CYTOCHROME *C* MATURATION AND REDOX HOMEOSTASIS IN URANIUM-REDUCING BACTERIUM *SHEWANELLA PUTREFACIENS*

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CYTOCHROME *C* MATURATION AND REDOX HOMEOSTASIS IN URANIUM-REDUCING BACTERIUM *SHEWANELLA PUTREFACIENS*

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This thesis is dedicated to my mother Judith, father Forrest and sister Therese. Thank you for your immeasurable love, patience and support.

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LIST OF SYMBOLS AND ABBREVIATIONS

C3965	*S. oneidensis* MR-1R cytochrome c_3 insertional mutant (*cctA*::Gm^R)
Ccm	System I cytochrome *c* maturation pathway
CcmB	Permease subunit of the CcmAB ABC-type transporter required for System I cytochrome *c* maturation
CCMB1	*S. putrefaciens* 200R *ccmB* point mutant (H108Y), also designated Urr14
Δ*ccmB*	*S. putrefaciens* 200R in-frame *ccmB* deletion mutant
DNA	deoxyribose nucleic acid
E. coli	*Escherichia coli*
E'_0	Reduction potential at standard conditions
FeRB	iron-reducing bacteria
LB	Luria-Bertani growth medium
NRFA1	*S. oneidensis* MR-1R nitrite reductase NrfA insertional mutant (*nrfA*::pCRNR)
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
SM	synthetic minimal medium
S. oneidensis	*Shewanella oneidensis* strain MR-1
S. putrefaciens	*Shewanella putrefaciens* strain 200
SRB	sulfate-reducing bacteria

TEA	terminal electron acceptor
Tris	Tris(hydroxymethyl)aminomethane
UV-VIS	ultra violet – visible electromagnetic spectrum
w/v	weight per unit volume
DMSO	Dimethyl sulfoxide
TMAO	Trimethylamine oxide
Fe(III)-cit	Ferric citrate
Mn(III)-pyro	Manganese(III) pyrophosphate
URB	Uranium-reducing bacteria
Urr	Uranium reduction-deficient phenotype
Urr14	*S. putrefaciens* 200R *ccmB* point mutant (H108Y), also designated CCMB1

SUMMARY

Microbial metal reduction contributes to biogeochemical cycling, and reductive precipitation provides the basis for bioremediation strategies designed to immobilize radionuclide contaminants present in the subsurface. Facultatively anaerobic γ-proteobacteria of the genus *Shewanella* are present in many aquatic and terrestrial environments and are capable of respiration on a wide range of compounds as terminal electron acceptor including transition metals, uranium and transuranics. *S. putrefaciens* is readily cultivated in the laboratory and a genetic system was recently developed to study U(VI) reduction in this organism. U(VI) reduction-deficient *S. putrefaciens* point mutant Urr14 (hereafter referred to as CCMB1) was found to retain the ability to respire several alternate electron acceptors. In the present study, CCMB1 was tested on a suite of electron acceptors and found to retain growth on electron acceptors with high reduction potential (E'_0) [O_2, Fe(III)-citrate, Mn(IV), Mn(III)-pyrophosphate, NO_3^-] but was impaired for anaerobic growth on electron acceptors with low E'_0 [NO_2^-, U(VI), dimethyl sulfoxide, trimethylamine *N*-oxide, fumarate, γ-FeOOH, SO_3^{2-}, $S_2O_3^{2-}$]. Genetic complementation and sequencing analysis revealed that CCMB1 contained a point mutation (H108Y) in a CcmB homolog, an ABC transporter permease subunit required for *c*-type cytochrome maturation in *E. coli*. The periplasmic space of CCMB1 contained low levels of cytochrome *c* and elevated levels of free thiol equivalents (-SH), an indication that redox homeostasis was disrupted. Anaerobic growth ability, but not cytochrome *c* maturation activity, was restored to CCMB1 by adding exogenous disulfide bond-containing compounds (e.g., cystine) to the growth medium. To test the possibility

that CcmB transports heme from the cytoplasm to the periplasm in *S. putrefaciens*, H108 was replaced with alanine, leucine, methionine and lysine residues via site-directed mutagenesis. Anaerobic growth, cytochrome *c* biosynthesis or redox homeostasis was disrupted in each of the site-directed mutants except H108M. The results of this study demonstrate, for the first time, that *S. putrefaciens* requires CcmB to produce *c*-type cytochromes under U(VI)-reducing conditions and maintain redox homeostasis during growth on electron acceptors with low E'_0. The present study is the first to examine CcmB activity during growth on electron acceptors with widely-ranging E'_0, and the results suggest that cytochrome *c* or free heme maintains periplasmic redox poise during growth on electron acceptors with $E'_0 < 0.36$V such as in the subsurface engineered for rapid U(VI) reduction or anoxic environments dominated by sulfate-reducing bacteria. A mechanism for CcmB heme translocation across the *S. putrefaciens* cytoplasmic membrane via heme coordination by H108 is proposed.

CHAPTER 1

INTRODUCTION

Uranium is the most abundant naturally occurring actinide. Nuclear energy and weapons development during the 20th century elevated uranium concentrations in the biosphere. Uranium exists in the environment primarily in the U(VI) and U(IV) oxidation states and uranium mobility is highly dependent on the oxidation state. The U(VI) uranyl oxocation (UO_2^{2+}) is water soluble and mobile in the environment while the reduced U(IV) mineral uraninite ($UO_{2(s)}$) is sparingly soluble and less mobile. Microbial U(VI) reduction is therefore the basis of an alternative remediation strategy of uranium-contaminated environments, especially the terrestrial subsurface.

Despite the potential benefits of microbial U(VI) reduction as an effective bioremediation strategy, the molecular mechanism of microbial U(VI) reduction remains poorly understood. A variety of metal-reducing microorganisms reduce U(VI), including members of the genera *Desulfovibrio* (49, 67, 78), *Geobacter* (9), *Anaeromyxobacter* (68, 85), *Pyrobaculum* (33), *Thermus* (34), *Deinococcus* (22), *Clostridium* (21), *Desulfiosporosinus* (75) and *Shewanella* (23, 48, 80). Metal-reducing members of the genus *Shewanella* are attractive models for mechanistic studies of microbial U(VI) reduction because they are capable of growing anaerobically on a variety of compounds as terminal electron acceptor. In addition, the genome sequence of *S. oneidensis* and 14 other *Shewanella* species is available.

A genetic system to study U(VI) respiration by *S. putrefaciens* was recently developed (80, 81). A library of chemically-generated, U(VI) reduction-deficient point mutants were isolated on agar plates containing U(VI) as electron acceptor. U(VI) reduction-deficient mutants were identified by the absence of insoluble U(IV) on colony surfaces after anaerobic growth on U(VI)-amended agar medium. In the present study, the anaerobic growth rates of a U(VI) reduction mutant strain (CCMB1) were determined on a suite of electron acceptors with widely ranging E'_0. The mutated gene in CCMB1 was identified via genetic complementation and DNA sequencing. Site-directed mutagenesis was conducted to determine the molecular mechanism of CcmB, an ABC transporter subunit required by *S. putrefaciens* for a) U(VI) reduction, b) cytochrome *c* maturation and c) anaerobic respiration of electron acceptors with low E'_0.

Anaerobic respiration by bacteria

Microorganisms conserve the energy released in redox reactions by forming high-energy phosphoanhydride bonds. Adenosine-5'-triphosphate (ATP) (derived from adenosine-5'-diphosphate (ADP) and P_i) serves as the principal carrier of energy to drive biosynthetic reactions. Generation of adenosine-5'-triphosphate (ATP) from adenosine-5'-diphosphate (ADP) and P_i is a vital process of all living organisms. The two basic mechanisms of ATP generation are substrate level phosphorylation and electron transport chain-linked (oxidative) phosphorylation (52). Substrate-level phosphorylation is coupled to the oxidation of organic substrates and high-energy phosphoryl bonds are formed during the intermediate steps. Hydrolysis of the high-energy phosphoryl bond is coupled to transfer of the phosphate group to ADP. Electron transport chain-linked phosphorylation involves coupling ATP synthesis to the flow of electrons between

membrane-bound electron carriers. Electron flow proceeds from donors with more negative redox potentials to acceptors with more positive redox potentials. Respiratory chains generate ATP by linking electron flow between electron transport chain components to proton translocation and formation of a proton motive force and membrane potential (24, 27, 57). Respiratory chain components are localized in the cytoplasmic membrane of bacteria to allow a net flux of protons from the cytoplasm to the exterior of the cell. The outer envelope of gram-negative bacteria is composed of three layers: cytoplasmic membrane, peptidoglycan and outer membrane (52). The cell membrane is a phospholipid bilayer containing integral and membrane-associated proteins. The outer membrane is more commonly referred to as the lipopolysaccharide layer and is composed of phospholipids, polysaccharides and proteins. The outer membrane is permeable to small molecules transported through porins. The periplasmic space is the area confined by the exterior of the cytoplasmic membrane and interior of the outer membrane. The peptidoglycan layer confers strength and rigidity to the cell membrane and lies within the periplasmic space. The formation of a proton motive force and membrane potential requires that the cytoplasmic membrane (in bacteria) or inner membrane (in mitochondria) be impermeable to OH^- and H_3O^+.

In the chemiosmotic hypothesis (58), energy obtained from electron transport is coupled to proton translocation from the cytoplasm to the periplasmic space, thereby generating an electrochemical gradient across the cytoplasmic membrane. Controlled release of the protons back into the cell through a membrane-bound ATPase harnesses the electrochemical potential for ATP synthesis. The free energy of electron transfer is determined by the redox potential of the electron donor/acceptor pairs. Electron donors

such as nicotinamide-adenine dinucleotide (NADH) or flavin-adenine dinucleotide (FADH) may be produced by cytosolic catabolic reactions (e.g., glycolysis, citric acid cycle). In addition, exogenous organic or inorganic compounds may be taken up, oxidized and the resulting electrons donated directly to membrane bound respiratory systems.

A typical oxidation-reduction reaction may be described as follows:

$A_{red} \rightarrow A_{ox} + n$ electrons (oxidation reaction)

$B_{ox} + n$ electrons $\rightarrow B_{red}$ (reduction reaction)

The tendency for a redox reaction to proceed towards the reduction of the desired electron acceptor (B) is determined by the reduction potential at standard conditions (E'_0) of the individual reactions. E'_0 (given at 1 atm pressure, 25°C and pH 7 with respect to a hydrogen electrode) describes the tendency of a substance to act as an electron donor or acceptor. E'_0 for the major redox couples involved in microbial processes are displayed in the redox tower given in Figure 1.1. The stronger reductants are at the top of the tower and the stronger oxidants are at the bottom. The standard free energy of the reaction is a function of the difference between standard reduction potentials. For example, electron transport chain components mediating the reduction of oxygen by H_2 have E'_0 between those of $2H^+$ and oxygen. ΔE_0 for the reduction of oxygen (+0.82 V) by H_2 (-0.42 V) is 1.24 V and the standard free energy of change for the transfer of 2 electrons from H_2 to ½ O_2 is –237 kJ/mol (79):

(1.1) $\Delta G_0 = -nF\Delta E_0$

n = number of electrons transferred per mole of reactants

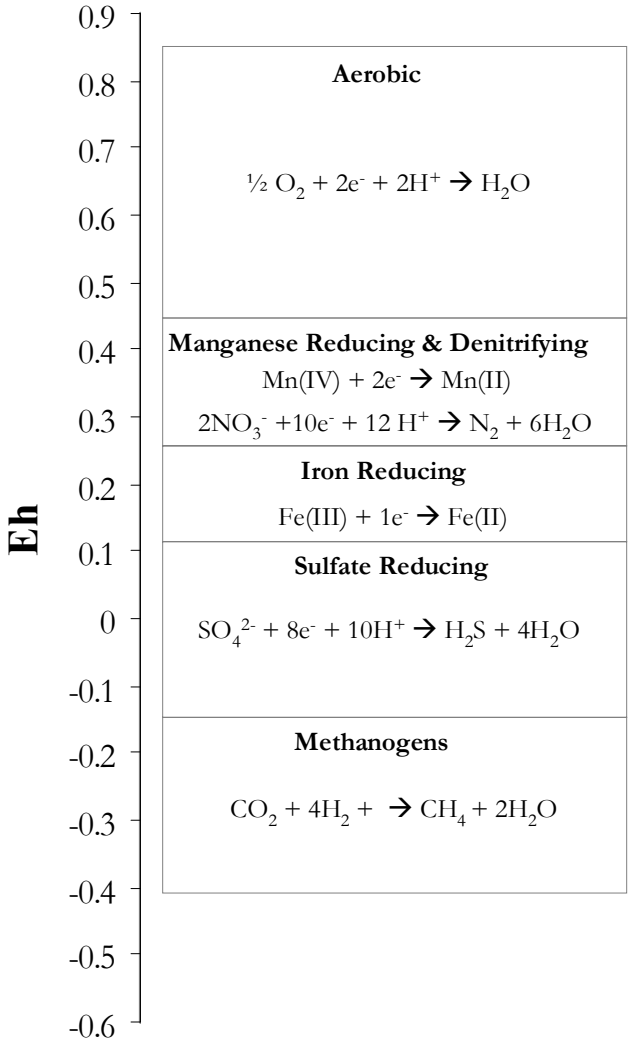

Figure 1.1. Ambient redox potential (Eh) vs. the predominant respiratory process in a redox-stratified environment.

F = Faraday constant = 96,485 Coulomb/mol

E. coli reduces various electron acceptors during respiration including oxygen, fumarate, NO_3^-, NO_2^-, dimethyl sulfoxide (DMSO) and trimethylamine oxide (TMAO) (35, 36). As *E. coli* shifts from aerobic to anaerobic respiration, changes are observed in cellular protein composition. The most profound changes are found in respiratory chain components, including modification of cytochrome content (74). These changes result in the expression of electron transport chain components with low reduction potential.

Electron transport chain components include dehydrogenases, flavoproteins, iron-sulfur proteins, quinones and cytochomes. Dehydrogenases are located at the beginning of the respiratory chain and serve as the primary oxidase of electron donors. *E. coli* contains several dehydrogenases, including formate, lactate, NADH and hydrogenase. Flavoproteins are enzymes with a covalently bound riboflavin moiety. The flavin prosthetic group is redox reactive and also translocates protons. The quionones are highly hydrophobic non-protein-containing molecules that link dehydrogenases and various electron transport chain components. Quinones contain isoprenoid side chains, rendering them lipid soluble. *E. coli* produces three types of quinones: methylnapthoquinone (menaquinone), dimethylmenaquinone, and ubiquinone. All three quinones serve as hydrogen atom acceptors and electron and proton donors (52).

Cytochromes are respiratory proteins usually involved in electron transfer at the terminal end of the electron transport chain (31, 41, 76). Cytochromes contain tetrapyrole heme prosthetic groups that each consists of an iron-complexed (chelated) porphyrin ring (Figure 1.2). There are four major cytochrome types: *a*, *b*, *c* and *d*. Axial heme iron coordination is the primary mechanism of heme attachment to cytochromes.

Amino acid side chains in cytochromes (e.g., histidine imidazole or methionine methylsulfanyl) provide axial heme ligation to one or both of the two remaining coordination spheres of heme iron. *c*-type cytochromes are the only cytochromes that covalently bind the porphyrin ring structure to the apoprotein. The porphyrin ring is attached via a thioether bond between the vinyl side chain of the heme and conserved cysteine residues (of the signature CXXCH motif) in the apocytochrome. Reduction potential and spectral properties of cytochromes are determined by the heme ligands, holo-cytochrome heme architecture and quarternary protein interactions (17, 54, 70, 82). The spectral properties of cytochromes are used to determine cytochrome content and composition in *E. coli*. Reduced cytochromes yield three absorption peaks, designated α (~552 nm), β (~524 nm) and γ (~419 nm). The peak in the α region is used to identify different cytochrome types (1, 8, 25, 88). Anaerobic respiration is actively studied in members of the genus *Shewanella*, facultatively anaerobic γ-proteobacteria that are able to grow on an extraordinary array of compounds as electron acceptor including O_2, NO_3^-, NO_2^-, DMSO, TMAO, fumarate, Fe(III), Mn(IV), SO_3^{2-}, $S_2O_3^{2-}$, $S_4O_6^{2-}$, and S^0. The presence of many *c*-type cytochromes with wide-ranging midpoint redox potential in *Shewanella* spp. (e.g., forty-two *c*-type cytochromes are predicted in the *S. oneidensis* genome) are thought to confer, in part, respiratory versatility and may provide a competitive advantage at the oxic-anoxic interface in sediments or in dynamic redox environments (14, 15, 28, 77). *c*-type cytochromes are localized to the outer face of the outer membrane of *Shewanella* to reduce electron acceptors with low solubility such as Fe(III) and Mn(IV) (5, 18, 63). *c*-type cytochromes are also part of branched electron transport pathways in *S. oneidensis*. For example, cytochrome CymA is required by *S.*

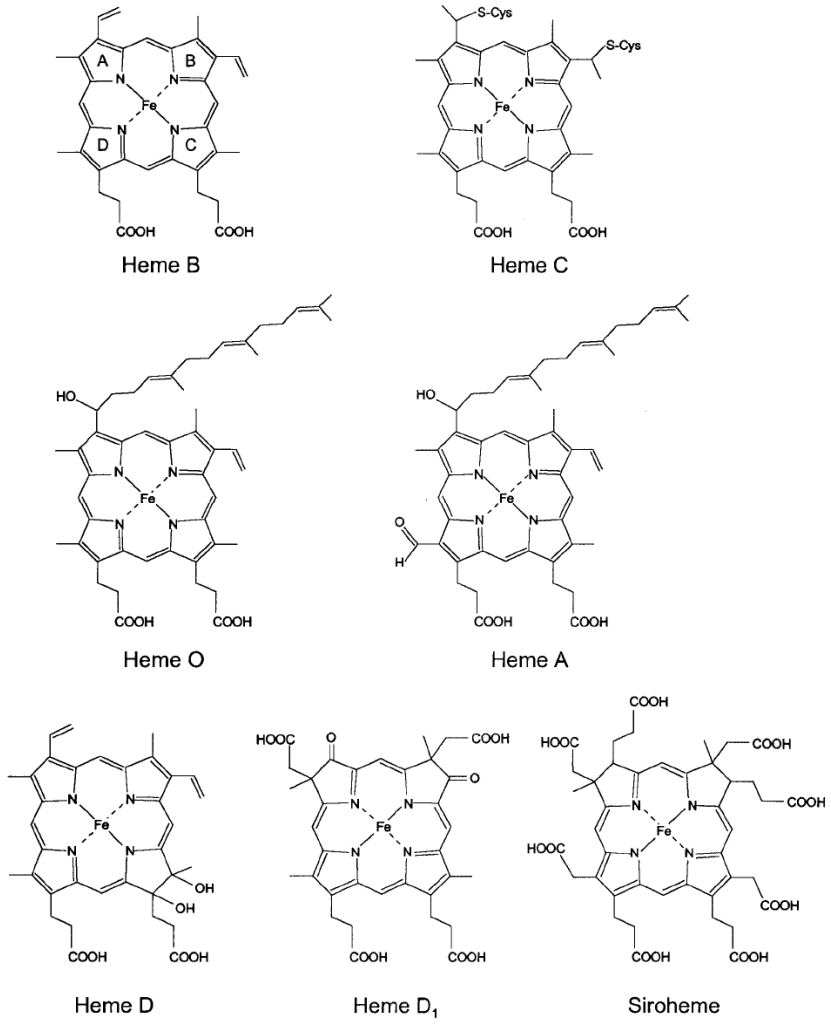

Figure 1.2. Heme cofactors of bacterial cytochromes. In reduced heme, Fe is present as Fe^{2+}; in oxidized heme, it is present as Fe^{3+}. Note that heme C is bound covalently by two thioether bonds to cysteinyl residues of the polypeptide. The nomenclature of porphyrin rings A to D is identified for heme B (76).

oneidensis for anaerobic growth on Fe(III), NO_3^-, NO_2^-, DMSO, fumarate and As(V) (61, 62, 72) (Figure 1.3).

