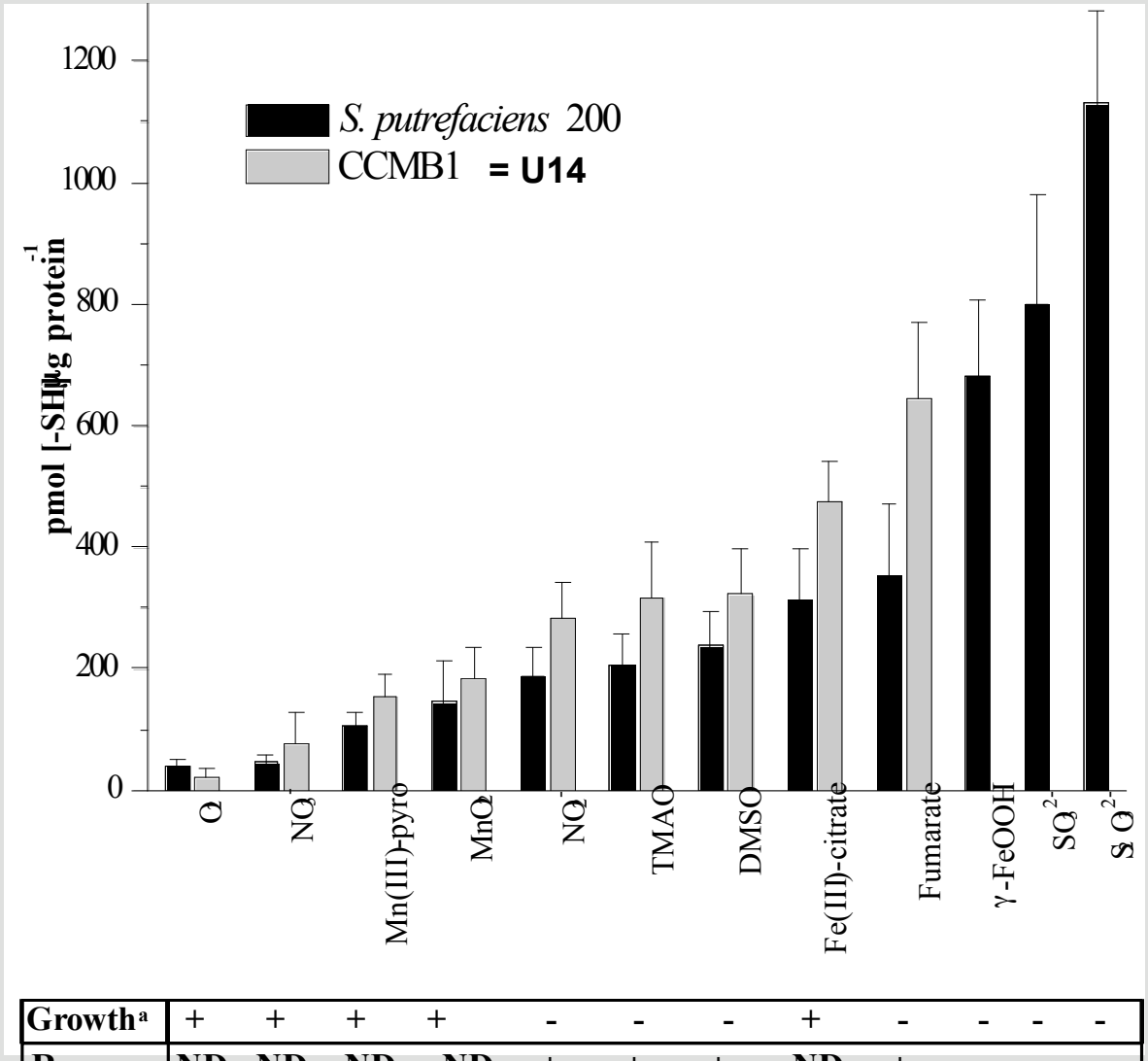
Periplasm of U14 is overly reduced:
Thiol content of U14 periplasm is 25-50% greater than wild-type



Chemical complementation of U14: Anaerobic growth of U14 is rescued by addition of cystine or oxidized glutathione



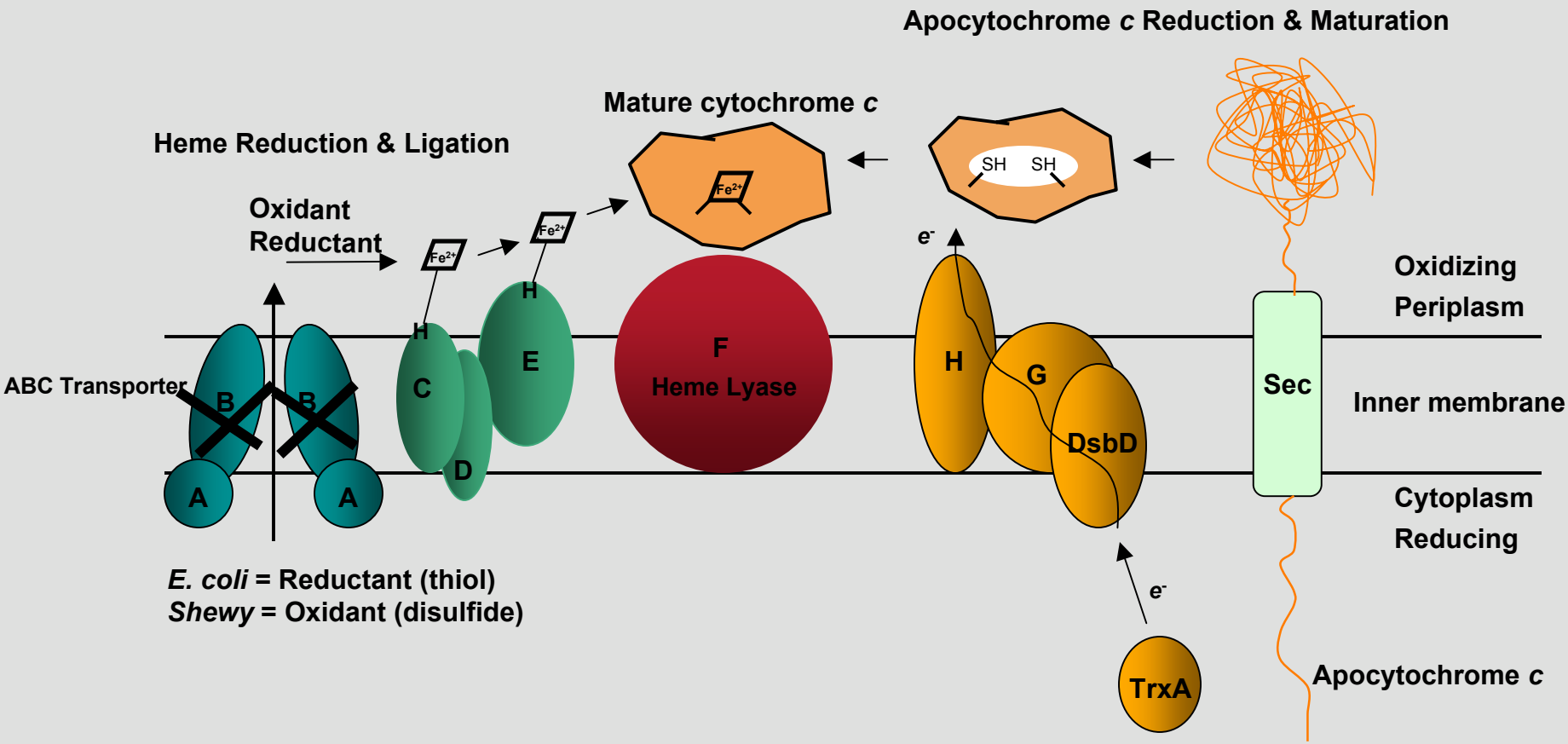
Chemical complementation (rescue) of U14 anaerobic respiratory deficiencies via addition of cystine to growth medium