Uranium geochemistry

Uranium has the greatest atomic mass of the naturally-occurring elements on Earth. Uranium is a member of the actinide series of elements and undergoes α radioactive decay. Three isotopes occur naturally (^{234}U, ^{235}U and ^{238}U) with isotopic prevalence of 0.0055%, 0.7200% and 99.2745%, respectively (71). The half-life of the most prevalent isotope (^{238}U) is approximately 4.7 x 10^9 years. Uranium is found in Earth's crust at an abundance of 3 μg/g, approximately as abundant as silver and boron but more abundant than tin or mercury (39). Uranium concentrations in natural groundwater rarely exceed 20 μg/L, but may range up to 120 μg/L in locations near uranium ore deposits. Uranium is found in several oxidation states, including U^{3+}, U^{4+}, U^{5+}, U^{6+} and U^{7+}. U^{4+} and U^{6+} are the most stable. In oxidizing environments and at circumneutral pH, uranium is found in the 6+ oxidation state as the uranyl ion (UO_2^{2+}). In reducing environments and at circumneutral pH, uranium is found in the 4+ oxidation state as uraninite (UO_2). At pH > 5, U(IV) is sparingly soluble (10^{-8} M) while U(VI) is highly soluble (10^{-4} M) (69). Complexation reactions influence U(VI) mobility in subsurface environments. U(VI) forms complexes with inorganic ligands (e.g., hydroxyl, carbonate, phosphate, sulfate) and organic ligands (e.g., acetate, malonate, citrate, oxalate); the carbonate complexes are particularly important in natural systems open to atmospheric CO_2. In a typical groundwater system (CO_2 partial pressure of $10^{-2.0}$ bar and pH > 5), carbonate may replace the hydroxyl functional group (-OH) in aqueous uranyl complexes. Replacement of -OH with carbonate increases U(VI) solubility and limits

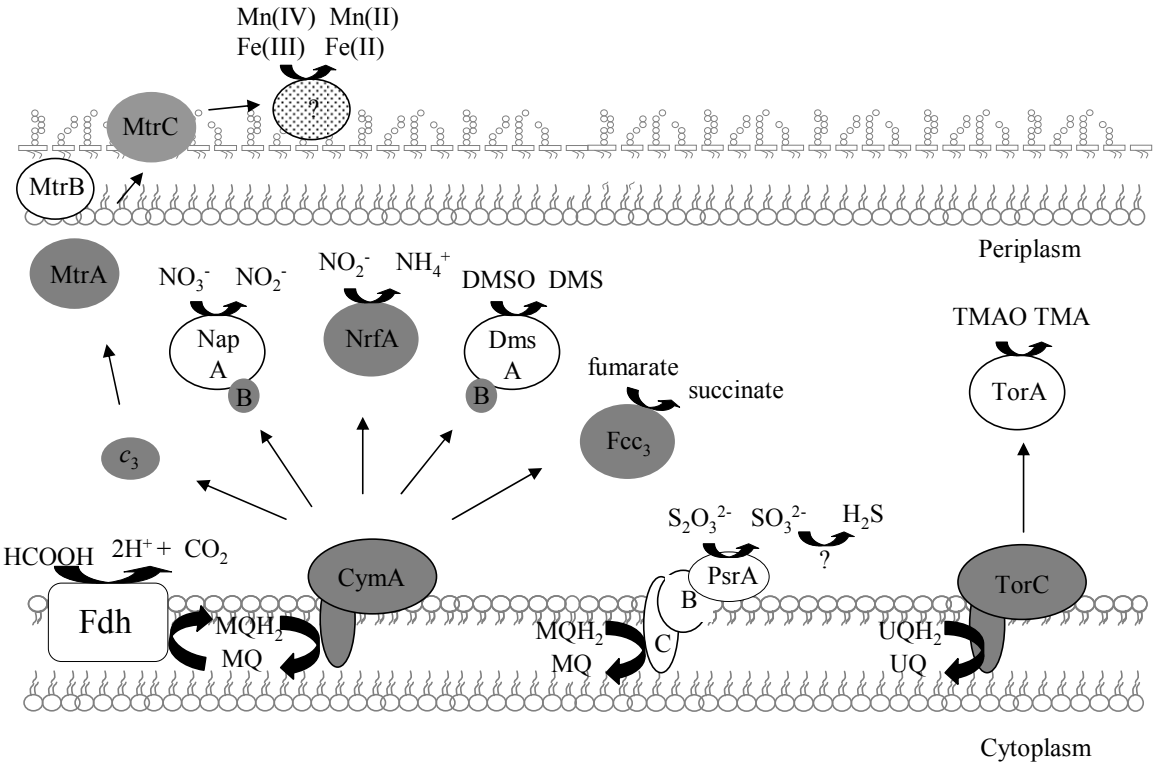

Figure 1.3. Branched electron transport chains in *S. oneidensis*. *c*-type cytochromes are shaded (72).

adsorption (thereby enhancing uranium mobility) (39). Carbonate also facilitates U(IV) oxidation reactions by enhancing U(VI) dissolution and detachment from U(IV) surfaces under aerobic conditions (13). U(IV) on the other hand, is and relatively non-reactive with complexants. The uranium Eh-pH diagram (Figure 1.4) illustrates uranium oxidation state and speciation over a range of ambient redox potential (Eh) and pH in a typical groundwater system. Soluble uranyl complexes are found at high Eh while insoluble uraninite is favored at low Eh. Uranyl ion and uranyl complexes with hydroxyl or hydroxyl carbonate are stable at pH < 7 while uranyl dicarbonate or uranyl tricarbonate complexes are stable at pH > 7.

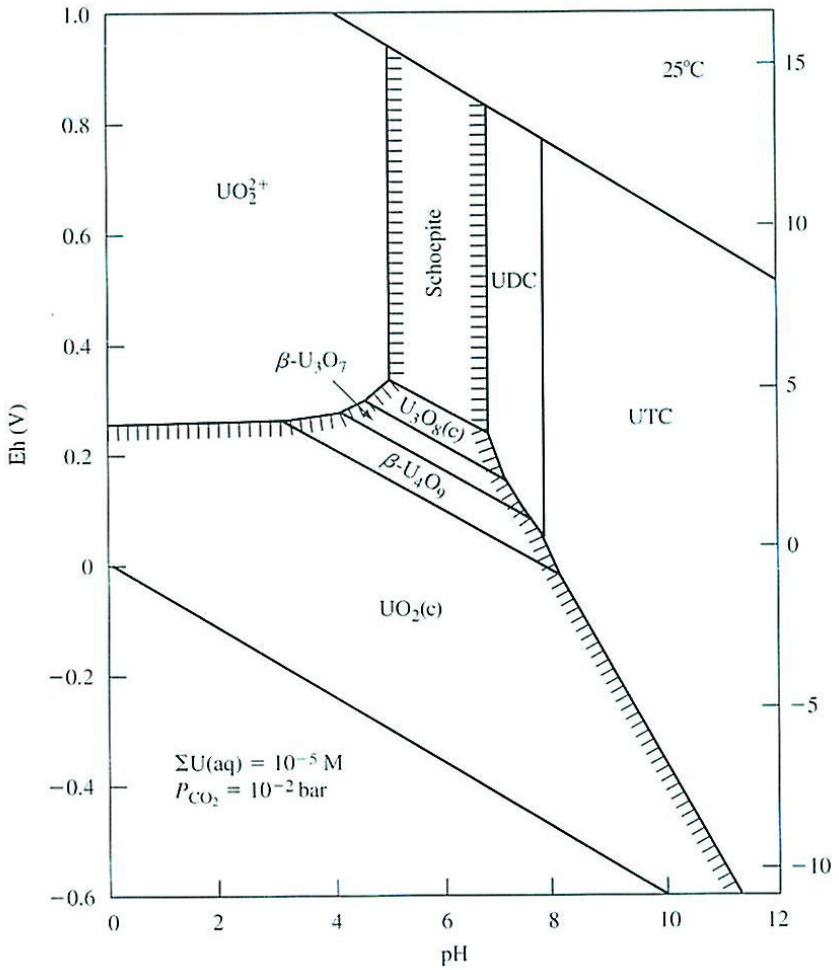

Figure 1.4. Eh-pH diagram of aqueous uranium in the system U-O_2-CO_2-H_2O at 25°C and 1 bar total pressure. Solid/aqueous boundaries (stippled) for U=10^{-5}M, UDC=$UO_2(CO_3)_2^{2-}$ and UTC=$UO_2(CO_3)_3^{4-}$. Schoepite is insoluble U(VI) (UO_3) (39).

Sources of uranium contamination and toxicity

Waste uranium mine and mill tailings have accumulated during the past six decades due to the demand for fissile ^{235}U in nuclear reactors. Since only 0.72% of uranium ore consists of ^{235}U, the remaining isotopes must be removed. The uranium isotopes are physically separated and the enriched ^{235}U is used as fuel for nuclear reactions. Depleted uranium is stored or incorporated into ship keel weights and aircraft counterbalances. Uranium is extracted from open cut and underground mines and subsurface uranium-containing minerals are leached *in situ* with sulfuric acid or bicarbonate (42). During milling operations, uranium is further purified from the ore body by crushing, grinding, extracting with acid, bicarbonate and kerosene, precipitating with ammonia, and drying the resulting yellowcake (U_3O_8) product (26). The waste ore slurry is pumped to a tailings dam. U_3O_8 is then converted to gaseous uranium hexafluoride (UF_6) and isotopes of different mass are separated via ultracentrifugation. Enriched $^{235}UF_6$ is converted to $^{235}UO_2$ by vaporization and hydrolysis to $^{235}UO_2F_2$, followed by H_2-catalyzed reduction at high temperature. Enriched uraninite is pressed and sintered to form ceramic pellets and packaged into fuel rods. Environmental contamination may occur following a breach at any stage of uranium processing. *In situ* leach mining is perhaps most problematic due to immediate mobilization of soluble U(VI) into the groundwater (60). Soluble U(VI) may also enter the subsurface from compromised tailings ponds or waste storage drums. The uranium concentration in groundwater at underground mines can range up to 400 μg/L and leachates from mill tailings often contain 10-20 mg/L uranium (39).

Terrestrial and anthropogenic inputs from rivers contributes to elevated concentrations of uranium in marine environments (42). Uranium concentrations range from 2 to 3.7 μg/L in seawater (39). Uranium is immobilized under reducing conditions in marine sediments, where up to 70% of the U(VI) input is immobilized as U(IV) (89). In addition to enzymatic microbial U(VI) reduction, H_2S, and CH_4 sorbed to Fe^{3+}-oxide mineral surfaces and clays, chemically reduce U(VI) in sediments and Fe^{3+}- and SO_4^{2-}-reducing bacteria may participate in these processes by supplying Fe^{2+} and H_2S, respectively (43, 65). U(VI) may be re-oxidized, however, when oxygenated overlying water is introduced via physical disturbances such as convection or bioturbation. U(IV) may also be chemically oxidized by Fe^{3+} or Mn^{4+} and the products of Fe^{2+}- and Mn^{2+}-oxidizing bacteria may supply these reactants (12, 44, 73). U(VI) complexes may also adsorb to Fe^{3+}-oxide surfaces without reduction thereby removing the uranium from the aqueous phase (38).

Uranium is considered a radiological hazard that may increase the incidence of cancer, but the greatest risk to public health is chemical toxicity. Ingested uranium precipitates in kidney glomerulous tubules causing renal failure (30). Uranyl ascorbate complexes may cleave the phosphodiester backbone of supercoiled DNA, however, it is not yet known if uranium-induced DNA hydrolysis is mutagenic in humans (87). Uranium mine workers may inhale toxic uranium dust and receive harmful levels of exposure to the uranium radioactive decay product radon. The U.S. Environmental Protection Agency (EPA) has set a maximum contaminant level (MCL) for drinking water at 30 μg/L total uranium. As a result, the U.S. Department of Energy (DOE) is

charged with remediating over 7,000 uranium-contaminated sites, including an estimated 1.7 trillion gallons of groundwater and 40 million cubic meters of soil (10).

Uranium remediation strategies

Efforts to remove uranium and prevent U(VI) mobility in the environment have been largely unsuccessful. Pump-and-treat methods pump uranium-contaminated water to the surface where chemical processes (e.g., ion exchange) are employed to remove U(VI) from solution. Common adsorbents in such systems include amidoxime (29) and dihydroxyazobenzene derivatives (40). Costs associated with pumping groundwater, chemical treatment and soil excavation may be prohibitive, especially when handling low-level and wide-spread uranium waste. The resulting contaminant levels after such treatments often fail to meet regulatory compliance. For these reasons, pump-and-treat and chemical treatments have not been applied to contaminated environments containing low-level and widespread waste.

Innovative technologies are under development to remediate uranium waste i*n situ*. Zero valent iron (Fe^0) is an example of a permeable reactive barrier to prevent U(VI) migration through the vadose zone (59). U(VI) may adsorb to iron corrosion products or undergo surface-catalyzed reduction to U(IV) followed by U(IV) surface precipitation (64). Phytoremediation is based on the ability of plants to remove U(VI) from arable soil by accumulating uranium in roots (a process termed rhizofiltration) (20). U(VI) may also be removed via bioadsorption to plant, fungi, and microbial biomass. U(VI) adsorbs to immobilized microorganisms such as *Pseudomonas* and *Bacillus* and is subsequently eluted with a carbonate/EDTA solution. The binding capacity of the cells is regenerated, and the biosorbing columns are reused (53). *Citrobacter, Deinococcus,*

Rahnella, and *Bacillus* display organophosphate activity that forms the basis of a promising strategy to immobilize U(VI) in oxic environments (3, 4, 32, 50, 51, 56). Phosphatases localized to the outer aspect of the outer membrane cleave extracellular organic phosphate and the inorganic phosphate combines with U(VI) forming an insoluble mineral such as autunite.

Microbial uranium reduction

Microbial U(VI) reduction has recently received attention as an attractive alternate strategy for remediation of uranium-contaminated subsurface environments. A variety of metal-reducing microorganisms reduce U(VI), including members of the genera *Desulfovibrio* (49, 67, 78, 83), *Geobacter* (9), *Anaeromyxobacter* (46, 85) *Pyrobaculum* (33), *Thermus* (34), *Deinococcus* (22), *Clostridium* (21), *Desulfiosporosinus* (75) and *Shewanella* (23, 48, 80).

The suitability of U(VI) as a terminal electron acceptor is highly dependent on the E'_0 of available uranyl species. U(VI) is commonly found as uranyl ion (UO_2^{2+}), and UO_2^{2+} readily forms -hydroxy and -carbonate complexes at circumneutral pH. Complexes containing carbonate generally have higher E'_0 than the $UO_2^{2+}{}_{(aq)}/UO_{2(s)}$ couple and uranyl carbonate complexes containing calcium have lower E'_0 than the $UO_2^{2+}{}_{(aq)}/UO_{2(s)}$ couple. The following half-reactions demonstrate the effect of complexation on E'_0:

(1.2) $UO_2(CO_3)_3^{4-} + 3H^+ + 2e^- \rightarrow UO_{2(s)} + 3HCO_3^-$ $E'_0 = -17$ mV

(1.3) $UO_2(CO_3)_2^{2-} + 2H^+ + 2e^- \rightarrow UO_{2(s)} + 2HCO_3^-$ $E'_0 = -62$ mV

(1.4) $UO_2^{2+}(aq) + 2e^- \rightarrow UO_{2(s)}$ $E'_0 = -70$ mV

(1.5) $CaUO_2(CO_3)_3^{2-} + 3H^+ + 2e^- \rightarrow UO_{2(s)} + 3HCO_3^- + Ca^{2+}$ $E'_0 = -76$ mV

(1.6) $UO_2CO_3 + 6H^+ + 2e^- \rightarrow UO_{2(s)} + HCO_3^-$ $E'_0 = -78$ mV

(1.7) $Ca_2UO_2(CO_3)_3 + 3H^+ + 2e^- \rightarrow UO_{2(s)} + 3HCO_3^- + 2Ca^{2+}$ $E'_0 = -181$ mV

where pH=7.4, $P_{CO2}=10^{-3.5}$, U(VI)=10^{-8}M, $Ca^{2+}=10^{-1.5}$M (6, 19, 39, 84).

Uranyl tricarbonate has the highest E'_0 of the species in equations 1.2-1.6 and is theoretically the most favorable electron acceptor. Uranyl complexes containing calcium have lower E'_0 and are reduced at lower rates (7). Uranyl reduction coupled to hydrogen or organic carbon oxidation is thermodynamically favorable for microbial respiration. Free energy yield is proportional to the $\Delta E'_0$ between the two half-reactions (according to equation 1.1):

(1.8) $H_2 + UO_2(CO_3)_3^{4-} + H^+ \rightarrow UO_{2(s)} + 3HCO_3^-$ $\Delta G_0 = -77$ kJ/mol

(1.9) $HCOOH + UO_2(CO_3)_3^{4-} + H^+ \rightarrow CO_2 + UO_{2(s)} + 3HCO_3^-$ $\Delta G_0 = -77$ kJ/mol

(1.10) $¼Acetate+UO_2(CO_3)_3^{4-}+H_2O+¾H^+ \rightarrow UO_{2(s)}+3½HCO_3^-$ $\Delta G_0 = -51$ kJ/mol

(1.10) $Ethanol + UO_2(CO_3)_3^{4-} \rightarrow Acetaldehyde + 3HCO_3^-$ $\Delta G_0 = -35$ kJ/mol

(1.11) $Lactate+UO_2(CO_3)_3^{4-}+2H^+ \rightarrow Pyruvate+UO_{2(s)}+3HCO_3^-$ $\Delta G_0 = -33$ kJ/mol

Assuming the thermodynamic efficiency of electron transport is 42% and the free energy required to synthesize 1 mole of ATP is approximately 32 kJ/mole, uranyl tricarbonate respiration is favorable by coupling with reductants with E'_0 below ethanol ($E'_0 = -0.2$ V).