Growth[a]	+	+	+	+	-	-	-	+	-	-	-	-
Rescue	**ND**	**ND**	**ND**	**ND**	+	+	+	**ND**	+	-	-	-

Working Hypothesis:

Cytochrome *c* maturation in *S. putrefaciens* requires that the CcmB permease secrete an <u>oxidant</u> to maintain proper redox poise in periplasm during growth on electron acceptors with low (but not high) E_0'

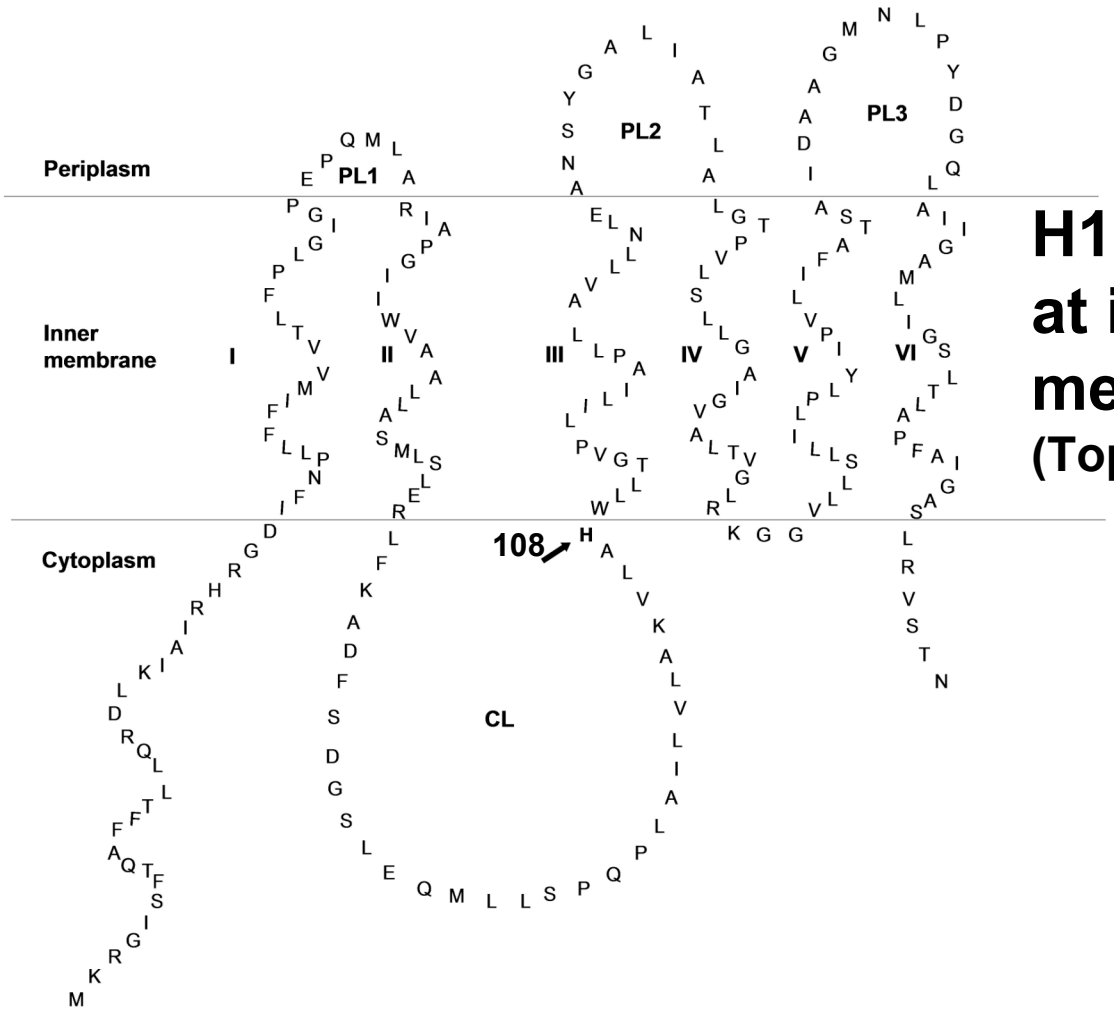


U14 contains a mutation in cytochrome *c* maturation gene *ccmB* at position 108 (H108Y)

Shewy
E. coli
Land plants
Red algae

FIG X. CcmB sequence of *S. putrefaciens* (Shepu) and E. coli (Ecoli), orthologous Ccb206 of *A. thaliana* (Arath), Orf206 of *T. aestivum* (Triae), Orf277 of *M. polymorpha* (Marpo) and YejV of *C. merolae* (Cymer). Identical residues are shaded. H108 of *S. putrefaciens* 200 and corresponding identical residues are boxed. Predicted transmembrane domains in *S. putrefaciens* are indicated by bars above the sequence.

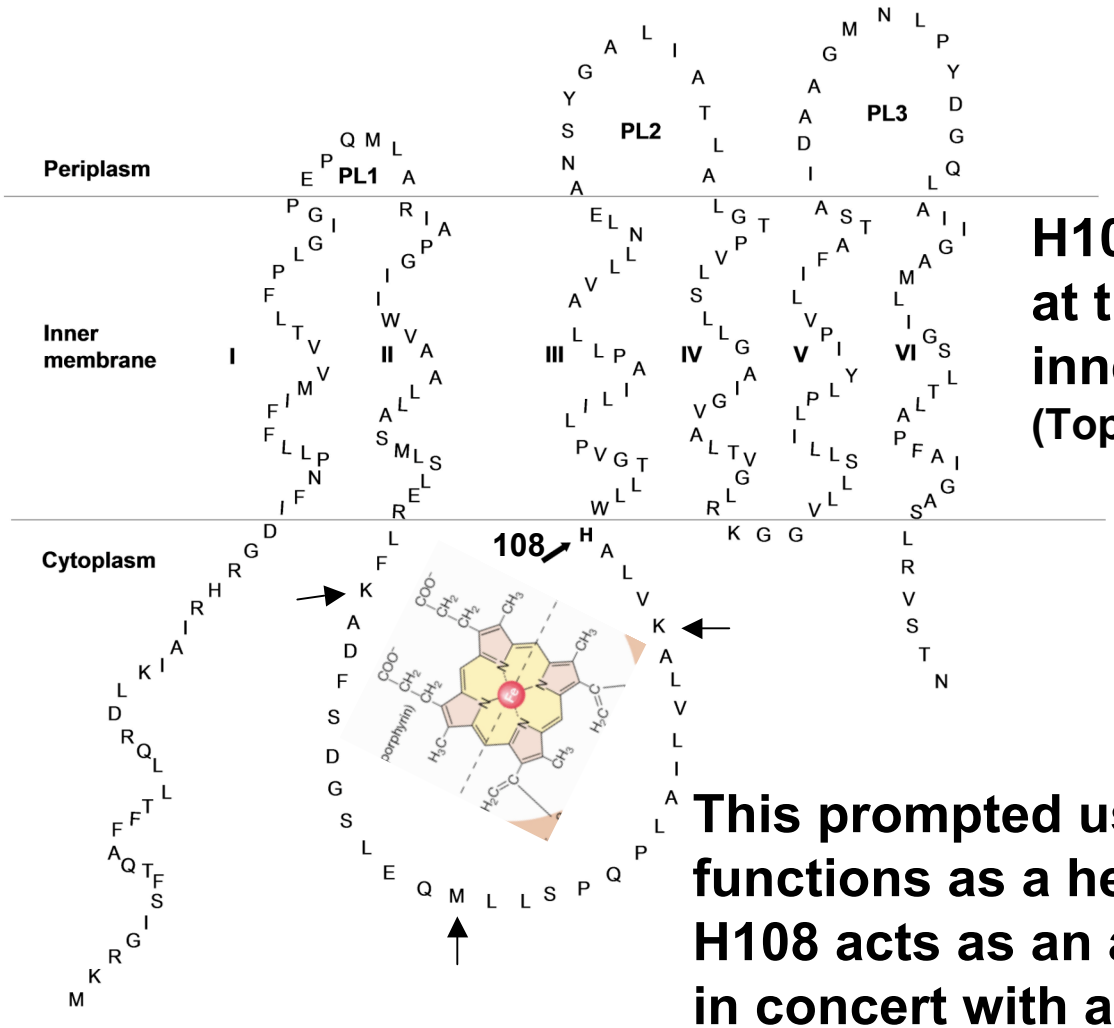
H108 is one of only six aa residues conserved across domain lines



H108 is predicted to reside at interface between inner membrane and cytoplasm

(Topology prediction via TopPred2)

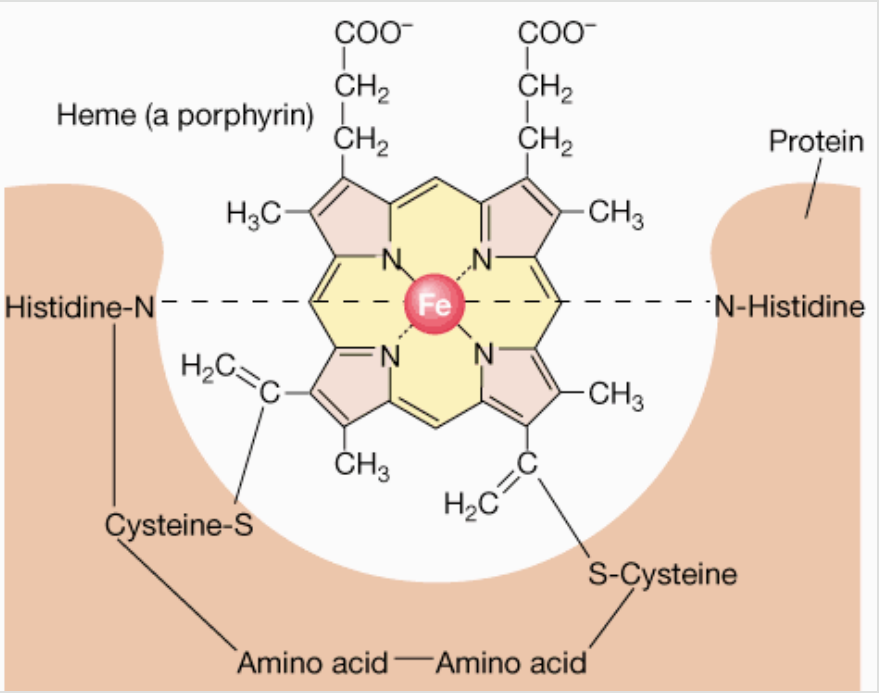
Predicted topology of CcmB in cytoplasmic membrane



H108 is predicted to reside at the interface between the inner membrane and cytoplasm
(Topology prediction via TopPred2)

This prompted us to hypothesize that CcmB functions as a heme transporter and that H108 acts as an axial ligand for heme binding, in concert with a distal ligand in cytoplasmic loop (H, K, M, Y or C)…….

Cytochrome structure



- **As a general rule in all hemoproteins, the energies of the Fe d-orbitals are controlled by the ligand field strength of heme axial ligands**

- **Only H, M, K, Y or C amino acids contain side chains that are strong field ligands able to maintain Fe in low-spin so that Fe does not structurally rearrange during Fe3+/Fe2+ redox transition**

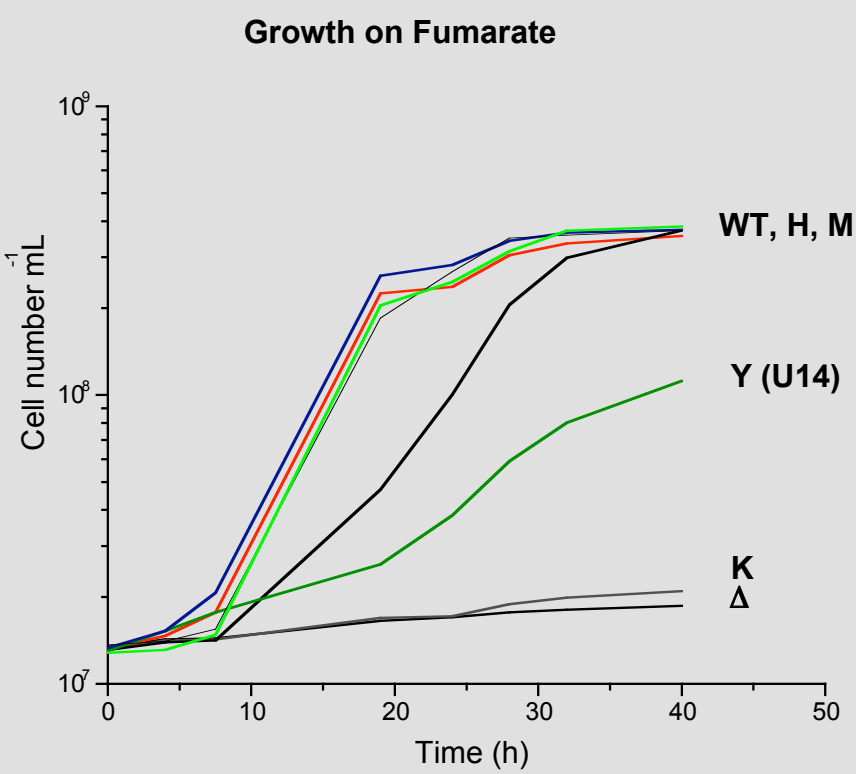
10 combinations of H, M, K and C are possible, but only 4 observed in nature:

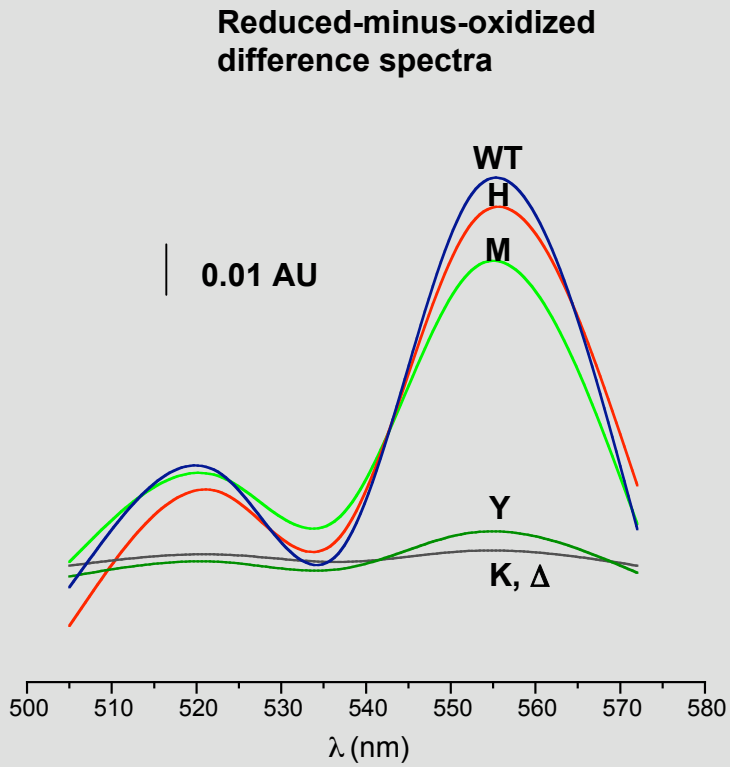
H-H			
H-M	**M-M**		
H-K	**M-K**	**K-K**	
H-C	**M-C**	**K-C**	**C-C**

CXXCH (HAO)
H = imparts more negative potential to heme

CXXCK (NrfA)
K (OTR)
K = imparts more positive potential to heme

To test this hypothesis, site-directed H108 mutants of CcmB were constructed and examined for anaerobic growth and c-type cytochrome maturation activity:

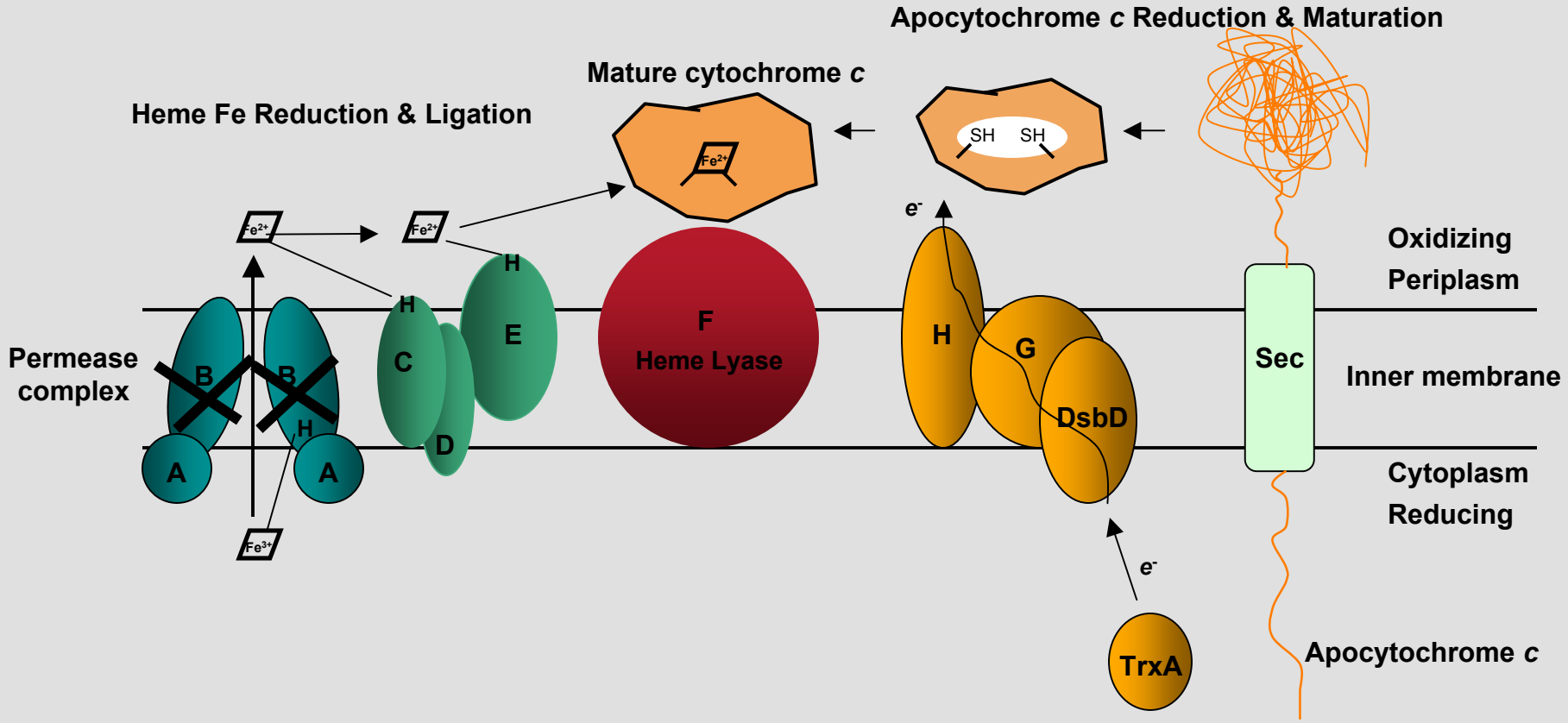




Cytochrome *c* maturation in *S. putrefaciens* 200

Histidines on CcmB, CcmC and CcmE carry out two functions:

1. Shuttle heme from cytoplasm, thru CM to periplasmic CcmF for ligation to apocytochrome
2. Maintain heme Fe at proper E'_0 for Ccm F ligation reaction