Organic or inorganic ligands bound to U(VI) dramatically affect uranium solubility and bioavailability for reduction. For example, U(VI) is bound by citrate with varying strength as a function of pH (66). *Desulfovibrio desulfuricans* more rapidly reduces U(VI) bound to monodentate aliphatic complexes such as acetate, while *Shewanella alga* more rapidly reduces U(VI) bound to multidentate aliphatic complexes such as malonate, citrate and oxalate (23). These differences may reflect different

molecular mechanisms of U(VI) reduction by SRB and FeRB, such as a requirement for ligand exchange between the U(VI) substrate and terminal reductase. Microbial U(VI) reduction is also inhibited by competitive terminal electron acceptors. In carbonate-containing, redox-stratified groundwater at circumneutral pH, electron acceptors with E'_0 greater than –17 mV (e.g., O_2, Mn(IV), NO_3^-, Fe(III)) are reduced prior to $UO_2(CO_3)_3^{4-}$. On the other hand, U(VI) is reduced prior to SO_4^{2-} and CO_2.

Recent field experiments demonstrate the utility of microbial U(VI) reduction for bioremediation of the contaminated subsurface. U(VI) reduction was stimulated by injecting exogenous electron donors (e.g., acetate) into a contaminated aquifer in Rifle, CO (2). U(VI) was reduced concomitantly with Fe(III) and 16S rRNA gene and phospholipid fatty acid analyses (PFLA) demonstrated that FeRB (members of the genus *Geobacter*) were the predominant members of the microbial community. Uranium concentrations in reduced groundwater samples fell to less than 10^{-8} M. After U(VI) concentrations in the groundwater dropped below detection limits, acetate-oxidizing SRB (e.g., *Desulfotomaculum*, *Desulfosporosinus*) dominated the microbial community.

Subsequent electron donor injection field studies at the Department of Energy Field Research Center (FRC, Oak Ridge National Laboratory) indicated that SRB and URB community structure is modified by the choice of electron donor (86). The subsurface field site was hydraulically controlled by a duel-loop well recirculation system. High levels of aluminum and calcium were chemically precipitated and the pH was buffered by carbonate addition before electron donor amendment. Ethanol was added as an electron donor to promote denitrification and removal of the competing electron acceptor nitrate. Ethanol injection was continued and U(VI) levels decreased

concurrent with sulfate reduction. In contrast to the Rifle, Colorado experiment, URB and SRB population activities overlapped. URB apparently did not out-compete SRB for ethanol at the FRC.

The first enzyme displaying U(VI) reductase activity (cytochrome c_3) was isolated from U(VI)-reducing (but non-respiring) *Desulfovibrio vulgaris* (47, 67). Cytochrome c_3 couples H_2 oxidation to U(VI) reduction *in vitro*. Cytochrome c_7 of *Geobacter sulfurreducens* also displays U(VI) reductase activity *in vitro*, however, both cytochrome c_3 and c_7 mutant strains retain U(VI) reduction capability indicating that either cytochrome c_3 and c_7 do not reduce U(VI) *in vivo* or that *D. vulgaris* and *G. sulfurreducens* contain multiple U(VI) reductases (45).

$UO_{2(s)}$ is observed in both the periplasmic space and extracellular milieu after U(VI) reduction by *S. putrefaciens* (44, 55). In *S. oneidensis*, many *c*-type cytochromes are required for Fe(III) and Mn(IV) reduction. Mutant strains lacking all *c*-type cytochromes ($CcmC^-$), outer membrane cytochromes ($OmcA^-$ and $MtrC^-$) and a periplasmic cytochrome ($MtrF^-$) were recently constructed and tested for U(VI) reduction activity (55). $MtrF^-$ retained near wild-type U(VI) reduction rates. $OmcA^-$, $MtrC^-$ and double mutant $OmcA^-$-$MtrC^-$ retained 50% wild-type U(VI) reduction rates and $CcmC^-$ was unable to reduce U(VI). $UO_{2(s)}$ was observed predominantly in the periplasm in $MtrC^-$ and double mutant $OmcA^-$-$MtrC^-$ in electron micrographs. Only purified MtrC displayed U(VI) reducing activity *in vitro*. These results suggest that *c*-type cytochromes are essential for U(VI) reduction, that outer membrane and periplasmic cytochromes *c* reduce U(VI) and that outer membrane MtrC plays a major role in extracellular U(VI) reduction by *S. oneidensis*.

A genetic system to study U(VI) respiration by *S. putrefaciens*

A genetic system was recently developed to study U(VI) respiration by *S. putrefaciens* (81). A set of *S. putrefaciens* U(VI) reduction-deficient (Urr) mutants were generated by treating wild-type cultures with the chemical mutagen ethyl methane sulfonate (EMS). Approximately 18,000 colonies arising from EMS-treated cells were transferred to agar growth medium supplemented with U(VI) carbonate as electron acceptor. Plates were incubated under microaerobic conditions for 3-4 days and colonies were examined for production of a brown precipitate (presumably U(IV)) on their surface. Strains unable to form the U(IV) precipitate were tested for anaerobic growth in liquid medium supplemented with U(VI) as electron acceptor. Cell growth was monitored via direct counts of acridine orange-stained cells and U(VI) depletion was measured spectrophotometrically via an Arsenazo III-based assay (37). Strains displaying a Urr phenotype on the rapid plate assay were also unable to respire U(VI) in anaerobic liquid growth medium. Each Urr mutant strain was subsequently tested for their ability to grow on other compounds as terminal electron acceptor. All Urr strains isolated also lacked the ability to respire NO_2^-, and Urr mutant Urr14 retained the ability to respire all electron acceptors except U(VI) and NO_2^- (80). These results suggest that the electron transport chains terminating with the reduction of NO_2^- and U(VI) share common respiratory components.

Genetic complementation of Urr14

A clone library of wild-type chromosomal DNA fragments cloned into the broad-host-range cosmid pVK100 (16) was mobilized into mutant Urr14 for genetic complementation. A 32 kb DNA fragment (D14) restored NO_2^- and U(VI) respiratory

capability to the Urr14 transconjugant (11). *Hind*III digestion of fragment D14 yielded three fragments of size 14 kb (D14-1), 13 kb (D14-2) and 5 kb (D14-3). Each of these fragments was cloned into cosmid pVK100 and mobilized into Urr14 via tri-parental conjugation. Only transconjugant Urr14-D14-2 displayed wild-type NO_2^- and U(VI) reduction activity. Subsequent subcloning of D14-2 with *Bam*HI fragments yielded 4 fragments of size 6 kb (D14-2A), 4 kb (D14-2B), 2 kb (D14-2C) and 1 kb (D14-2D) but U(VI) and NO_2^- reduction by Urr14 was not restored in Urr14 transconjugates containing these fragments. The results of the D14-2 subcloning experiments suggest that the gene complementing Urr14 contains a *Bam*HI recognition sequence.

RESEARCH OBJECTIVES

The main objective of the present study was to identify genes required for U(VI) reduction by *S. putrefaciens*. Two experimental strategies were followed: 1) genetic complementation of *S. putrefaciens* mutants unable to reduce U(VI) but capable of growth and reduction activity on several alternate electron acceptors and 2) inactivation of respiratory chain components postulated to be involved in electron transport to U(VI) and testing the resulting knockout mutants for anaerobic growth deficiencies.

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CHAPTER 2

PERIPLASMIC *C*-TYPE CYTOCHROME C_3, BUT NOT NITRITE REDUCTASE NRFA, IS INVOLVED IN U(VI) REDUCTION BY *S. ONEIDENSIS*

Abstract

Members of the genus *Shewanella* are capable of anaerobic respiration with uranium [U(VI)] as terminal electron acceptor. The genes and gene products required for U(VI) respiration, however, are poorly understood. Cytochrome c_3 is widely distributed among sulfate reducing bacteria that are also capable of reducing U(VI). Cytochrome c_3 from *Desulfovibrio vulgaris* was the first enzyme identified that displays U(VI) reduction ability *in vitro* (29) and *D. desulfuricans* c_3 mutants were impaired for U(VI) reduction *in vivo* (42). In the present study, a *S. oneidensis cctA* (c_3 homolog) insertional mutant was found to reduce U(VI) at 60% the rate of the wild-type strain suggesting that *S. oneidensis* CctA is involved, but not required, for U(VI) reduction. In a previous study, a set of randomly-generated U(VI) reduction deficient (Urr) point mutants of *S. putrefaciens* were constructed (57). The Urr mutants displayed a variety of deficiencies for anaerobic growth on nitrate, nitrite, sulfite, thiosulfate, Fe(III), Mn(IV), fumarate or trimethylamine-N-oxide as electron acceptor. All Urr mutants were unable to grow on U(VI) and NO_2^-, including mutant Urr14, which retained the ability to grow on the other electron acceptors. These results suggest that the respiratory pathways leading to U(VI) and NO_2^- reduction in *Shewanella* share electron transport components. In the present study, however, a *S. oneidensis nrfA* (cytochrome *c* nitrite reductase structural gene)

insertional mutant was found to reduce U(VI) at wild-type rates suggesting that *S. oneidensis* NrfA is not involved in U(VI) reduction. A NO_2^- toxicity assay indicated that NO_2^- is toxic above 2.0 mM and inhibits anaerobic growth by *S. putrefaciens*.

Introduction

Cytochrome c_3 from *D. vulgaris* was the first enzyme identified that displayed U(VI) reductase activity *in vitro* (29) and *D. desulfuricans* c_3 mutants were impaired for U(VI) reduction *in vivo* (42). Cytochrome c_3 transfers electrons from hydrogenase to sulfite reductase (SiR) in sulfate-reducing bacteria (SRB) (27, 43, 53) (Figure 2.1). In SRB, ATP sulfurylase activates sulfate by forming adenosine-5'-phosphosulfate (APS). APS is subsequently reduced by ApsR to sulfite and adenosine monophosphate (AMP) (55). The dissimilatory sulfate reducing system also includes a transmembrane complex containing a nine-heme cytochrome *c* (HcA) and a 16-heme, high molecular weight cytochrome *c* (HmcA) that delivers electrons from hydrogenase to ApsR and may pump protons from the cytoplasm to the periplasm to generate PMF (16, 17). Cytochromes c_3 from different SRB have low primary sequence similarity yet their three-dimensional structure show a common topology organized around a four-heme cluster with similar spatial orientation.

Cytochromes c_3 in *Desulfovibrio* are classified into two types based on their biological and structural properties. Type I (TpI-c_3), also designated "basic" due to its high isoelectric point (pI), is the most abundant c_3 in *Desulfovibrio* species, has a molecular weight of approximately 13 kDa, is characterized by several conserved lysine

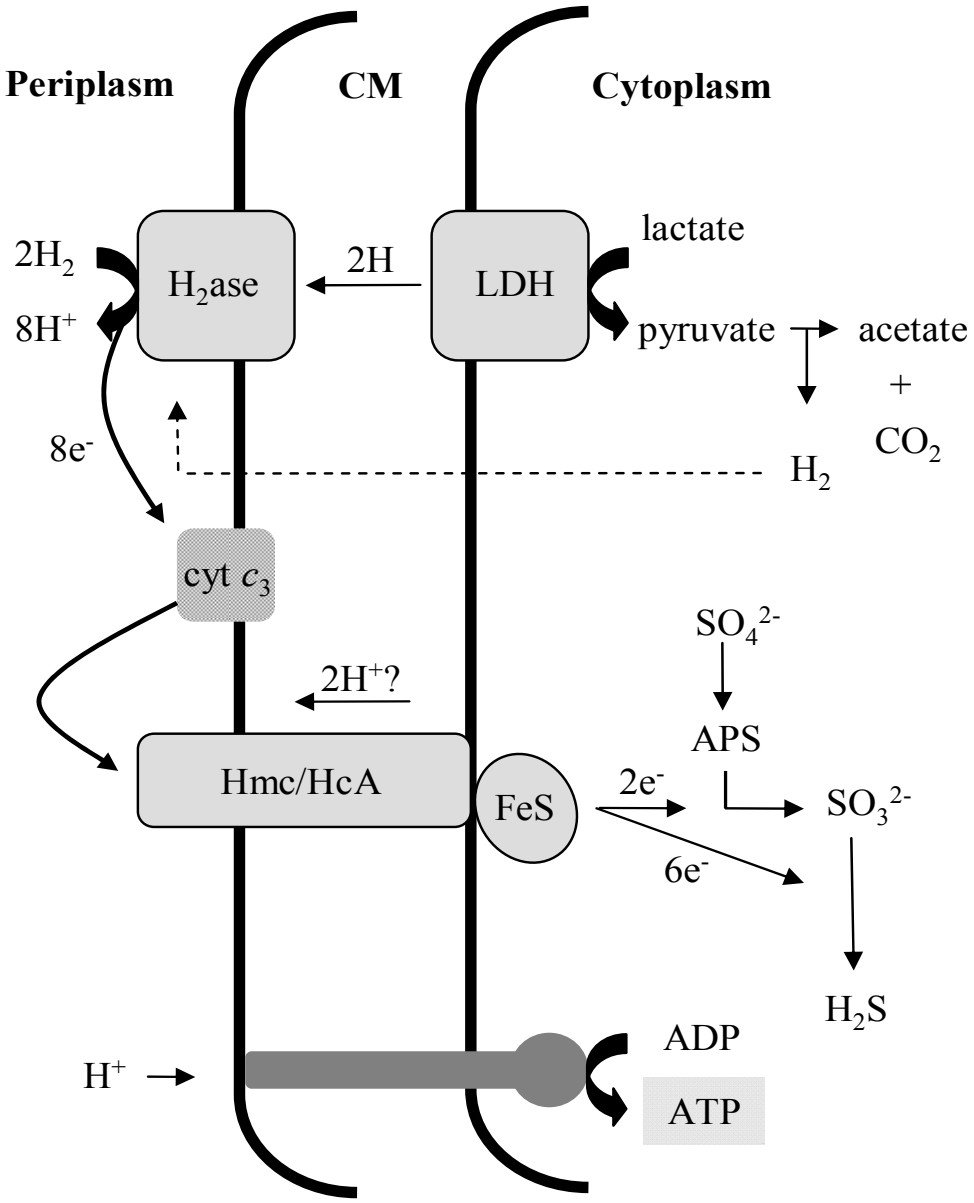

Figure 2.1. Dissimilatory sulfate reduction pathway. In addition to external hydrogen (H_2), H_2 originating from the catabolism of organic compounds such as lactate and pyruvate can fuel hydrogenase (H_2ase). Adapted from (30).

residues at the proposed hydrogenase docking site near heme 4, is loosely attached to the periplasmic face of the cytoplasmic membrane and is encoded by a monocistronic ORF (45, 56). Type II (TpII-c_3), on the other hand, is designated "acidic" due to its low pI, has a molecular weight of approximately 14 kDa, lacks the lysine patch near heme 4, has a solvent-exposed negative surface charge near heme 1 and is membrane-bound. TpII-c_3, *hcA* and *hmcA* are transcriptionally coupled. Purified TpI-c_3 accepts electrons from hydrogenase 50-fold more efficiently than TpII-c_3 and is most likely a physiological redox partner with hydrogenase in SRB (45). TpII-c_3 may receive electrons from TpI-c_3 and reduce components of the transmembrane complex (i.e., HcA, HmcA) or other electron transport chain components that depend on H_2 oxidation.

Members of the genus *Shewanella* are the only facultative anaerobes that contain cytochrome c_3 orthologs. c_3 orthologs in *Shewanella* are similar to the c_3 cytochromes of *Desulfovibrio* in heme content and E'_0, but the high-resolution (0.97 Å) crystal structure of the c_3 ortholog in *S. oneidensis* (CctA) reveals that CctA may share characteristics with both TpI-c_3 and TpII-c_3 in *Desulfovibrio* (28). As in TpII-c_3, CctA lacks the basic lysine patch, and Heme 1 is the most solvent-exposed heme with several acidic residues nearby including D7, E11, E16, D21, D27, E31 and D66. On the other hand, *cctA* is monocistronic and a soluble periplasmic protein. In contrast to a defined electron path in TpI-c_3 or TpII-c_3 indicated by specific electron entry and exit points, CctA in *S. oneidensis* lacks identifiable intermolecular contacts. CctA also contains a high heme-to-amino acid ratio and appears to possess exceptional intramolecular flexibility (Figure 2.2). These findings suggest that CctA functions as a generic electron transport intermediate whereby productive collision between many redox partners may be possible.

Cytochrome c_3 from *Desulfovibrio vulgaris* was the first enzyme found to reduce U(VI) *in vitro* (29). A multi-protein complex containing cytochrome c_3 and hydrogenase catalyzed U(VI) reduction concurrent with H_2 oxidation. *D. desulfuricans* c_3 insertional mutant I2 was constructed and tested for U(VI) reduction activity (42). I2 reduced U(VI) at approximately 50% the rate of the wild-type strain, indicating that c_3 may play a role in U(VI) reduction but it is not the sole U(VI) reductase. Cytochrome c_3 may fortuitously reduce U(VI) due to the presence of heme cofactors with low E'_0. The *S. oneidensis* c_3 ortholog CctA primary sequence is 29% identical and 39% similar to the cytochrome c_3 of *D. vulgaris*. The 11.7 kDa cytochrome c_3 of *S. frigidimarina* (also designated CctA) is

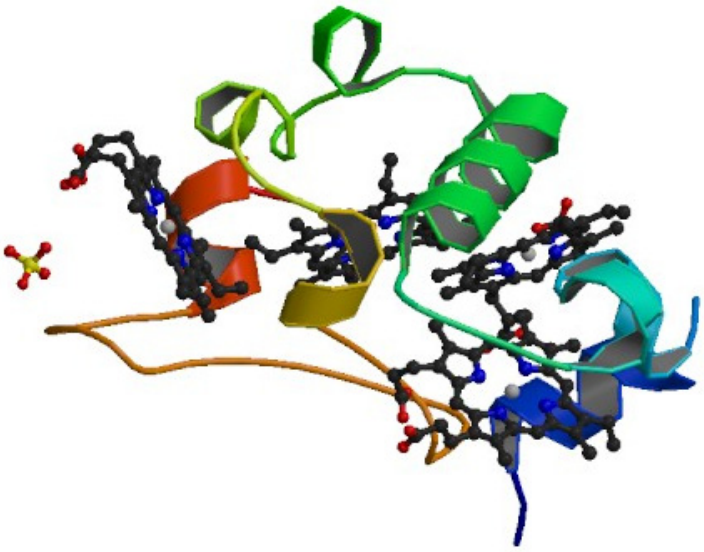

Figure 2.2. Ribbon structure of CctA in *S. oneidensis*. α-helices of the amino acid backbone are represented in red, yellow, green and blue. Heme prosthetic groups are represented as black ball-and-stick models. Note the high heme-to-amino acid ratio and apparent intramolecular flexibility among α-helices (28, 31).

required for anaerobic growth on Fe(III), but not NO_3^-, NO_2^-, TMAO, DMSO, fumarate, $S_4O_6^{2-}$ or SO_3^{2-} (19). *S. oneidensis* CctA is one of the most abundant periplasmic cytochromes during anaerobic growth and is capable of Fe(III) reduction when artificially reduced *in vitro* (28, 32, 54). *cctA* insertional mutants of *S. oneidensis* MR-1, however, retain anaerobic growth ability on Fe(III) and Mn(VI) *in vivo* (34). In the present study, a previously constructed *S. oneidensis cctA* mutant (C3965) is tested for U(VI) reduction ability to determine if CctA is required for U(VI) reduction by *S. oneidensis*.