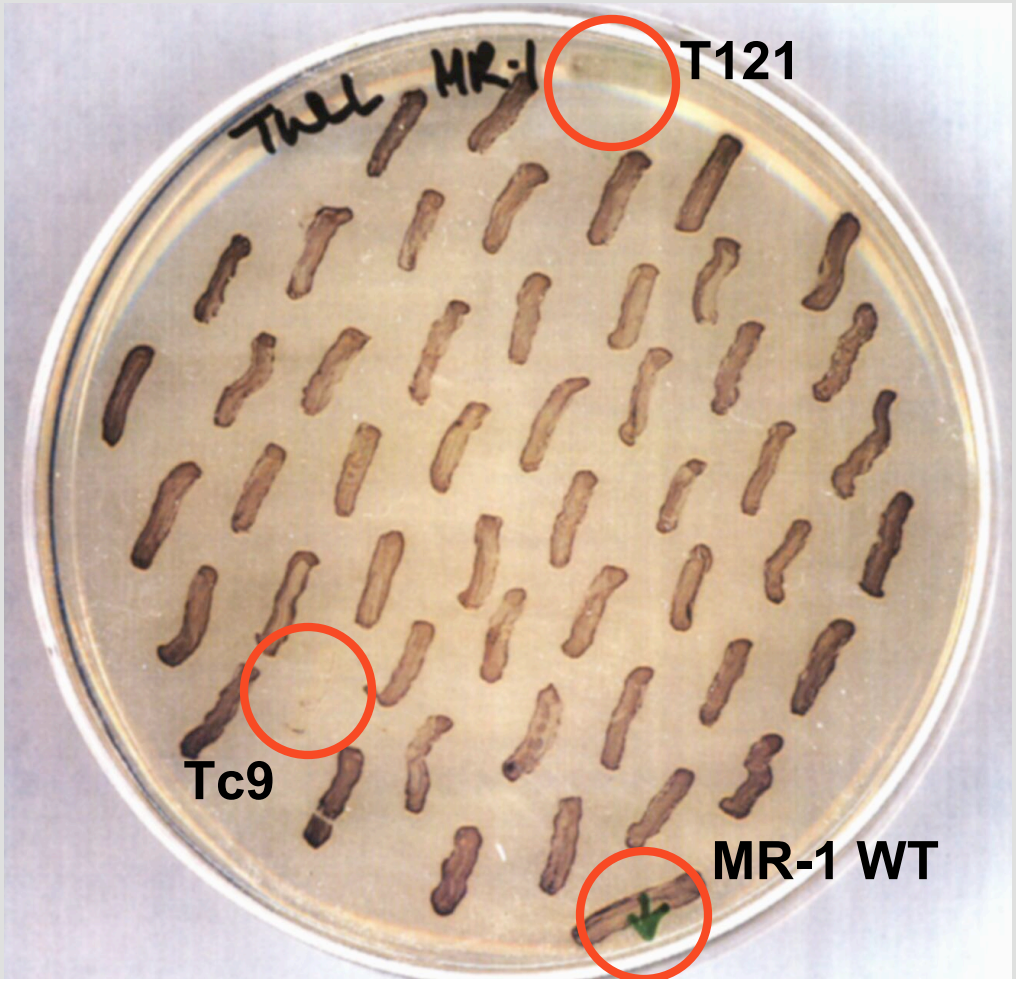
Practical Applications

Overly reduced aquifer (Fe2+, S2-) inhibits biosynthesis of *c*-type cytochromes (required for U(VI) reduction) by altering the periplasmic redox condition required for heme lyase (CcmF) activity

Overly oxidized aquifer?

Technetium reduction

Tc^{7+} clear, soluble ⟶ Tc^{4+} black precipitate



5,000 screened, 6 identified

Tcr mutants tested for anaerobic growth capability in liquid culture

Anaerobic Respiratory Capability of Tc(VII) Reduction-Deficient Mutants

Electron Acceptor / Electron Donor	Tc(VII)			O_2		Fum.		DMSO		TMAO		SO_3^-		$S_2O_3^-$		U(VI)	NO_2^-			NO_3^-			Fe(III)			Fe(III)Cit			Mn(IV)			Mn(III)		
	H	L	F	L	F	L	F	L	F	L	F	L	F	L	F	H	H	L	F	H	L	F	H	L	F	H	L	F	H	L	F	H	L	F
MR-1	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T121	-	-	-	+		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tc-9	-	-	+	+		+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Tc-13	-	-	-	+		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tc-14	-	-	-	+		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tc-16	-	-	-	+		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tc-17	-	-	-	+		-	-	-	-	+	+	+	+	-	-	+	+	+	+	+	+	-	+	-	-	+	+	+	+	-	+	+	+	-
Tc-18	-	-	+	+		+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+

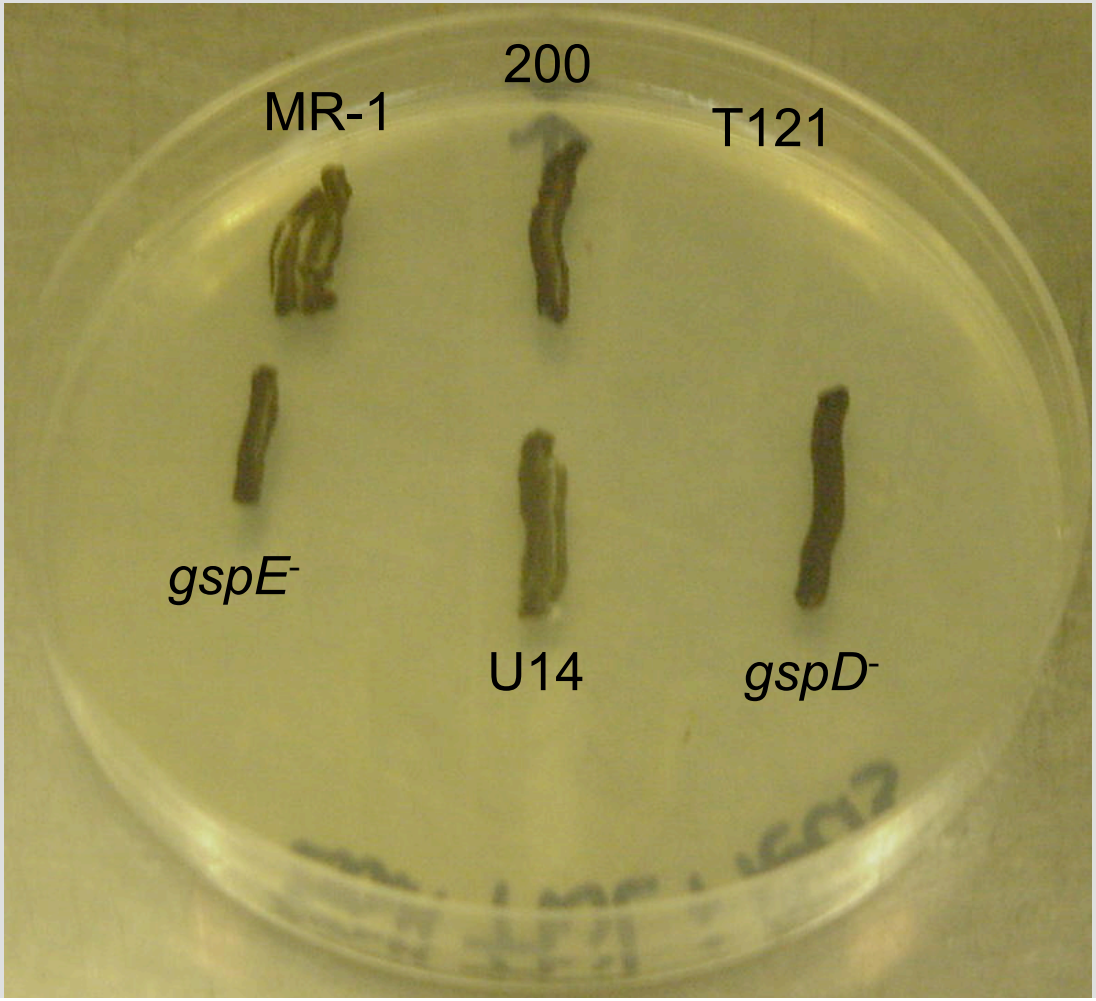
- Tc9 and Tc18 are unable to reduce Tc(VII) with H_2 as electron donor, but retain Tc(VII) reduction activity with formate

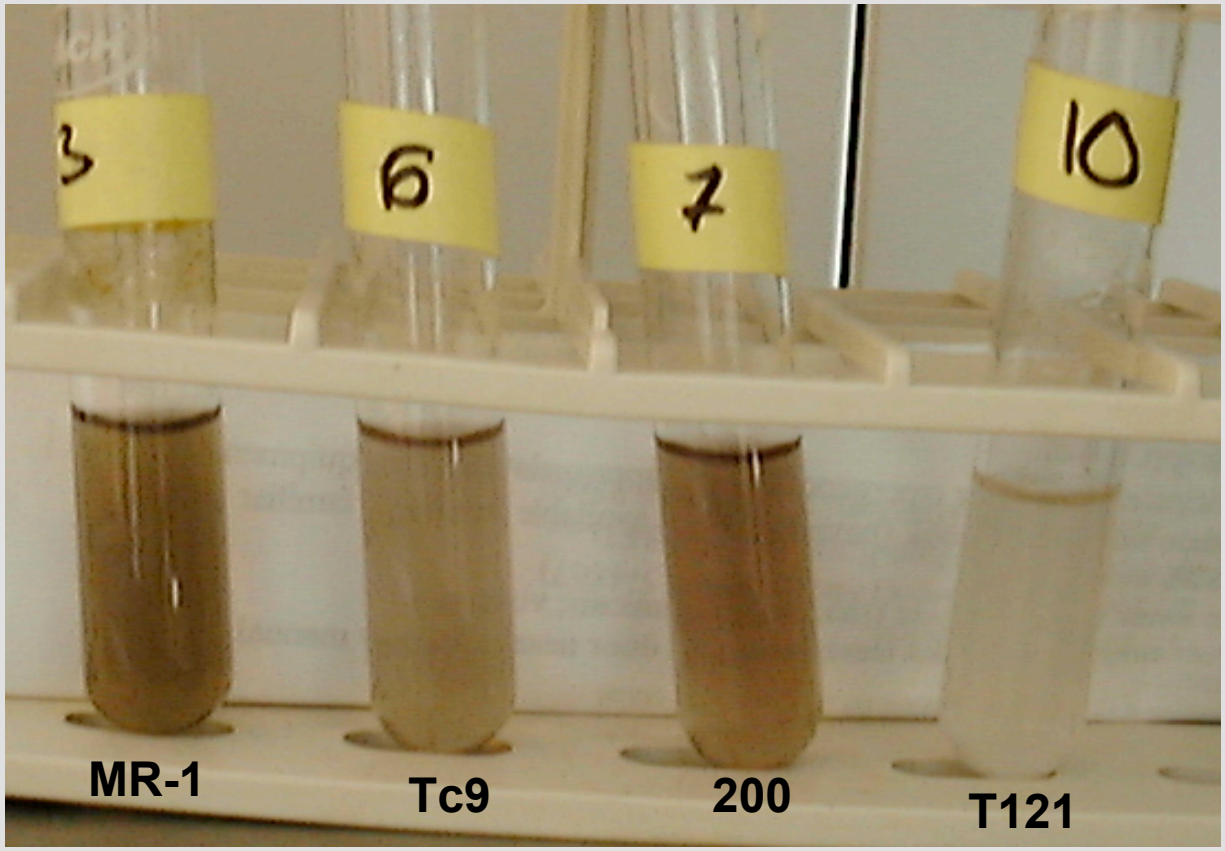
- Tc9 and Tc18 are also unable to reduce NO_3^-, Mn(III) or U(VI) with H_2 as electron donor:

Genetic complementation analysis to identify Tc(VII) reduction genes

Technetium reduction

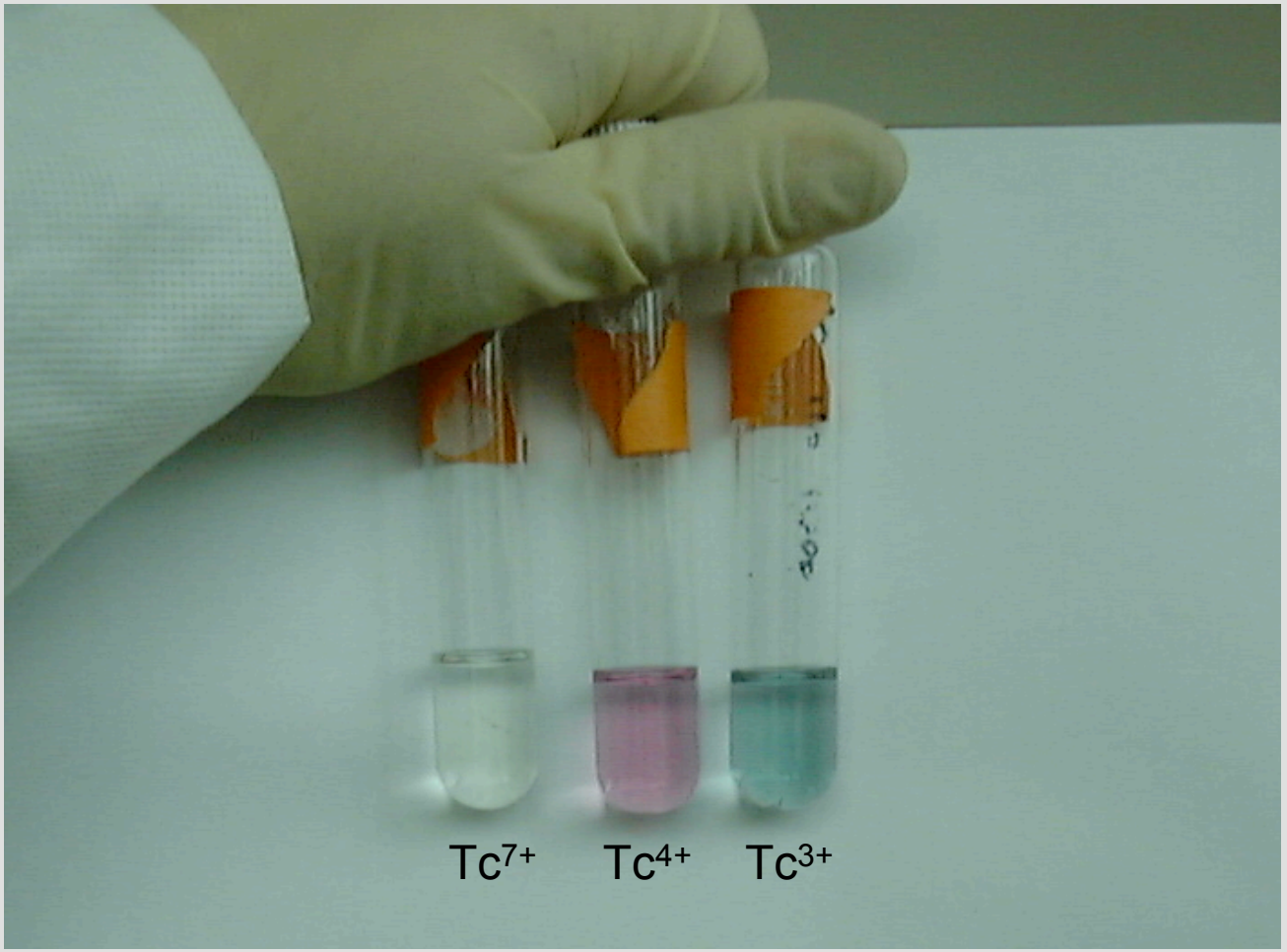
Tc^{7+} clear, soluble → Tc^{4+} black precipitate