Previous random mutagenesis and anaerobic growth experiments in *S. putrefaciens* indicated that the respiratory chains leading to U(VI) or NO_2^- reduction shared electron transport components (57, 58). Eight chemically-induced *S. putrefaciens* point mutants incapable of U(VI) reduction on agar plates (Urr) were isolated and subsequently tested for U(VI) reduction and anaerobic growth ability on a suite of 10 alternate electron acceptors in liquid culture. Each Urr strain retained the ability to grow on at least one electron acceptor besides O_2; however, the ability to grow on NO_2^- was abolished in all Urr strains.

Three types of respiratory NO_2^- reductases are known in bacteria: copper cofactor-containing NirK, cytochrome cd_1-containing NirS and *c*-type cytochrome NrfA (35). In the *E. coli* periplasm, NrfA catalyzes the 6-electron reduction of NO_2^- to NH_4^+ as the terminal reaction of a dedicated respiratory pathway termed ammonification (10, 21, 40). The NirK and NirS respiratory pathways, in contrast, reduce NO_2^- to nitric oxide (NO), which is subsequently reduced to N_2 via a nitrous oxide (N_2O) intermediate in a process known as denitrification (11, 18, 22). The *S. oneidensis* MR-1 genome was scanned for putative NO_2^- reductases via Basic Local Alignment Search Tool (BLAST)

analysis (2). Only *nrfA* was found in the *S. oneidensis* genome: locus SO3980 is 65% identical and 79% similar to *nrfA* in *E. coli*.

The Nrf respiratory pathway in *E. coli*, *Wollinella succinogenes* and *Desulfovibrio desulfuricans* are well characterized (8, 51). Three intermediate electron transport components link menaquinone oxidation to NrfA reduction in *E. coli*: NrfD, NrfC and NrfB. NrfD is a hydrophobic protein localized to the cytoplasmic membrane and may function as a quinol oxidase and electron donor to NrfC, a cytoplasmic membrane-spanning FeS protein. NrfC delivers electrons to the soluble periplasmic *c*-type cytochrome NrfB, the NrfA reductase (21). A single intermediate electron transport component is required to link menaquinone oxidation to NrfA reduction in *W. succinogenes*: NrfH, a transmembrane *c*-type cytochrome belonging to the NapC/NirT family of quinol oxidases (52). *nrfA* is transcriptionally coupled to its electron donor in *E. coli* and *W. succinogenes*, while *nrfA* in *S. oneidensis* is monocistronic. The *S. oneidensis* genome contains ORFs similar to both *nrfB* and *nrfH*: paralogous MtrA and MtrD are involved in Fe(III) and Mn(IV) reduction and have similar heme arrangement as NrfB (7, 47). NO_2^- reduction in *S. oneidensis* requires CymA, a putative quinol oxidase similar to NrfH, NapC and NirT (38, 50). *S. oneidensis* also requires CymA for anaerobic growth on Fe(III), NO_3^-, DMSO, As(V) and fumarate as electron acceptor (37, 38, 49). In addition to *nrfA* and *nrfB*, *S. oneidensis* contains two ORFs that show similarity to *nrfD* (paralogous *nrfD*-1 and *nrfD*-2) but no identifiable *nrfC*. Quinol oxidase CymA may transport electrons from menequinone or NrfD to NrfA and functionally replace NrfC in *S. oneidensis* (50).

NrfA crystal structures from *Sulfurospirillum deleyianum* (15), *E. coli* (5) and *Desulfovibrio desulfuricans* (9) were recently determined (Figure 2.3). The NrfA subunit contains five *c*-type heme prosthetic groups and forms a homodimer. Active site heme 1 iron is axially coordinated by lysine in a CXXCK heme-binding motif unique to NrfA homologs and the octaheme tetrathionate reductase (OTR) in *S. oneidensis* (36) (Figure 2.3). The five heme groups are tightly packed and arranged at near 90^{o} angles to each other. This heme packing motif is shared by cytochromes c_3 in cytochrome c_7 in *Geobacter* and hydroxylamine oxidoreductase (HAO) in *Nitrosomonas europaea* (3, 36). HAO oxidizes hydroxylamine (NH_2OH) to NO_2^- while NrfA reduces NO_2^- and NH_2OH to NH_4^+. Axial heme iron coordination may explain the difference in active-site oxidoreductase activity among this family of enzymes. Replacement of histidine in the CXXCH heme binding site by lysine raises the E'_0 of heme 1 and generates an electron sink in NrfA and OTR (3, 14, 36). In the present study, a *S. oneidensis nrfA* insertional mutant (NRFA1) is tested for growth on NO_3^- and NO_2^- to establish that NrfA is the functional NO_2^- reductase in *S. oneidensis*. NRFA1 is also tested for U(VI) reduction to determine if NrfA is required for U(VI) reduction by *S. oneidensis*.

Materials and methods

Bacterial strains, growth media and cultivation conditions. A previously isolated rifamycin resistant strain of *S. oneidensis* strain MR-1R (hereafter referred to as *S. oneidensis*) was routinely cultured in the presence of 50 μg ml^{-1} rifamycin in either Luria Broth or a defined salts growth medium (SM, pH 7.5) (12, 13, 39). *S. oneidensis* strains harboring cloning vectors were grown in media supplemented with appropriate antibiotics at the following concentrations: chloramphenicol (25 μg ml^{-1}) and gentamycin

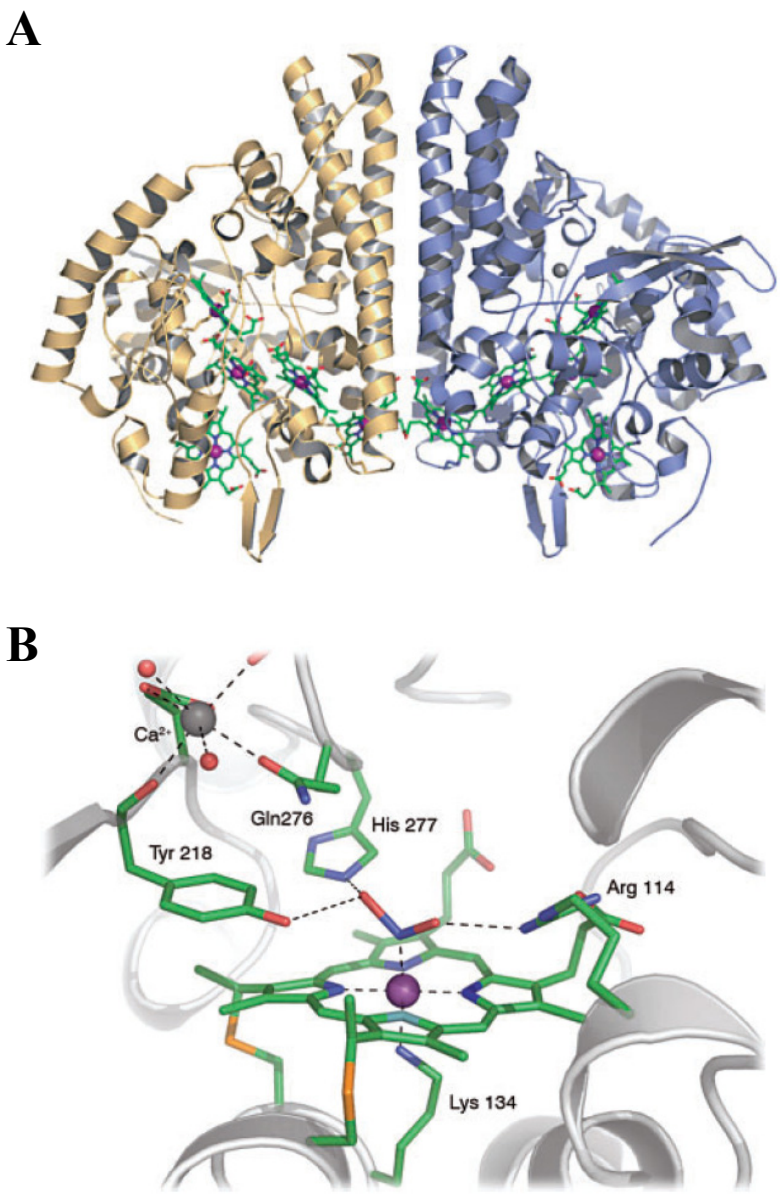

Figure 2.3. Ribbon structure of the cytochrome *c* nitrite reductase (NrfA) homodimer from *S. deleyianum* (A) and NrfA heme 1 with NO_2^- bound to the active site. Note the covalent attachment of heme to the polypeptide and heme iron coordination by Lys 134. Heme iron ligand His 277 is displaced by substrate binding (14).

(15 μg ml^{-1}) (see Table 2.1 for strains and plasmids used in the present study). *E. coli* strains were routinely cultured in Luria Broth supplemented with the appropriate antibiotics. Anaerobic growth experiments were carried out in 13-ml Hungate tubes (Bellco Glass, Inc.) containing 10 ml SM supplemented with sodium lactate (30 mM) and sealed with black butyl rubber stoppers under an N_2 atmosphere. Anoxic electron acceptors were added to the SM medium from filter-sterilized stocks to the following final concentrations: NO_3^-, (15 mM); NO_2^-, (0.3 mM); U(VI)-carbonate, (1.0 mM). A U(VI)-carbonate (30 mM) stock solution was prepared by dissolving 127 g/L uranyl acetate in a 1M sodium bicarbonate buffer (pH 8.0). The U(VI)-carbonate growth medium contained a final sodium bicarbonate concentration of 30 mM. Control experiments consisted of inoculating SM liquid growth medium with *S. oneidensis* cells held at 80°C for 30 minutes (heat-killed control) or omitting inocula (abiotic control).

Insertional mutagenesis of *nrfA* in *S. oneidensis*. *nrfA* was inactivated in *S. oneidensis* by chromosomal plasmid insertion. A 797 bp internal fragment of *nrfA* was PCR-amplified from the *S. oneidensis* chromosome with the nrfAINT primer set (nrfAINTF, gctcattagtgacgccaac; nrfAINTR, gctcattagtgacgccaac). The purified PCR product was cloned into pCR2.1 (Invitrogen) and inserted into pKNOCK-GmR at the *Kpn*I and *Xba*I endonuclease restriction sites. *E. coli* S17-1 λ-pir was transformed with the resulting plasmid construct, pCRNR, via electroporation. pCRNR was mobilized into *S. oneidensis* MR-1R by conjugation and insertional mutant NRFA1::pCRNR arising on LB agar plates containing 10 μg/ml gentamycin was confirmed by PCR amplifying *nrfA* and flanking DNA with the nrfAEXP primer set (nrfAEXPF, gtattcggtttcgggcatt; nrfAEXPR, gaactcggcgtcagtatttc) and noting a gel mobility shift by agarose gel electrophoresis.

Table 2.1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Description	Reference
Shewanella oneidensis		
MR-1R	Spontaneous Rif^r-derivative of *S. oneidensis*, Fe(III)- and Mn(IV)-reducing Oneida Lake isolate	(12, 39)
NRFA1	MR-1R *nrfA*:pCRNR	(57)
C3965	MR-1R, *cctA*::Gm^R	(34)
200R	Spontaneous Rif^r-derivative of *S. putrefaciens* (NCIB 12577)	(12)
Urr14	U(VI) reduction-deficient point mutant of 200R	(57)
Escherichia coli		
S17-1 λ-pir	*Prp thi rec*A *hsd*R; RP4-2 (Tn1:ISR1 tet::Mu Km::Tn7); λ*pir*	(44)
TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
Plasmids		
pCR2.1	TA cloning vector; Amp^R, Kmr^R	Invitrogen
pKNOCK-Gm^R	Broad-host-range suicide vector for targeted DNA insertion	(1)
pBBR1MCS	Broad-host-range cloning vector, 4.7-kb α-lac/multiple cloning site, Cm^r	(25)
pCRNR	pKNOCK-Gm^R with nrfAINT fragment cloned via *Kpn*I, *Xba*I	This study
pNRFA	pBBR1MCS with nrfAEXP fragment cloned via *Kpn*I, *Xba*I	This study

Insertion and correct orientation was verified by DNA sequencing.

A wild-type copy of *nrfA* was mobilized into NRFA1 to genetically complement the *nrfA*::pCRNR mutation *in trans*. *nrfA*, along with 848 bp upstream and 761 bp downstream, was PCR-amplified from the *S. oneidensis* chromosome with the nrfAEXP primer set. Fragment nrfAEXP was cloned into pCR2.1 (Invitrogen) and inserted into pBBR1MCS at the *Kpn*I and *Xba*I endocuclease restriction sites. pNRFA was mobilized into NRFA1 via conjugation and NRFA1 colonies arising on LB agar containing 25 μg/ml chloramphenicol were tested for pNRFA uptake by plasmid purification and agarose gel electrophoresis.

Analytical techniques

U(VI) reduction assay. U(VI) reduction by *S. oneidensis* strains was measured in cell suspensions containing 2 X 10^8 cells/mL and 1.0 mM U(VI) in sodium bicarbonate buffer (30 mM $NaHCO_3$, 1 mM KCl, pH 8.0) in an atmosphere consisting of 10% H_2, 5% CO_2 and balance N_2. Samples were filtered (0.2 μm pore size) and centrifuged (10 minutes at 16,000 x g in a microcentrifuge) to remove cells and insoluble U(IV) prior to spectrophotometric U(VI) determination. U(VI) was measured colorimetrically with Arsenazo III reagent (26).

Anaerobic cell growth. S. oneidensis cultures were monitored for anaerobic growth on NO_3^- as electron by simultaneously measuring cell number and NO_2^- concentration. Three parallel yet independent anaerobic cultures of each strain were prepared and data was expressed as the arithmetic mean from the triplicate incubations. Acridine orange-stained cells were counted directly via epifluorescence microscopy (Nikon Diaphot 300 microscope). NO_2^- was measured spectrophotometrically with sulfanilic acid-N-1-naphthylethylene-diamine dihydrochloride reagent (33).

NO_2^- toxicity assay. NO_2^- was added to anaerobic SM medium containing 30 mM lactate at the following concentrations: 0.25, 0.50, 1.00, 2.00, 3.00, 5.00, 7.50, and 10.00 mM. Triplicate cultures were inoculated with *S. putrefaciens* (2 X 10^7cells/mL). The extent of NO_2^- reduction (expressed as % NO_2^- consumed) was determined after 72 hours.

Results

CctA is involved in H_2-dependent U(VI) reduction by *S. oneidensis*. *S. oneidensis* and *cctA* insertional mutant C3965 were tested for U(VI) reduction ability. C3965 reduced U(VI) at a rate of 4.85 X 10^{-14} M $cell^{-1}$ hr^{-1}, 60% that of the wild-type strain (Figure 2.4). U(VI) content in cultures containing heat-killed cells decreased at a rate 3% of the wild-

type strain. U(VI) adsorption to the cell surface of the metabolically inactive cells may contribute to the apparent U(VI) reduction in heat-killed *S. putrefaciens* cultures (20, 23, 58). U(VI) content in the uninoculated culture (abiotic control) did not significantly decrease, an indication that U(VI) did not precipitate via a purely chemical reaction. These results suggest that CctA plays a role in U(VI) reduction by *S. oneidensis* but is not the sole U(VI) reductase.

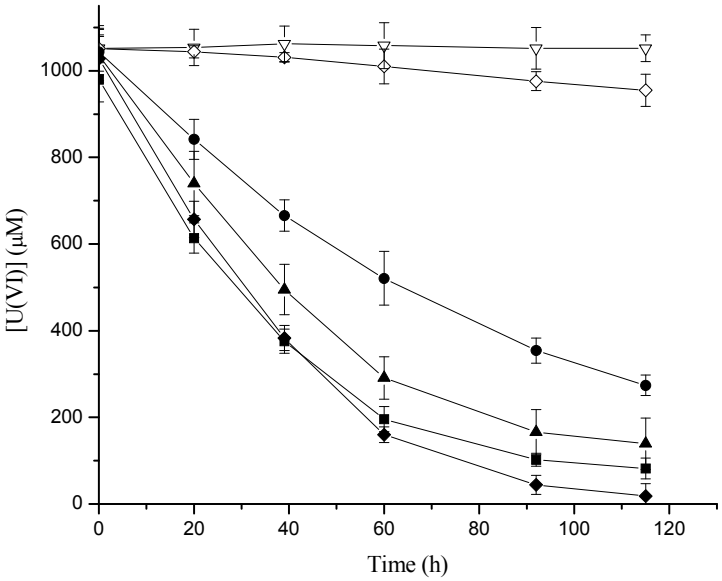

Figure 2.4. Reduction of 1.0 mM U(VI) by NRFA1 (-■-), NRFA1-pNRFA (-▲-), *S. oneidensis* wild-type (-♦-),C3965 (-●-), *S. oneidensis* heat-killed control (-◊-) and abiotic control (-▽-).

NrfA is not required for H_2-dependent U(VI) reduction by *S. oneidensis.* *S. oneidensis*, *nrfA* insertional mutant NRFA1 and NRFA1 genetic complement NRFA1-pNRFA were tested for U(VI) reduction ability. NRFA1 reduced U(VI) at a rate of 7.75 X 10^{-14} M hr^{-1} $cell^{-1}$, nearly identical to that of the wild-type strain and the positive control strain NRFA1-pNRFA (Figure 2.4). U(VI) content in cultures containing heat-killed cells decreased at a rate 3% of the wild-type strain. U(VI) adsorption to the cell surface of the metabolically inactive cells may contribute to the apparent U(VI) reduction in heat-killed *S. putrefaciens* cultures (20, 23). U(VI) content in the uninoculated culture (abiotic control) did not significantly decrease, an indication that U(VI) did not precipitate via a purely chemical reaction. These results suggest that NrfA is not required for U(VI) reduction by *S. oneidensis*.

NrfA is required by *S. oneidensis* for anaerobic growth on NO_2^-. *S. oneidensis*, *nrfA* insertional mutant NRFA1 and NRFA1 genetic complement NRFA1-pNRFA were tested for anaerobic growth on NO_3^- and NO_2^-. After stoichiometric reduction of NO_3^- to NO_2^-, growth of the wild-type strain and NRFA1-pNRFA continued on NO_2^- until all NO_2^- was depleted. NRFA1, on the other hand, was able to grow at wild-type rates on NO_3^- and to stoichiometrically convert NO_3^- to NO_2^- at a rate similar to the wild-type strain, yet was unable to sustain growth or deplete NO_2^- after all NO_3^- was reduced. NRFA1 cell density and NO_2^- concentrations remained unchanged after NO_3^- was completely reduced to NO_2^- (Figure 2.5). These results indicate that NrfA is required for growth of *S. oneidensis* on NO_2^-.