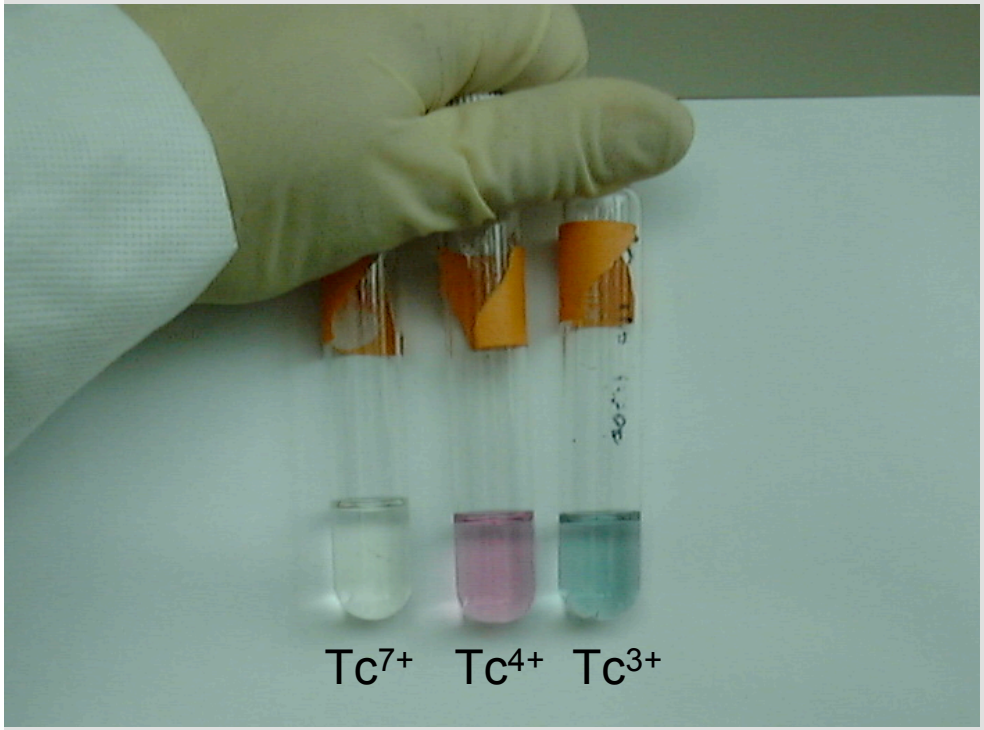
Tc(VII) reduction to Tc(IV) in anaeroibc salt buffer, but if buffer contains 50 mM bicarbonate…..

Tc(VII) is reduced step-wise to soluble Tc(III) via a soluble Tc(IV) intermediate

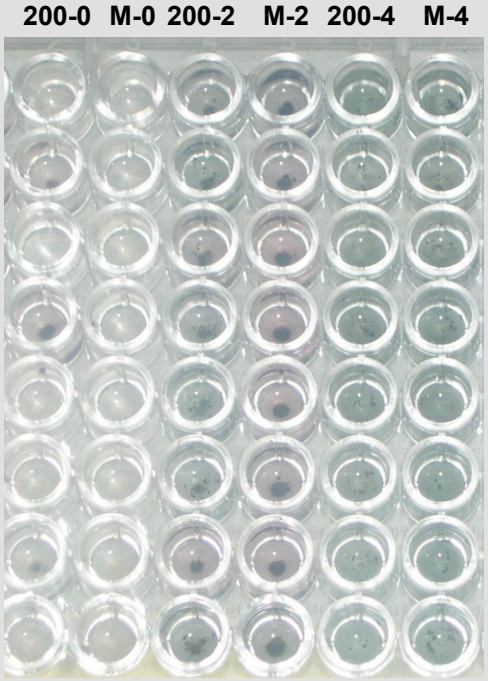


Time (hr) = 0 24 72

pH=8, bicarb buffer, 5% H2, 250 µM ammonium pertechnetate



Time (hr) = 0 24 72



Rapid screen for identification of Tc(IV) reduction-deficient mutants:

Mutagenize and identify mutants which remain pink-colored

WORKING HYPOTHESIS:

Tc(VII) reduction proceeds step-wise via two successive electron transfer reactions catalyzed by separate Tc(VII) and Tc(IV) reductases

Tc(VII) Reduction:

$Tc^{7+} \longrightarrow Tc^{4+} \longrightarrow Tc^{3+}$

Tc(III) Oxidation:

$Tc^{3+} \longrightarrow Tc^{4+} \longrightarrow Tc^{7+}$

Practical Applications

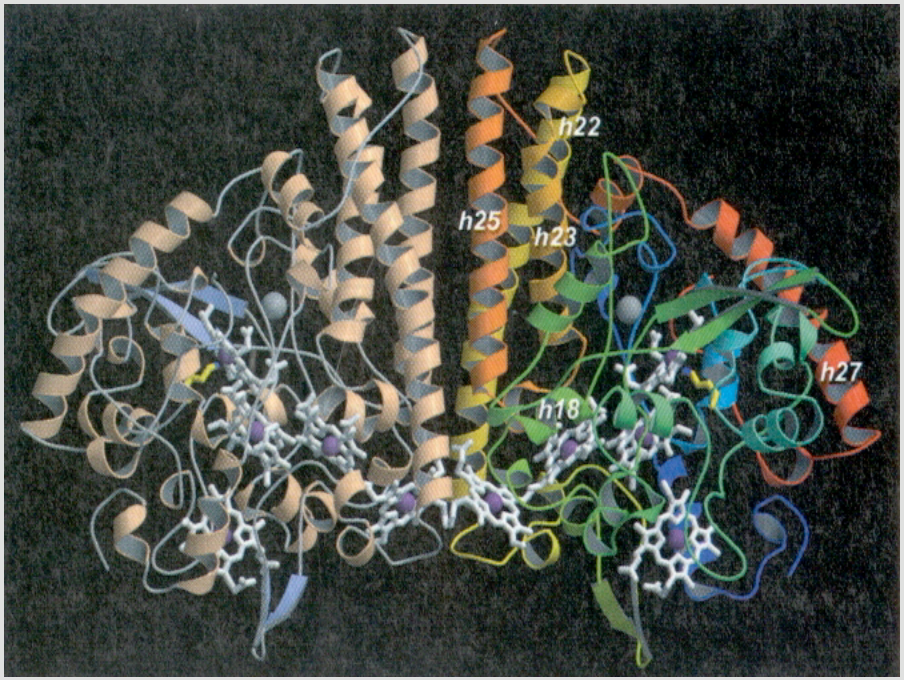
Uranium

Redox poise of aquifer environment affects biosynthetic pathway for synthesis of *c*-type cytochromes required for U(VI) reduction