S. putrefaciens Urr14 retains partial U(VI) and NO_2^- reduction activity in media containing subtoxic electron acceptor concentrations. Previously isolated point mutant

Urr14 was unable to reduce U(VI) or NO_2^- yet retained the ability to grow on a suite of alternate electron acceptors (57). In the present study, *S. oneidensis* strains lacking the nitrite reductase NrfA retained U(VI) reduction capability. To investigate the possibility that Urr14 is more susceptible than the wild-type strain to NO_2^- toxicity, Urr14 was tested for NO_2^- reduction in growth medium containing subtoxic electron acceptor concentrations (i.e., 10-fold below the toxic level). To determine the toxic level of NO_2^- for anaerobic growth of *S. putrefaciens*, a toxicity assay was conducted with the wild-type strain. *S. putrefaciens* was unable to reduce NO_2^- when added directly to anaerobic growth medium containing > 3.0 mM NO_2^- (Figure 2.6). Urr14 reduced NO_2^- at rates < 30% of the wild-type strain in cultures containing 300 μM NO_2^- (Figure 2.7). These results suggest that Urr14 is more susceptible to NO_2^- toxicity than the wild-type strain and that an additional impairment in Urr14 may prevent growth at wild-type rates on NO_2^-.

Discussion

The main objective of the present study was to determine if the electron transport chain components CctA or NrfA are required for U(VI) reduction by *S. oneidensis*. Previous studies suggested that cytochrome c_3 and the nitrite reductase NrfA are involved in U(VI) reduction by *D. desulfuricans* and *S. putrefaciens* (29, 42, 57). *Shewanella oneidensis* cytochrome c_3 (CctA) insertional mutant strain C3965 was tested for U(VI) reduction and was found to reduce U(VI) at rates 60% that of the wild-type strain. c_3 mutants of *D. desulfuricans* G20, on the other hand, reduce U(VI) at only 30% wild-type

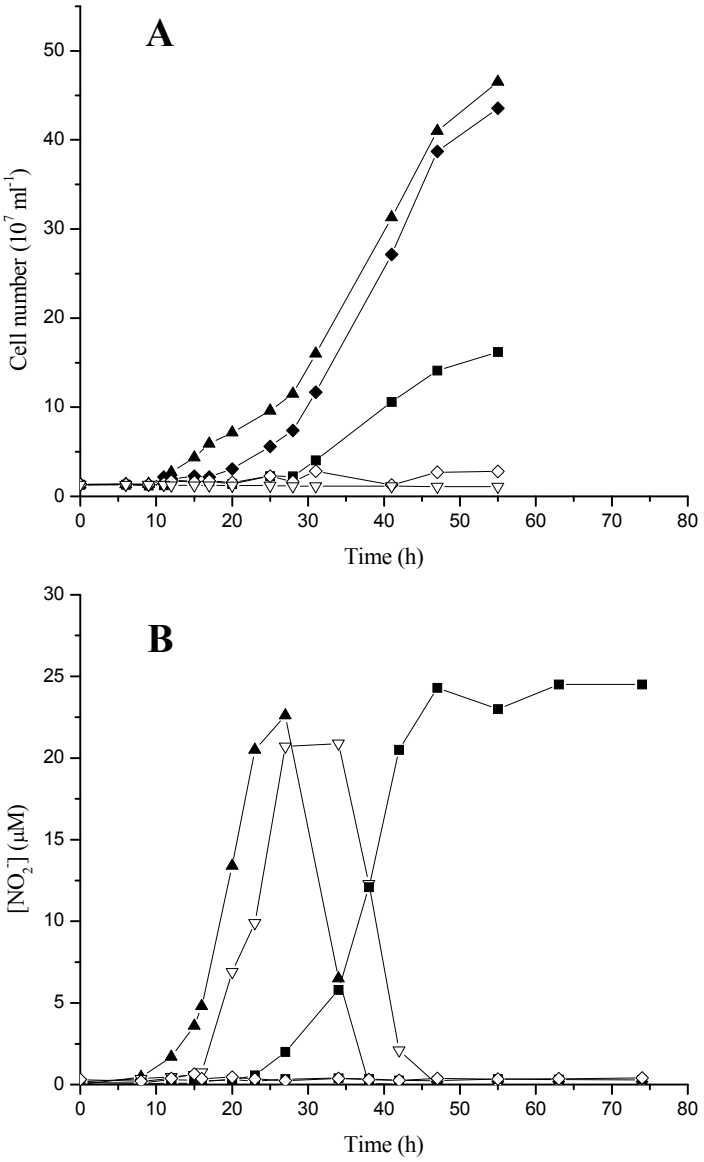

Figure 2.5. Anaerobic growth of NRFA1 on nitrate (A) and corresponding NO_2^- content (B). *S. oneidensis* wild-type (-♦-), NRFA1 (-■-), NRFA1-pNRFA (-▲-), *S. oneidensis* heat-killed control (-◊-) and abiotic control (-▽-).

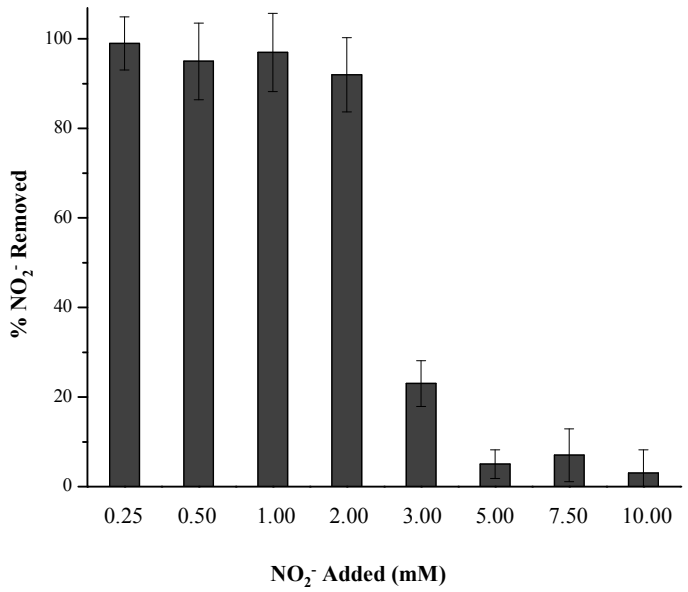

Figure 2.6. Nitrite toxicity assay. NO_2^- was added to anaerobic *S. putrefaciens* cultures containing excess lactate at the indicated concentrations and the extent of NO_2^- reduction (expressed as % NO_2^- removed from solution) was determined after 72 hours. Results are based on three parallel, yet independent incubations.

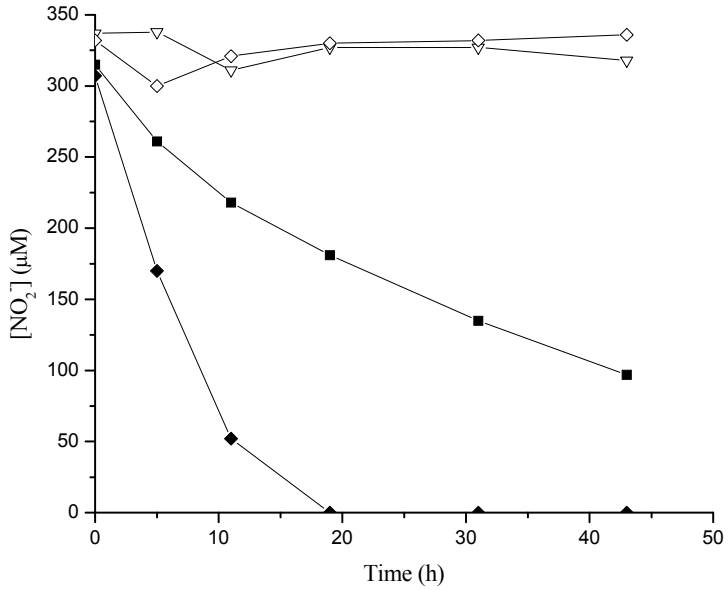

Figure 2.7. Reduction of 300 μM NO_2^- by Urr14 at subtoxic electron acceptor concentrations. Urr14 (-■-), *S. oneidensis* wild-type (-♦-), *S. oneidensis* heat-killed control (-◊-) and abiotic control (-▽-).

rates with H_2 as electron donor (41, 42). These results suggest that CctA is involved in U(VI) reduction but that *S. oneidensis* strains that lack CctA continue to reduce U(VI). Structural comparison of c_3 cytochromes from *D. desulfuricans*, *D. vulgaris* (4, 28, 45, 56) indicate that TpII-c_3 (acidic) is involved in U(VI) reduction by *Desulfovibrio*. CctA in *S. oneidensis*, however, shares structural characteristics with TpI-c_3 and TpII-c_3, suggesting that CctA may accept electrons from multiple electron donors (28). CctA insertional mutants are not significantly impaired for anaerobic growth on any electron acceptor, suggesting that CctA may also donate electrons to multiple redox partners, possibly including U(VI) (34).

Previous studies with U(VI) reduction-deficient mutant Urr14 indicated that U(VI) and NO_2^- reduction pathways share electron transport components (57). The *S. oneidensis* genome contains the cytochrome *c* nitrite reductase NrfA. In the present study, insertional mutant NRFA1 was found to reduce U(VI) at rates identical to the wild-type strain, indicating that NrfA is not required for U(VI) reduction in *S. oneidensis*. The redox partner of NrfA in *S. oneidensis* is unclear. *S. oneidensis* lacks NrfB and NrfH homologs, but decaheme paralogs MtrA and MtrD are structurally similar to NrfB and CymA belongs to the NapC/NirT family of quinol oxidases. CymA is required for NO_2^- reduction by *S. oneidensis* and may functionally replace NrfH (50). CymA is required in at least five electron transfer pathways in *S. oneidensis* including nitrite and nitrate reduction (38, 50). Urr14 reduces nitrate at wild-type rates, however, suggesting that the mutation in Urr14 is downstream of CymA. An intermediate electron transport chain component downstream of CymA and upstream of NrfA in *S. oneidensis* is presently unknown. MtrA and MtrD are upregulated during U(VI) reduction and MtrA mutant SR-

524 reduces U(VI) at only 30% the wild-type rate (6). SR-524 should be tested for NO_2^- reduction to determine if MtrA is required for both U(VI) and NO_2^- reduction by *S. oneidensis*.

In addition to coupling with electron donor oxidation for energy conservation, dissimilatory ammonification via NrfA protects bacteria from oxidative damage (24, 46, 48). To determine if NO_2^- reduction deficiency in U14 is due to electron acceptor toxicity, Urr14 was tested for NO_2^- reduction in media containing electron acceptor concentrations 10-fold below the toxic level in *S. putrefaciens*. U14 remained NO_2^- reduction deficient, however, U14 reduced NO_2^- at rates of about 30% that of the wild-type strain. In a previous study, Urr14 reduced NO_2^- at rates < 5% of the wild-type strain in cultures containing NO_2^- near the toxic level (58). NO_2^-, due to NO_3^- reduction, apparently accumulated to deleterious levels in Urr14 cultures at a rate greater than NO_2^- was detoxified via reduction to NH_4^+. These results suggest that point mutant Urr14 is more susceptible to NO_2^- toxicity but the primary impairment in Urr14 remains unknown. The identification of the Urr14 mutation and its role in U(VI) and NO_2^- respiration are the focus of future investigations.

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CHAPTER 3

A conserved histidine in cytochrome *c* maturation permease CcmB of *Shewanella putrefaciens* is required for anaerobic growth below a threshold standard redox potential

Abstract

Shewanella putrefaciens strain 200 respires a wide range of compounds as terminal electron acceptor. The respiratory versatility of *Shewanella* is attributed in part to a set of *c*-type cytochromes with widely varying mid-point redox potentials (E'_0). A point mutant of *S. putrefaciens*, originally designated Urr14 and here renamed CCMB1, was found to grow at wild-type rates on electron acceptors with high E'_0 [O_2, NO_3^-, Fe(III)-citrate, MnO_2 and Mn(III)-pyrophosphate] yet was severely impaired for growth on electron acceptors with low E'_0 [NO_2^-, U(VI), DMSO, TMAO, fumarate, γ-FeOOH, SO_3^{2-} and $S_2O_3^{2-}$]. Genetic complementation and nucleotide sequence analyses indicated that the CCMB1 respiratory mutant phenotype was due to mutation of a conserved histidine residue (H108Y) in a protein that displayed high homology to *E. coli* CcmB, the permease subunit of an ABC transporter involved in cytochrome *c* maturation. Although CCMB1 retained the ability to grow on electron acceptors with high E'_0, the cytochrome content of CCMB1 was < 10% of the wild-type strain. Periplasmic extracts of CCMB1 contained slightly greater concentrations of the thiol functional group (-SH) compared with the wild-type strain, an indication that the E_h of the CCMB1 periplasm was abnormally low. A *ccmB* deletion mutant was unable to respire anaerobically on any

electron acceptor, yet retained aerobic respiratory capability. These results suggest that the mutation of a conserved histidine residue (H108) in CCMB1 alters the redox homeostasis of the periplasm during anaerobic growth on electron acceptors with low (but not high) E'_0. This is the first report of the effects of Ccm deficiencies on bacterial respiration of electron acceptors whose E'_0 nearly span the entire redox continuum.

Introduction

Cytochromes with covalently attached heme (*c*-type cytochromes) are often major components of anaerobic electron transport chains of prokaryotic microorganisms. *c*-type cytochromes displaying widely varying midpoint reduction potentials (E'_0) may confer respiratory versatility to prokaryotes residing in redox-stratified environments (15, 17, 21). The genome of the metal-respiring γ-proteobacterium *Shewanella oneidensis* MR-1, for example, encodes 42 putative *c*-type cytochromes, several of which are involved in electron transfer to metals and other anaerobic electron acceptors with E'_0 values virtually spanning the entire redox continuum encountered by microorganisms in the environment. (28, 39, 48) The molecular mechanism of bacterial metal respiration, however, remains poorly understood.

In several metal-respiring bacteria, *c*-type cytochromes are involved in electron transport to oxidized forms of metals and radionuclides. In *Geobacter sulfurreducens*, outer membrane *c*-type cytochromes OmcB and OmcF are required for respiration on solid Fe(III)-oxides, and diheme *c*-type cytochrome MacA is an intermediate electron carrier (8, 31, 37). Metal-respiring members of the genus *Shewanella* also require outer membrane *c*-type cytochromes OmcA and OmcB (MtrC) for respiration on U(VI) and solid Fe(III)- or Mn(IV)-oxides (3, 41, 46). *c*-type cytochromes involved in periplasmic

electron transport in *S. oneidensis* include MtrA and CymA (43, 52). CymA oxidizes quinol and transfers electrons to downstream components of the electron transport pathway terminating with the reduction of Fe(III), NO_3^-, NO_2^-, fumarate and DMSO (43, 55, 56). A purified cytochrome c_3-hydrogenase complex isolated from U(VI)-reducing *Desulfovibrio vulgaris* Hildenborough displays H_2-U(VI) oxidoreductase activity *in vitro* (40). In addition, cytochrome c_3 mutants of *Desulfovibrio desulfuricans* G20 are deficient in U(VI) reduction activity with H_2 as electron donor (49, 50).

Bacteria, archaea, and the mitochondria and chloroplasts of eukarya require maturation systems to complete *c*-type cytochrome synthesis. Cytochrome *c* maturation (Ccm) systems attach heme groups to the CXXCH motifs of apocytochrome *c* (i.e., immature cytochrome *c* with CXXCH heme-binding motifs not bound by heme) via stereospecific thioether covalent bonds. Three Ccm systems are currently known [for recent reviews, see (58) and (60)]. System I, found predominantly in α- and γ-proteobacteria, land-plant and protozoan mitochondria and some archaea, consists of eight dedicated components (CcmA-H). System II, commonly found in Gram-positive bacteria, cyanobacteria, β- and δ-proteobacteria, chloroplasts and some archaea, includes the major components ResA-C and CcdA. The third cytochrome *c* maturation system, System III, is restricted to fungal and animal mitochondria and consists of a single heme lyase component (CCHL).

Cytochrome *c* maturation System I is the most extensively studied cytochrome *c* maturation pathway. A schematic of System I cytochrome *c* maturation components is given in Figure 3.1. System I is organized into two branches that converge at heme lyase (CcmF): a heme delivery branch comprised of CcmA-E and a thioredoxin branch that

includes DsbD and CcmGH (60). The heme delivery branch transfers heme to apocytochrome *c* and includes ABC transporter subunits CcmABC, membrane protein CcmD and heme chaperone CcmE. The thioredoxin branch transfers reducing equivalents to the periplasm and ensures that the thiol groups of apocytochrome *c* remain reduced for heme attachment. Maintenance of redox homeostasis in the periplasm is crucial for optimal Ccm activity (2, 9).

A random point mutant of S. putrefaciens strain 200 (originally designated Urr14 and here renamed CCMB1) was isolated by combining chemical mutagenesis (ethyl methane

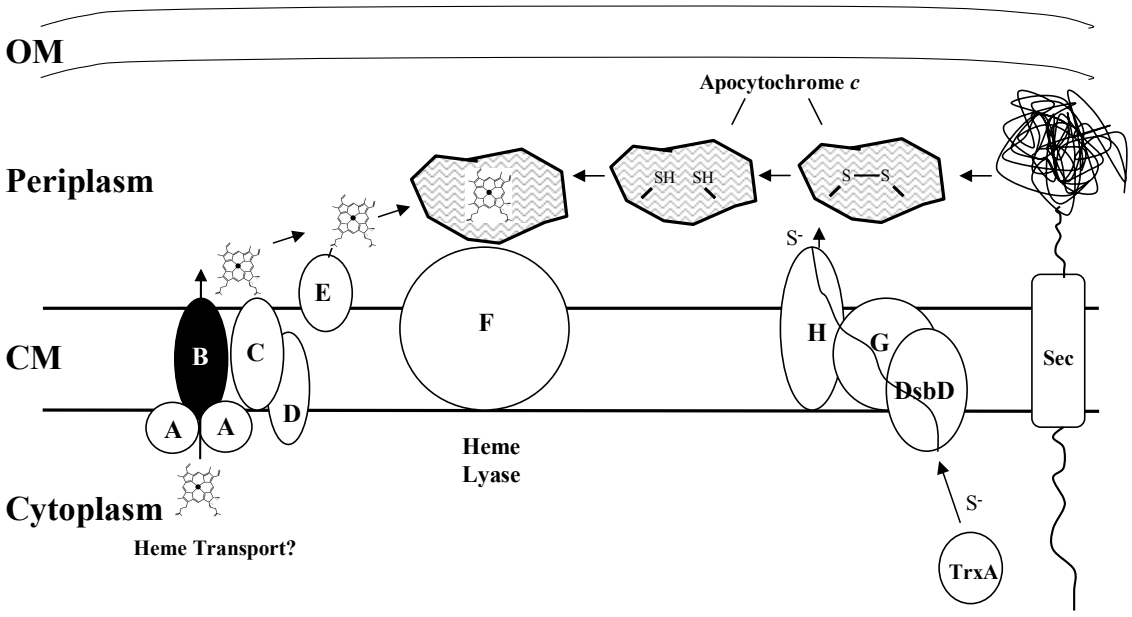


Figure 3.1. Schematic representation of System I cytochrome *c* maturation in *E. coli*. CcmABCDE deliver heme to apocytochrome *c* and heme lyase CcmF. CcmG, CcmH, DsbD and TrxA ensure that the thiol groups of apocytochrome *c* CXXCH motifs remain reduced. The Sec translocase exports the apocytochrome polypeptide from the cytoplasm to the periplasm. Cytochrome *c* and apocytochrome *c* (shaded) and CcmB (solid) are indicated.

sulfonate) procedures with a rapid mutant screen designed to detect respiratory mutants deficient in U(VI) reduction activity (62). Subsequent anaerobic liquid growth experiments demonstrated that CCMB1 was unable to respire U(VI) or NO_2^- as anaerobic electron acceptors, yet retained the ability to respire on O_2, NO_3^-, MnO_2 and Fe(III)-citrate. In the present study, the gene mutated in CCMB1 was identified.

Materials and methods

Bacterial strains, growth media and cultivation conditions. A previously isolated rifamycin resistant strain of *S. putrefaciens* strain 200 (hereafter referred to as *S. putrefaciens*) was routinely cultured in the presence of 50 μg ml^{-1} rifamycin in either Luria Broth or a defined salts growth medium (SM, pH 7.5) (16, 18, 44). Growth experiments consisted of triplicate cultures of each strain, and cell number, electron acceptor depletion or end-product accumulation data was expressed as the arithmetic mean of the three parallel yet independent incubations. Anaerobic growth experiments were carried out in 13-ml Hungate tubes (Bellco Glass, Inc.) containing 10 ml SM supplemented with sodium lactate (30 mM) and sealed with black butyl rubber stoppers under an N_2 atmosphere. Electron acceptor stocks were added at the following final concentrations (electron acceptor stock solutions were prepared as previously described (16, 62) except where indicated): NO_3^-, (20 mM); NO_2^-, (2.0 mM); U(VI)-carbonate, (1.0 mM) (62); Fe(III) citrate, (50 mM); γ-FeOOH (12), (40 mM); MnO_2 (as colloidal MnO_2), (10 mM) (51); Mn(III)-pyrophosphate, (10 mM) (33); TMAO, (30 mM); SO_3^{2-}, (10 mM); $S_2O_3^{2-}$, (10 mM); DMSO, (50 mM); fumarate, (35 mM). Aerobically-grown cells were inoculated in batch cultures vigorously aerated with atmospheric gas. U(VI) growth experiments were carried out in SM medium with the headspace gas consisting of 10%

H_2, 5% CO_2 and the balance N_2. Control experiments consisted of incubating wild-type *S. putrefaciens* in SM liquid growth medium with electron acceptor omitted or with heat-killed cells as inoculum. Antibiotics were added from filter-sterilized stocks at the following concentrations: chloramphenicol (25 μg ml^{-1}) and tetracycline (15 μg ml^{-1}).

Genetic complementation and nucleotide sequence analyses. Standard genetic procedures were used in genetic complementation and subcloning experiments (54). A previously constructed clone library of partially digested *Hin*dIII chromosomal DNA fragments of *S. putrefaciens* strain (harbored in broad-host-range cosmid pVK100 and maintained in *Escherichia coli* strain HB101) was mobilized into respiratory mutant CCMB1 following previously described tri-parental mating procedures that included helper strain *E. coli* HB101 (pRK2013) (16). To identify CCMB1 transconjugates with restored U(VI) reduction activity, Rif^r and Tet^r transconjugate colonies were screened for a positive U(VI) reduction phenotype on SM agar growth medium supplemented with U(VI)-carbonate (1 mM) (62). After screening approximately 1,000 transconjugates, a CCMB1 transconjugate (designated CCMB1-D14) with restored U(VI) reduction ability was identified and subsequently confirmed for wild-type U(VI) reduction activity in liquid culture.

Transconjugate CCMB1-D14 was found to contain a 34 kb complementing cosmid (designated pD14; see Figure 3.2 for subcloning strategy). D14 contained two internal *Hin*dIII restriction sites resulting in three *Hin*dIII fragments (D14-1, D14-2 and D14-3), each of which were ligated into cosmid pVK100. The three resulting recombinant cosmids were electroporated into *E. coli* HB101 and the three corresponding transformants were mated tri-parentally with HB101 (pRK2013) and CCMB1. Each

CCMB1 transconjugate was tested for anaerobic growth in liquid culture with U(VI) as electron acceptor. CCMB1 transconjugates containing complementing subclone D14-2 were restored for U(VI) reduction activity. Complementing subclone D14-2 was sequenced (6X coverage) with an ABI 3700 automated sequencer. Initial sequencing primers complementary to the unique cloning site of cosmid pVK100 included pvkF (gat cct ggt atc ggt ctg cga ttc cga ctc gtc) and pvkR (gta ctc ctg atg atg cat ggt tac tca cca ctg cga tcc). The nucleotide sequence of D14-2 has been submitted to the GenBank database under accession number DQ682922. Overlapping internal regions of D14-2 (designated DC1-to-10) were PCR-amplified and cloned into broad-host-range plasmid pBBR1MCS (34) for further genetic complementation analyses (see Table 3.1 for strains and plasmids used in the present study and Table 3.2 for primers and restriction sites used for subcloning). The altered nucleotide in mutant CCMB1 was identified via PCR amplification of the CCMB1 chromosomal region that corresponded to complementing fragment DC9 of wild-type *S. putrefaciens* (primers were identical to those used to PCR amplify the DC-9 region of wild-type *S. putrefaciens*). Nucleotide sequencing of the DC-9 region of CCMB1 was carried out as described above.

In-frame deletion mutagenesis of *ccmB*. *ccmB* was deleted in-frame from the *S. putrefaciens* chromosome according to recently described procedures (7). Briefly, 700 bp fragments (CCMB-A and CCMB-B, see Table 3.2) flanking *ccmB* were PCR-amplified with iProof™ high fidelity polymerase (Bio-Rad Laboratories, Hercules, CA), and CCMB-A and CCMB-B were fused by overlap

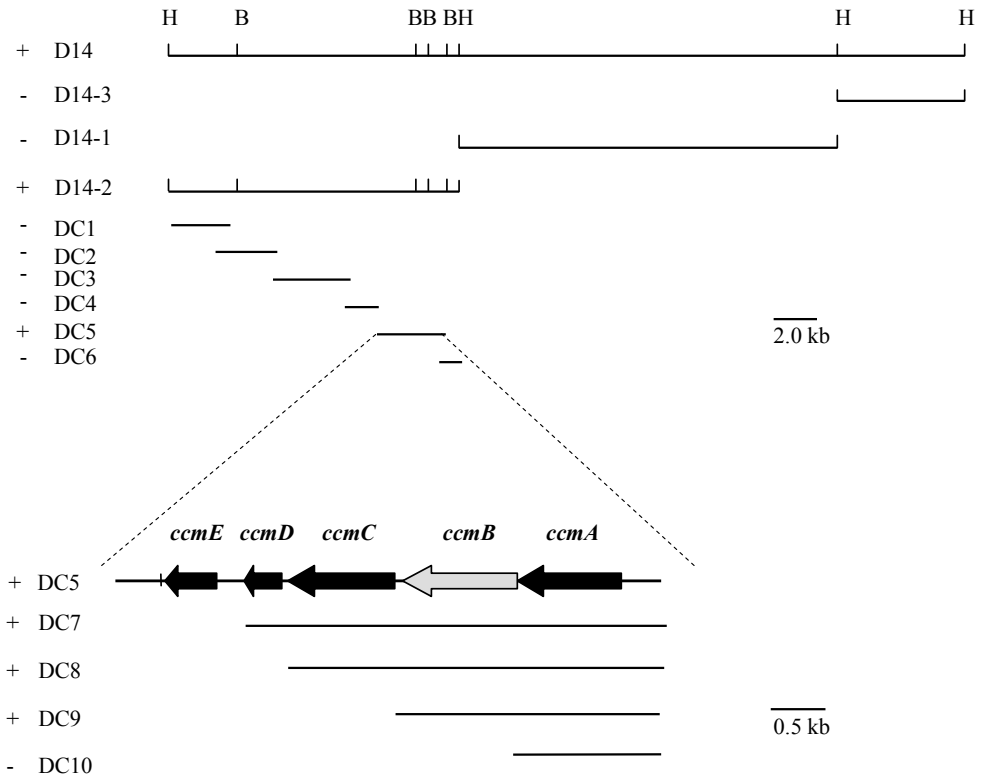


Figure 3.2. Schematic of subcloning strategy for CCMB1 complementing fragment D14. +, restores wild-type U(VI) reduction capability to CCMB1; -, does not restore wild-type U(VI) reduction capability to CCMB1. Fragments DC1-10 were PCR-amplified with primers designed from the D14-2 sequence (see Table 3.2). Restriction endonuclease cleavage sites H, *Hin*dIII; B, *Bam*HI. Note the different scale bars for fragment D14 and the amplicons of the *ccm* operon.

Table 3.1. Bacterial strains and plasmids used in the present study.

Strain or Plasmid	Description	Reference
Shewanella putrefaciens		
200R	Spontaneous Rif^r-derivative of *S. putrefaciens* (NCIB 12577)	(16)
CCMB1	U(VI) reduction-deficient *ccmB* point mutant (H108Y) of 200R	(62)
Δ*ccmB*	*ccmB* deletion mutant of 200R	This study
Escherichia coli		
S17-1	*thi pro recA hsdR* [RP4-2Tc::Mu-Km::Tn7] Mob$^+$	(57)
HB101	F$^-$ *hsdS20*(r$^-$ m$^-$) *recA13 ara-14 proA2 lacYl galK2 rpsL20 xyl-5 mtl-l supE44* λ, Sm^r	(5)
β2155	*ThrB1004 pro thi strA hsdS lacZDM15* (F ¢ *lacZDM15 lacIq trajD36 proA+ proB+*) Δ*dapA*::*erm pir*::RP4, Erm^r, Km^r	(14)
Plasmids		
pRK2013	ColE1 replicon containing RK2 transfer region; Km^r, Tra$^+$	(25)
pVK100	Broad-host-range cosmid, 23-kb, Tc^r Km^r Tra$^+$	(32)
pKO2.0	Suicide vector, 4.5-kb, R6K replicon, *mob*RP4, *sacB*, Gm^r	(7)
pD14	pVK100 with cloned 34-kb partially digested chromosomal *Hin*dIII fragment D14	This study
pD14-1	pVK100 with cloned 15-kb D14-derived *Hin*dIII subclone fragment D14-1	This study
pD14-2	pVK100 with cloned 13-kb D14-derived *Hin*dIII subclone fragment D14-2	This study
pD14-3	pVK100 with cloned 6-kb D14-derived *Hin*dIII subclone fragment D14-3	This study
pBBR1MCS	Broad-host-range cloning vector, 4.7-kb α-lac/multiple cloning site, Cm^r	(34)
pDC1-10	pBBR1MCS with cloned PCR-amplified fragments DC1 thru DC10	This study

Table 3.2. PCR primers used in the present study.

Fragment	Primer	Restriction Enzyme	Primer Sequence[a]	ORFs Included
DC1	DC1F	*Bam*HI	GACTGGATCCGTTACGTGAAGCAACCATG	*rpsH*, *rplF*, *rplR*, *rpsE*, *rpmD*, *rplO*
	DC1R	*Sal*I	GACTGTCGACCATGCTTAGGATGGTCCCGC	
DC2	DC2F	*Hin*dIII	GACTAAGCTTGAAGTCACCATCGCAAATCG	*rpmD*, *rplO*, *secY*, *rpmJ*
	DC2R	*Xho*I	GACTCTCGAGCTCACGACGTAAGTCACC	
DC3	DC3F	*Hin*dIII	GACTAAGCTTGCTCAAATAGATACACTACG	*rpsM*, *rpsK*, *rpsD*, transp
	DC3R	*Xho*I	GACTCTCGAGGCATTGTGCTGAGAGATCC	
DC4	DC4F	*Bam*HI	GACTGGATCCGCTGATCGTCGAGCTTTAC	transp, *rpoA*, *rplQ*
	DC4R	*Sal*I	GACTGTCGACGATGTTGTGAGGCTCTGAAC	
DC5	DC5F	*Hin*dIII	GACTAAGCTTGATTATCATTTAGTATAATTC	*ccmA*, *ccmB*, *ccmC*, *ccmD*, *ccmE*
	DC5R	*Xho*I	GACTCTCGAGCAATGTTCCTAATGCAAATAC	
DC6	DC6F	*Bam*HI	GACTGGATCCGAACCCTCTATCTCAAGCCG	*scyA*
	DC6R	*Sal*I	GACTGTCGACGACTAGCGTTGAAGTGATCG	
DC7[b]	DC7F	*Xho*I	GACTCTCGAGGAGCGCATACAGTAGAAGCG	*ccmA*, *ccmB*, *ccmC*, *ccmD*
DC8[b]	DC8F	*Xho*I	GACTCTCGAGCTGATAGAATCGAATTGC	*ccmA*, *ccmB*, *ccmC*
DC9[b]	DC9F	*Xho*I	GACTCTCGAGCGTTCAGGATCCGCATA	*ccmA*, *ccmB*
DC10[b]	DC10F	*Xho*I	GACTCTCGAGCTGAGTAAAGCTGATGCC	*ccmA*
CCMB-A	CCMB-A1	*Sal*I	GACTGTCGACCAACATCTCTGCTGGCATCGT	partial *ccmC*
	CCMB-A2	none	GTCTAGATTATCGCTTCATATAAGGTCAAGCATAGTACTAACTAAAATGTGGAAATGGTTACACCC	
CCMB-B	CCMB-B1	none	GGGTGTAACCATTTCCACATTTTAGTTAGTACTATGCTTGACCTTATATGAAGCGATAATCTAGAC	partial *ccmA*
	CCMB-B2	*Spe*I	GACTACTAGTGGTTATTGGAACCACATCAAATTGCTG	

[a] Restriction endonuclease recognition sites are underlined
[b] Reverse primer sequence identical to that used with fragment DC5

extension PCR generating fragment CCMB-C. CCMB-C was cloned into pKO2.0 with *Bam*HI and *Spe*I restriction endonucleases and electroporated into *E. coli* strain β2155. pKO2.0-CCMB-C was mobilized into recipient 200R via bi-parental mating. A plasmid integrant was identified by PCR analysis and the mutation was resolved on LB agar containing sucrose (10%). The in-frame deletion of *ccmB* in *S. putrefaciens* (Δ*ccmB*) was verified by PCR amplification with primers flanking the deletion (forward, CGCTTGTTATGATGAGTACCG and reverse, CCTTGGTGGAGGCAGACTCAT) and DNA sequencing. A schematic of the in-frame deletion mutagenesis procedure is given in Figure 3.3.

Analytical techniques. Cell growth was monitored by simultaneously measuring cell number and electron acceptor depletion or end product production. Acridine orange-stained cells were counted directly via epifluorescence microscopy (Nikon Diaphot 300 microscope). Fe(III) reduction was monitored by measuring HCl-extractable Fe(II) with the ferrozine technique (59). MnO_2 reduction was monitored by measuring MnO_2 depletion with the benzidine blue colorimetric assay (6). Mn(III)-pyrophosphate depletion was monitored spectrophotometrically at 480 nm (33). U(VI) was measured colorimetrically with Arsenazo III reagent (35). NO_2^- was measured spectrophotometrically with sulfanilic acid-*N*-1-naphthylethylene-diamine dihydrochloride reagent (42). Growth on O_2, TMAO, DMSO, fumarate, $S_2O_3^{2-}$ and SO_3^{2-} was monitored by cell growth only. Free thiol equivalents [exposed thiol functional groups (-SH) free to react with Ellman's reagent] were determined with 5,5'-dithio-bis-2-nitrobenzoic acid (DTMB) (19) standardized with reduced glutathione per μg protein.

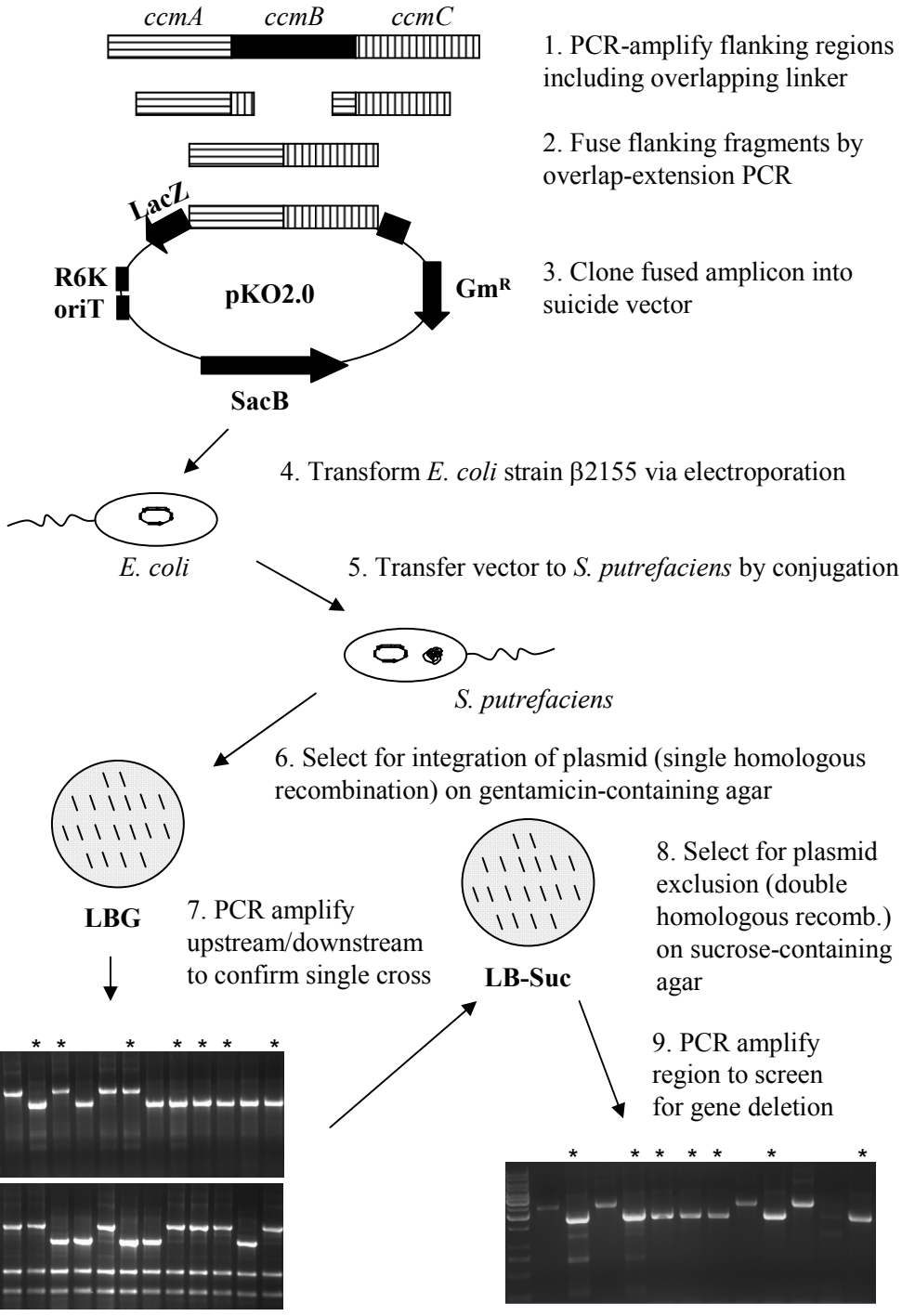


Figure 3.3. In-frame *ccmB* deletion mutagenesis strategy.