Technetium

In presence of bicarbonate, soluble Tc(VII) is reduced step-wise to a soluble Tc(III) end-product via a soluble Tc(IV) intermediate

Crystal structure of *Sulfurospirillum deleyianum* NrfA



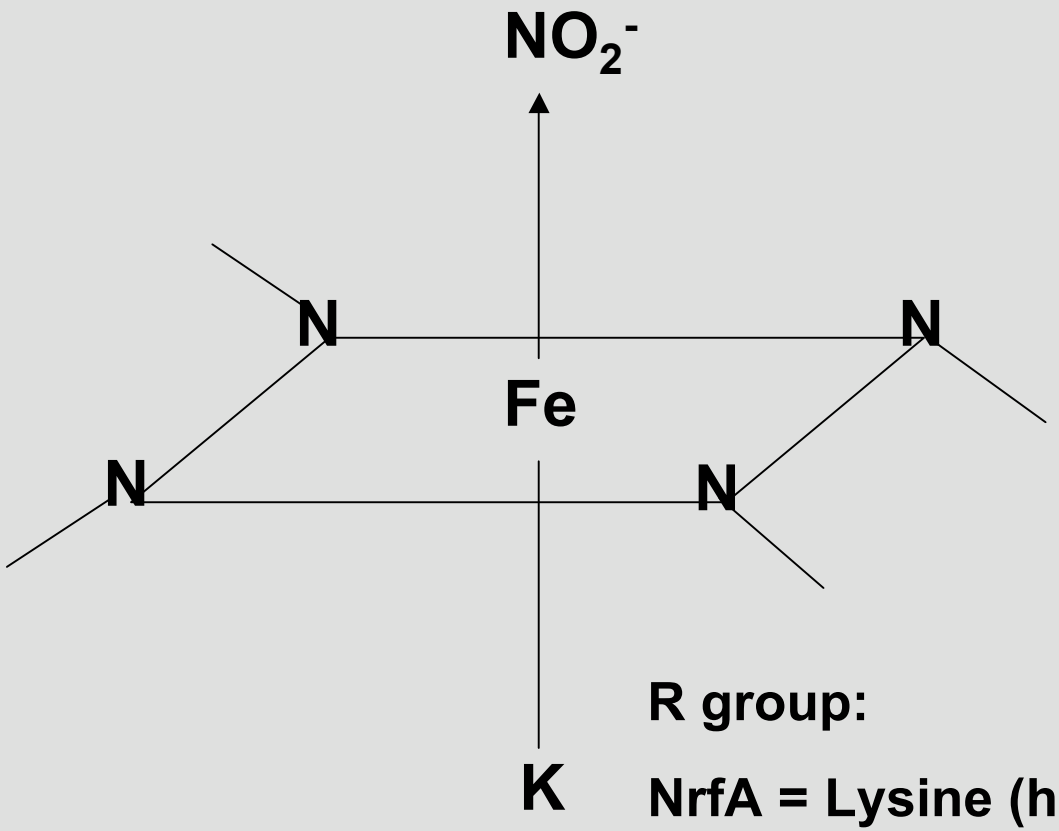
- homodimer with 5 close-packed hemes per monomer

- orientation of 5 heme groups is nearly identical to hydroxylamine oxidoreductase of *Nitrosomonas europaea* (NH_2OH oxidized to NO_2^-)

- active site heme of NrfA is K-coordinated as opposed to H-coordinated HAO in *N. europaea* (electron donor as opposed to electron sink)

- substitute H for K in NrfA (Heme Fe has more negative potential), NrfA is dead: e- transfer from 4 other hemes can not occur because redox potential is too negative.

NrfA Heme Ligation

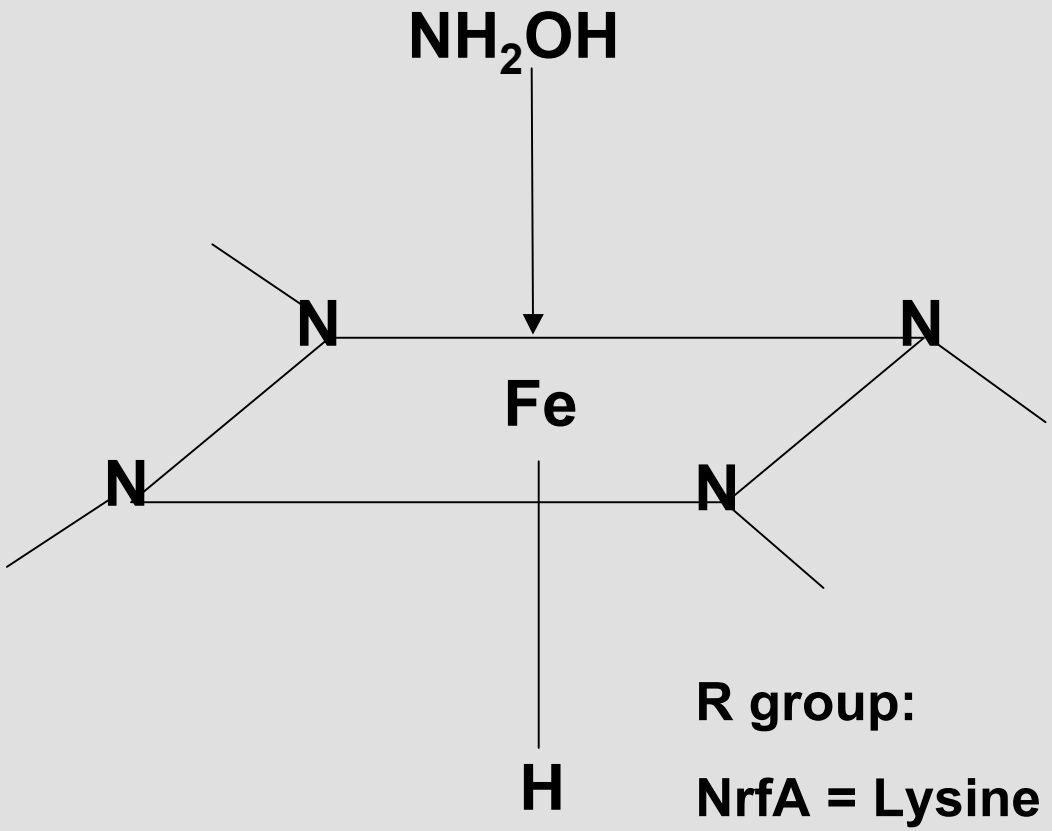


R group:

NrfA = Lysine (heme Fe is more +)

HAO = Histidine (heme Fe is more -)

HAO Heme Ligation



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