Protein concentration was determined with Coomassie Plus Bradford assay (Pierce Biotechnology) standardized with bovine serum albumen (BSA). Homologous and orthologous protein sequences were identified by BLAST analysis and aligned with ClustalW (1, 29). Membrane topology was predicted *in silico* with worldwide web-interfaced software HMMTOP and TopPred2 (10).

Cytochrome detection. Wild-type and CCMB1 mutant cell cultures were grown on the array of electron acceptors in SM liquid medium supplemented with lactate (30 mM) as carbon and energy source. Cells were harvested during late logarithmic growth phase and washed three times in anaerobic phosphate-buffered saline (PBS; 130 mM NaCl, 50 mM sodium phosphate, pH 7.2, 4°C). Cell extracts were prepared as in previously described procedures (20). Briefly, cells were resuspended in cold extraction buffer (50 mM sodium phosphate, 2 mg/ml polymyxin B sulfate, 300 mM NaCl, 5 mM EDTA, pH 7.2, 4°C). The suspension was stirred gently for 1 h at 4°C and centrifuged (Beckman Coulter Optima L-100 XP Ultracentrifuge 40,000 x *g* for 20 min at 4°C). Extracts from each sample were collected and specific cytochrome content was determined by measuring dithionite-reduced-minus-ferricyanide-oxidized difference spectra on a Shimadzu UV-1601 spectrophotometer operated in the split-beam mode. Estimates of total cytochrome content were based on the difference between absorbance values at the peak and trough of the Soret peak standardized per mg total protein (11, 45). Protein fractions were separated by SDS-PAGE on a linear gradient gel (4% to 20% resolving) with 200 μg/ml total protein loaded per well (36). Gels were stained for heme peroxidase activity by incubating in a solution containing 3, 3-dimethoxybenzidine-peroxide (1 μg ml^{-1}) and H_2O_2 (30% stock solution, 0.0025 μl ml^{-1}) (26).

Results

Genetic complementation analysis of *S. putrefaciens* respiratory mutant CCMB1. A transconjugate (designated CCMB1-D14) displaying wild-type U(VI) reduction activity was identified by re-applying the respiratory mutant screen that was originally used to identify U(VI) reduction-deficient mutant CCMB1 (Figure 3.4) (62). Subsequent subcloning and anaerobic growth experiments demonstrated that only those subclones containing a wild-type copy of *ccmB* restored anaerobic growth capability to CCMB1 (Figure 3.2). The CCMB1 chromosomal region corresponding to the wild-type DC9 subclone was PCR-amplified and sequenced with 6-fold coverage. A single nucleotide transversion (H108Y) was identified in *ccmB* at amino acid position 108 (Figure 3.5).

Sequence analysis of *S. putrefaciens ccmB*. *ccmB* is the second gene in a five gene cluster that includes *ccmA-E* (Table 3.3). The *ccm* gene cluster is conserved among all

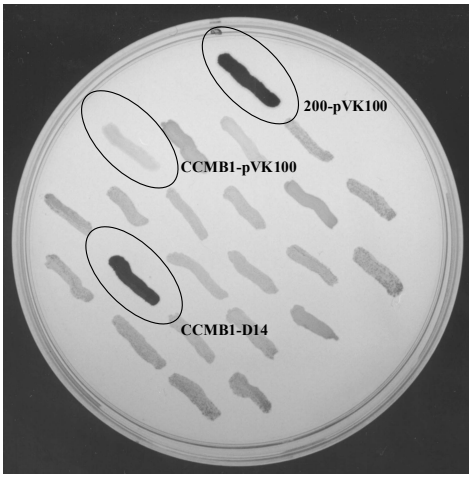


Figure 3.4. U(VI) reduction screening plate from which complementing transconjugate CCMB1-pD14 (second colony from left in fourth row) was identified. Strain 200-pVK100 (colony in top row) and CCMB1-pVK100 (first colony from left in second row) were included as U(VI) reduction-positive and U(VI) reduction-negative control strains, respectively.

Shewanella species sequenced to date and, in *S. putrefaciens* and *S. oneidensis* MR-1, is flanked by divergently transcribed *c*-type cytochrome *scyA* and an operon encoding RNA polymerase subunits. Gene assignments are based on The Institute for Genomic Research's annotation of the MR-1 genome sequence (www.tigr.org) (28). The *ccm* gene cluster has been studied most extensively in *E. coli*, and the translated amino acid sequences from the *ccmABCDE* genes of *S. putrefaciens* share high similarity with these proteins (40-61% identity, 62-74% similarity; Table 3.3).

Topology prediction analysis indicated that CcmB contains six transmembrane helices (Figure 3.5). Based on similarity to previous results of C-terminal tagging analysis and prior topology models in *E. coli*, the N- and C-termini of CcmB in *S. putrefaciens* are both predicted to face the cytoplasm (13, 27). Residue H108 is predicted to reside at the interface of a cytoplasmic loop and the third transmembrane helix. Residue H108 of *S. putrefaciens* CcmB is highly conserved among α-, β- and γ- proteobacteria (Figure 3.5) and is one of six CcmB residues strictly conserved among several land-plant mitochondrial orthologs such as those found in *Marchantia polymorpha*, *Arabidopsis thaliana*, *Triticum aestivum* and *Oenothera berteriana* and in the red alga *Cyanidioschyzon merolae* (22).

Respiratory mutant CCMB1 retains the ability to respire on electron acceptors with high (but not low) reduction potentials (E'_0). To determine the overall respiratory capability of CCMB1, anaerobic growth experiments were carried out on a set of 13 electron acceptors. CCMB1 retained the ability to grow at wild-type rates with O_2, MnO_2, Mn(III)-pyrophosphate, NO_3^-, and Fe(III)-citrate, yet was severely impaired for growth

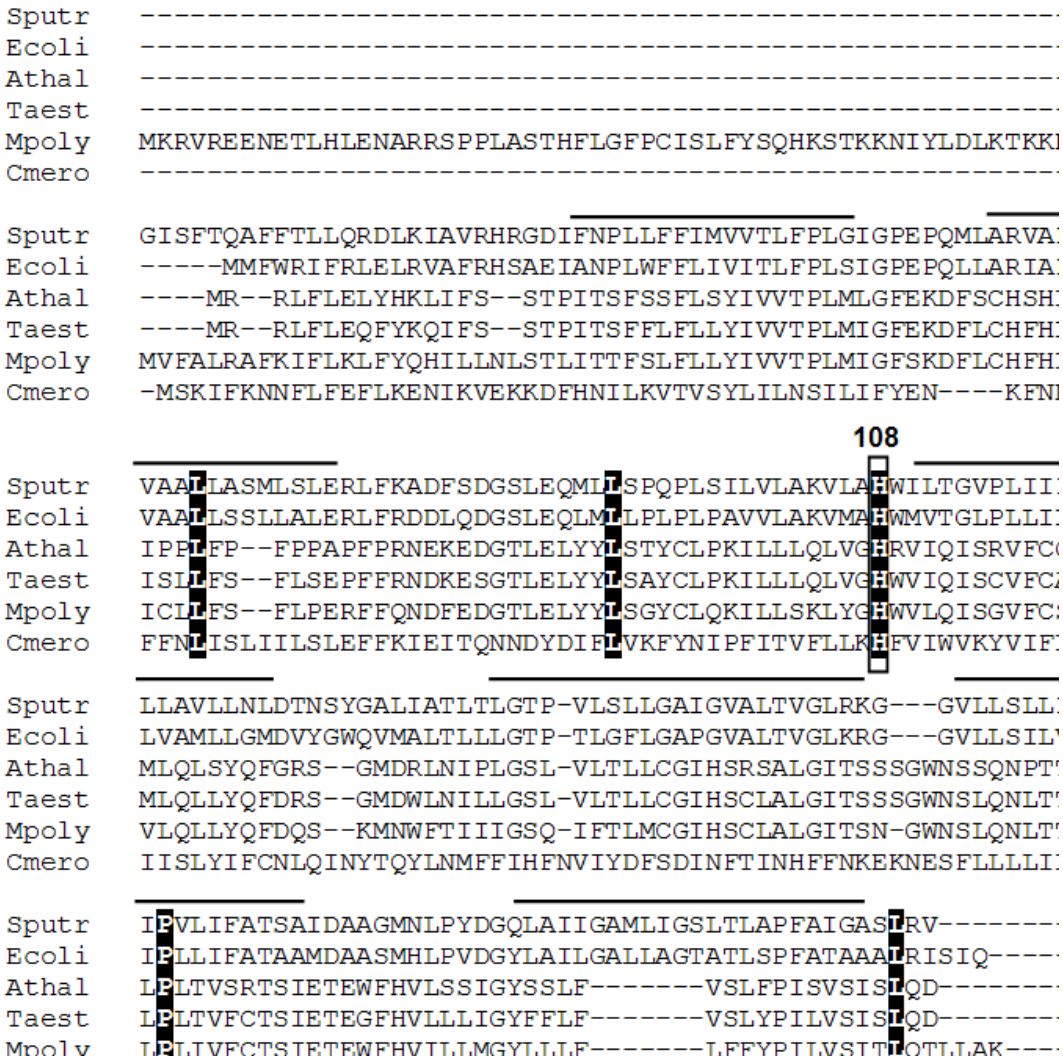


Figure 3.5. Multiple alignment of *S. putrefaciens* CcmB amino acid sequence with orthologs in three domains of life. *S. putrefaciens* (Sputr), *E. coli* (Ecoli), orthologous Ccb206 of *Arabidopsis thaliana* (Athal), Orf206 of *Triticum aestivum* (Taest), Orf277 of *Marchantia polymorpha* (Mpoly) and YejV of *Cyanidioschyzon merolae* (Cmero). Identical residues are highlighted. H108 of *S. putrefaciens* and corresponding identical residues are boxed. Predicted transmembrane domains in *S. putrefaciens* are indicated by bars above the sequence.

Table 3.3. Sequence analysis of *S. putrefaciens* CcmABCDE gene cluster.

ORF[d]	*Shewanella* spp.[a]			*Escherichia coli*[b]			GenBank[c]				
	Sim	ID	E-value	Sim	ID	E-value	Best Hit	Sim	ID	E-value	Putative Function
CcmA	84-100	71-100	10^{-101} - 10^{-122}	63	46	10^{-48}	*Colwellia psychrerythraea*	78	64	10^{-75}	ABC Transporter, ATP-binding subunit
CcmB	96-100	82-100	10^{-82} - 10^{-97}	73	61	10^{-73}	*Colwellia psychrerythraea*	87	72	10^{-90}	ABC Transporter, permease subunit
CcmC	93-100	84-99	10^{-96} - 10^{-134}	74	60	10^{-78}	*Vibrio fischeri*	83	66	10^{-99}	Heme delivery subunit; required for CcmE heme attachment
CcmD	81-100	69-100	10^{-19} - 10^{-32}	62	40	10^{-6}	*Mannheimia succiniciproducens*	71	57	10^{-12}	Membrane protein; CcmCDE complex assembly
CcmE	86-100	81-100	10^{-40} - 10^{-51}	69	54	10^{-18}	*Vibrio cholerae*	77	66	10^{-26}	Heme chaperone; covalently binds heme

[a] Percent similarity (Sim), percent identity (ID) and expect value (E-value) between *S. putrefaciens* Ccm amino acid sequences translated from sequenced clone library fragment D14-2. Ranges were determined for *S. putrefaciens* compared to the completed genome of *S. oneidensi*s MR-1 and draft genomes of *S. amazonensis* SB2B, *S. baltica* OS155, *S. denitrificans* OS217, *S. frigidimarina* NCIMB 400, *S. putrefaciens* CN-32, *Shewanella* sp. ANA-3, *Shewanella* sp. MR-4, *Shewanella* sp. PV-4 and *Shewanella* W3-18-1.

[b] Percent similarity (Sim), percent identity (ID) and expect value (E-value) from *S. putrefaciens* compared to *E. coli*

[c] Organism outside the genus *Shewanella* with the ortholog of highest similarity to *S. putrefaciens* (Best Hit) determined by BLASTP analysis of the GenBank non-redundant database

[d] *S. putrefaciens* open reading frames translated from chromosomal DNA fragment D14-2.

on NO_2^-, U(VI), DMSO, TMAO, fumarate, Fe(III)-oxide, SO_3^{2-} or $S_2O_3^{2-}$ as electron acceptor (Figure 3.6 and Figure 3.7). CCMB1 transconjugates containing plasmid pDC9 *in trans* displayed wild-type rates of growth and reduction of each electron acceptor. CCMB1 was unable to grow at wild-type rates on electron acceptors with E'_0 values below a threshold value of approximately 0.36 V (NO_2^-/NH_4^+ couple) (Figure 3.8). CCMB1 grew at wild-type rates on NO_3^- yet was unable to grow on NO_2^-. After stoichiometric reduction of NO_3^- to NO_2^-, growth of the wild-type strain continued on NO_2^- until all NO_2^- was depleted. CCMB1, on the other hand, was able to grow at wild-type rates on NO_3^- and to stoichiometrically convert NO_3^- to NO_2^- at a rate similar to the wild-type strain, yet was unable to sustain growth or deplete NO_2^- after all NO_3^- was reduced. CCMB1 cell density and NO_2^- concentrations remained unchanged after NO_3^- was completely reduced to NO_2^-.

CCMB1 contains low *c*-type cytochrome content. CCMB1 retained the ability to grow anaerobically on electron acceptors with high E'_0 [MnO_2, Mn(III)-pyrophosphate, NO_3^- and Fe(III)-citrate]. To determine if CCMB1 produced *c*-type cytochromes at wild-type levels during growth on these electron acceptors, the cytochrome content of CCMB1 cell extracts was determined via reduced-minus-oxidized difference spectra and by staining SDS-PAGE gels for covalently attached heme (Figure 3.9 and Table 3.4). For comparison, wild-type *S. putrefaciens* cells were grown and extracts were prepared under identical conditions. Prominent *c*-type cytochrome bands in the heme stain and pronounced reduced-minus-oxidized difference spectra at 552 nm were observed for wild-type cells grown on Fe(III)-citrate and fumarate. The cytochrome content of CCMB1, however, was nearly undetectable for cells grown on any electron acceptor

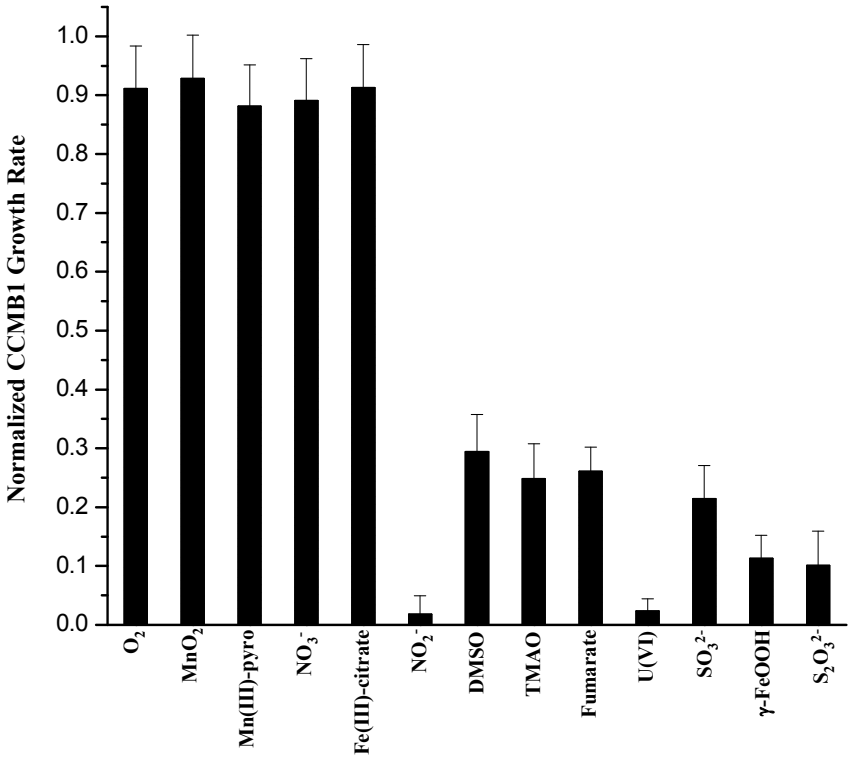


Figure 3.6. Growth rate of CCMB1 on a set of 13 electron acceptors (rates normalized to the wild-type strain *S. putrefaciens*). Wild-type growth rates (h^{-1}): O_2 (1.39), MnO_2 (0.71), Mn(III)-pyrophosphate (0.32), NO_3^- (0.38), Fe(III)-citrate (0.21), NO_2^- (0.23), DMSO (0.87), TMAO (0.36), fumarate (0.91), U(VI) (0.04), SO_3^{2-} (0.18), γ-FeOOH (0.25), $S_2O_3^{2-}$ (0.14). See Figure 3.7 for individual growth curves. Error bars represent the standard deviation of three parallel yet independent incubations.

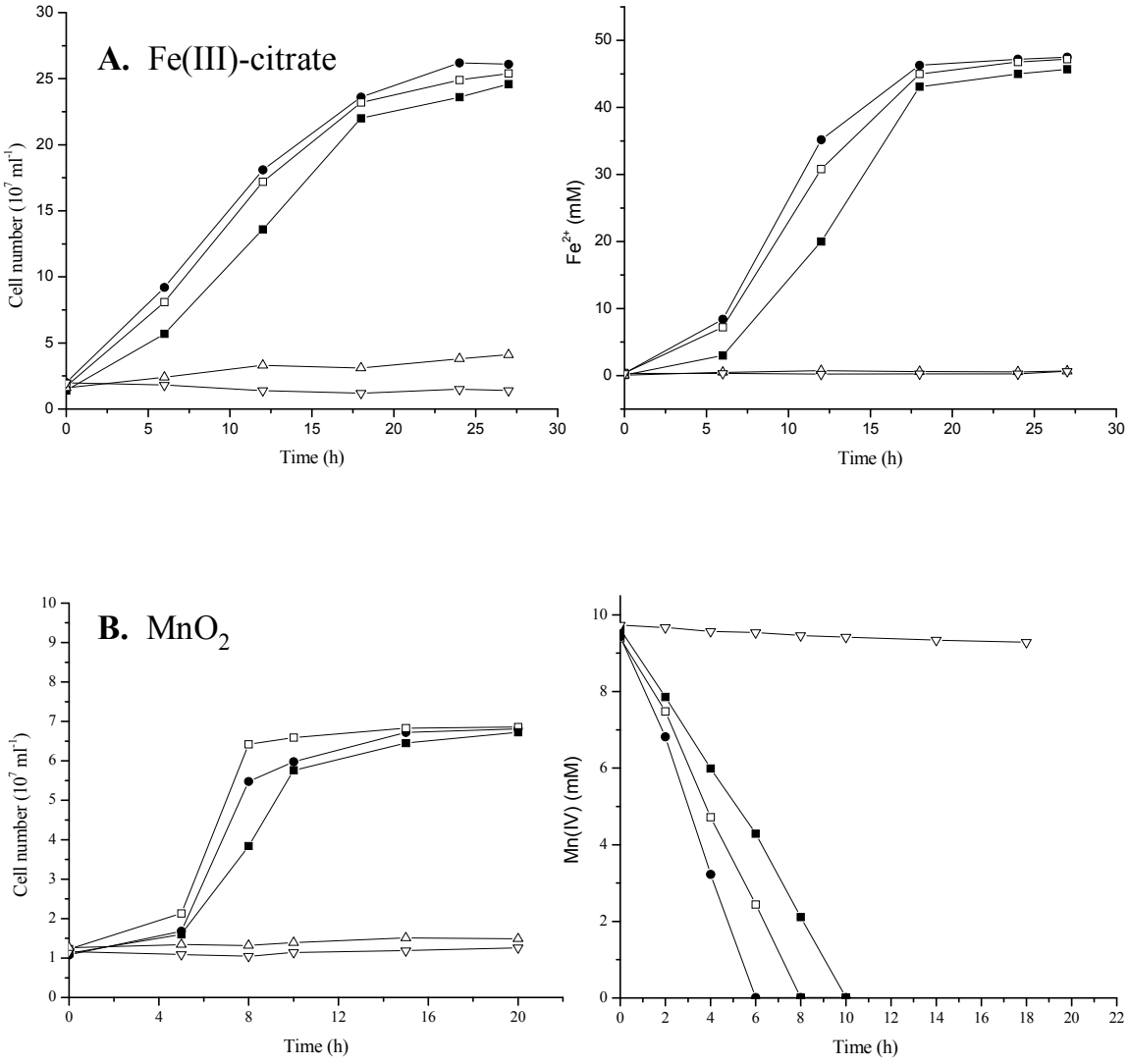


Figure 3.7. Anaerobic growth and corresponding electron acceptor depletion or end-product production of wild-type and mutant CCMB1 strains. A (Fe(III)-citrate), B (MnO_2), C [Mn(III)-pyrophosphate)], D (NO_3^-), E (γ-FeOOH), F [U(VI)], G (O_2), H (DMSO), I (TMAO), J (fumarate), K ($S_2O_3^{2-}$) and L (SO_3^{2-}). *S. putrefaciens* (pBBR1MCS) (-●-), mutant strain CCMB1 (pBBR1MCS) (-■-), CCMB1 (pDC9) (-□-). *S. putrefaciens* with electron acceptor omitted (-△-) and heat-killed cells (inoculum held at 80°C for 30 min prior to inoculation) (-▽-) were included as negative controls.

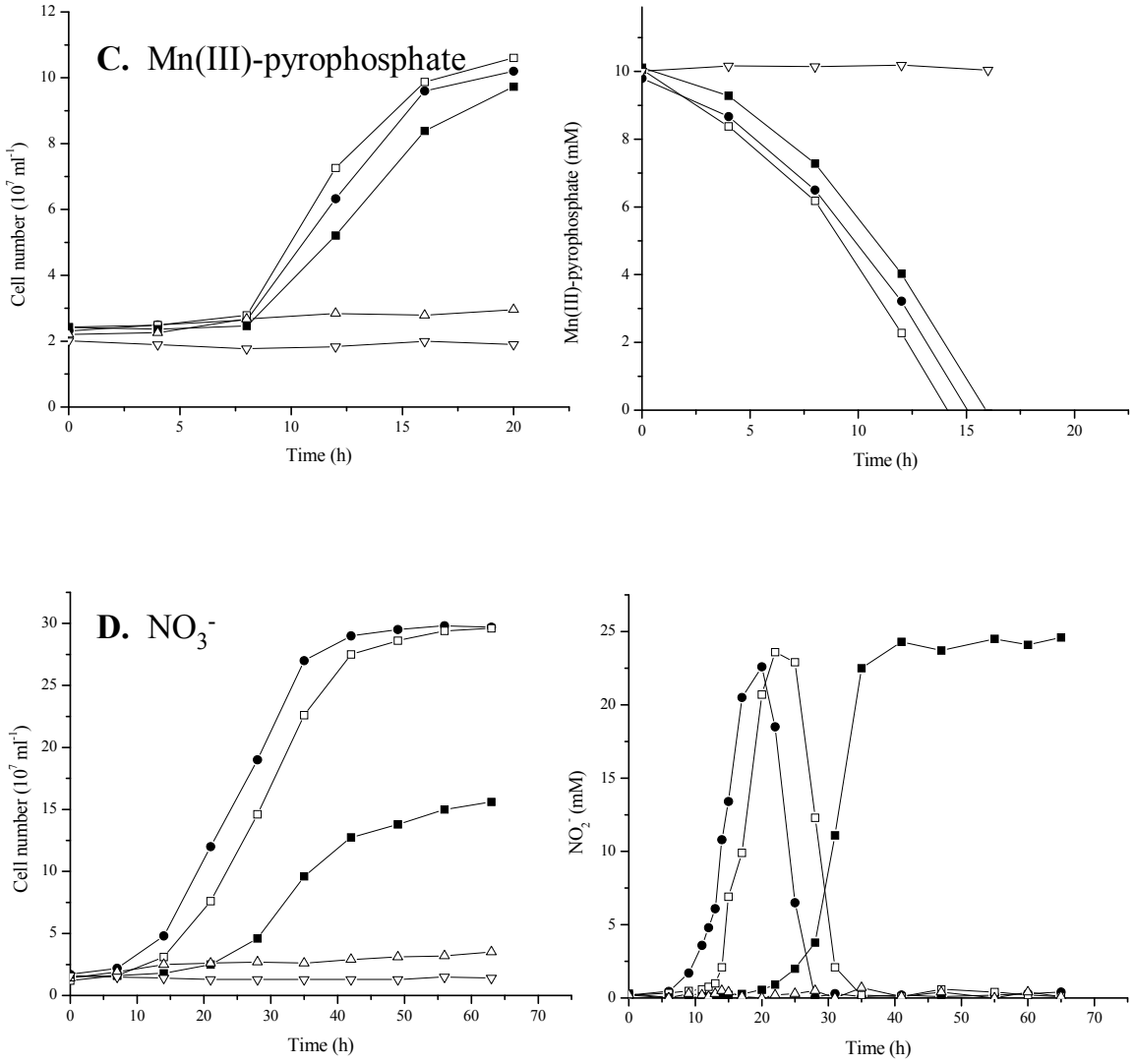
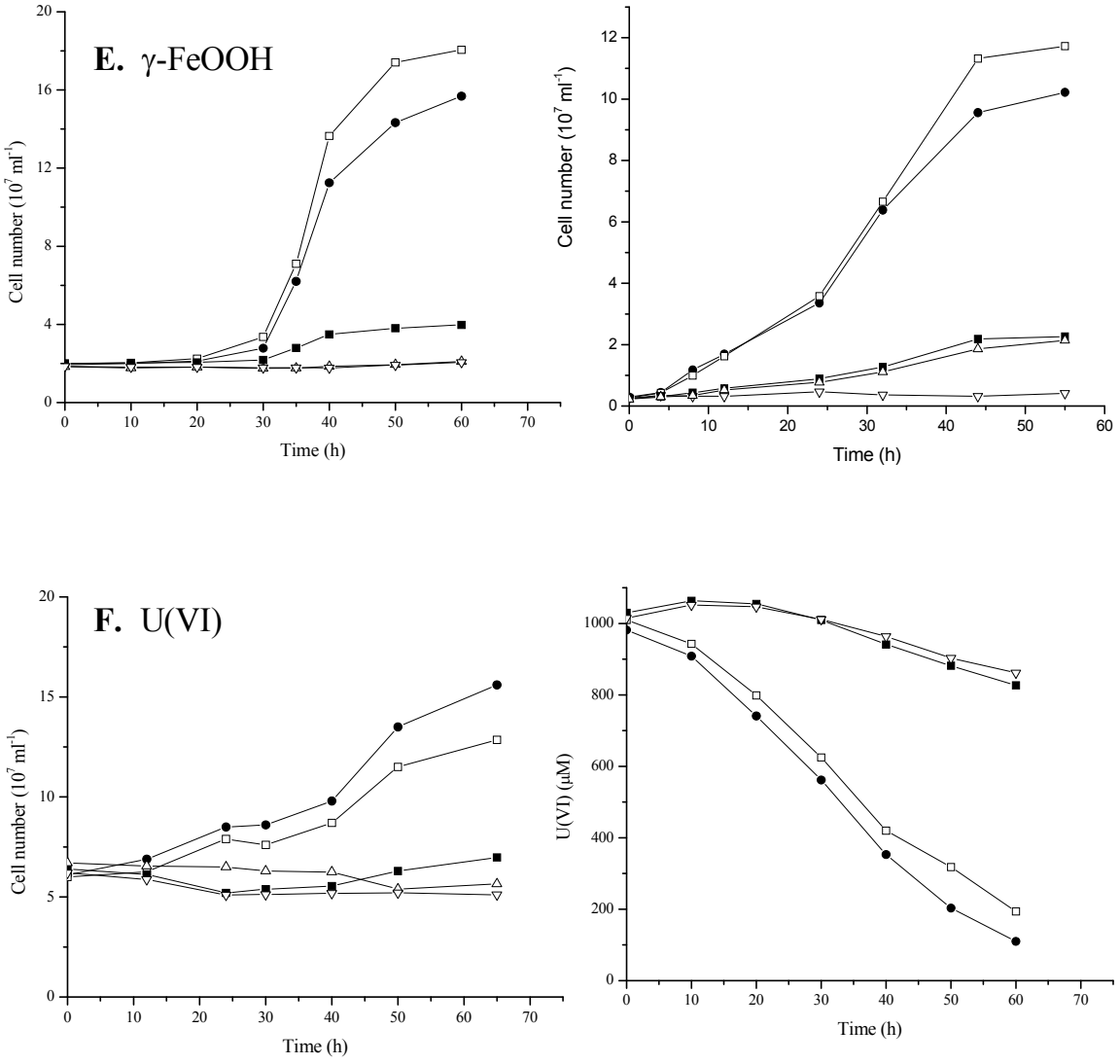


Figure 3.7 continued.



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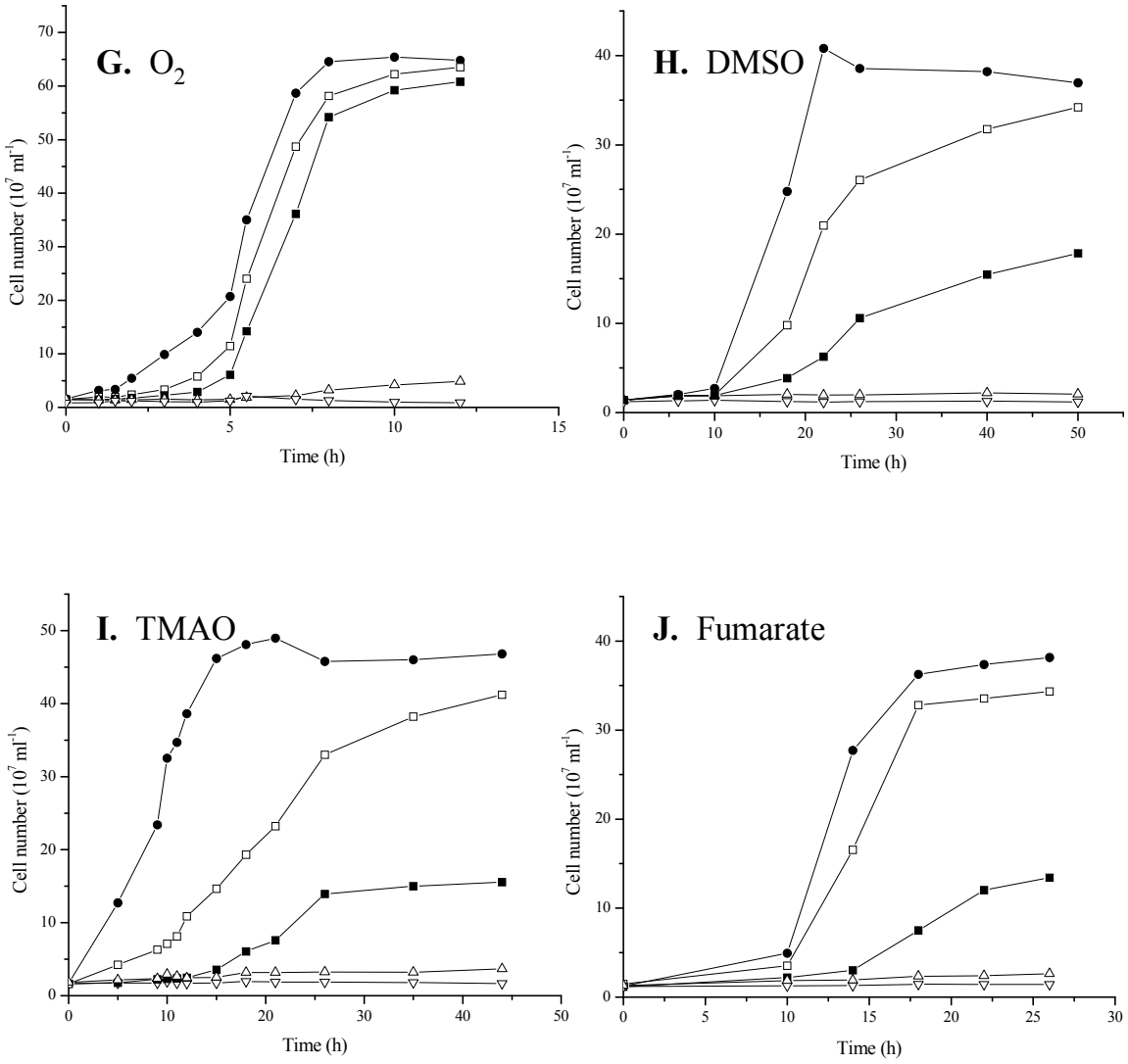

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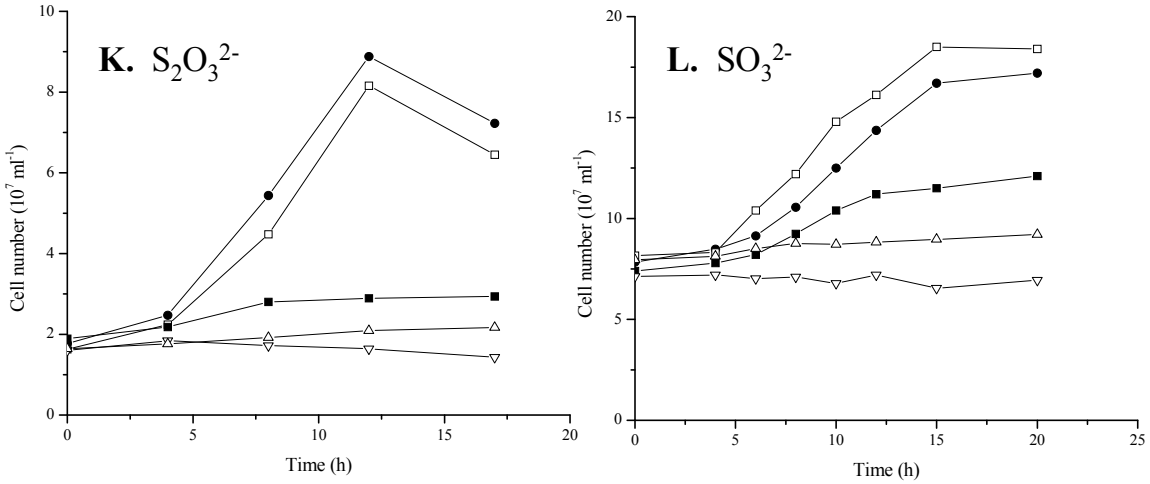


Figure 3.7 continued.

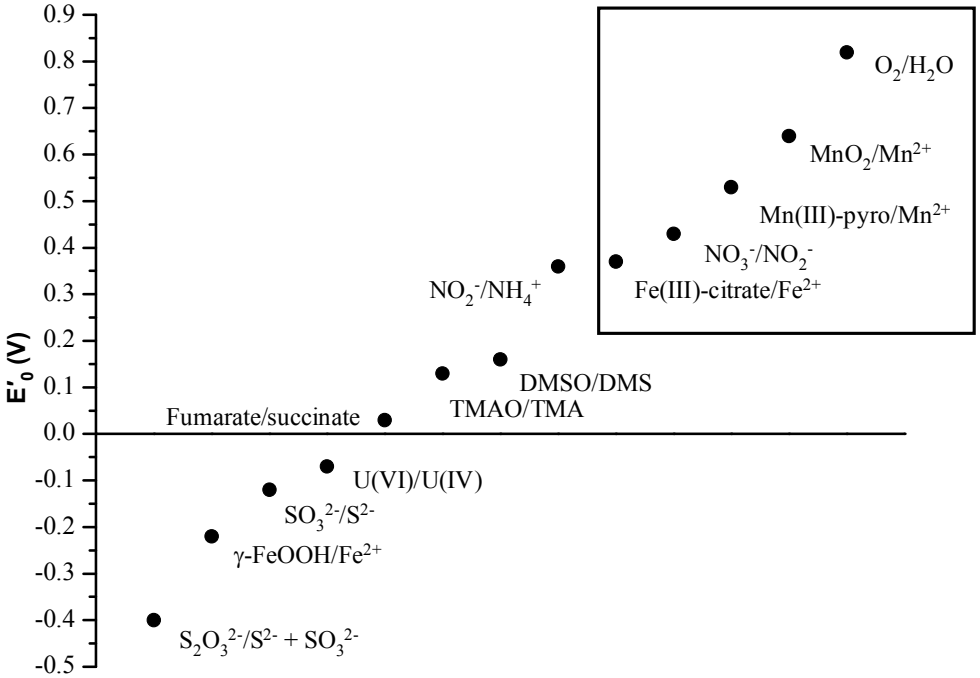


Figure 3.8. Reduction potential (E'_0) of electron acceptors included in the present study. E'_0 values were obtained from published values for the indicated couples at neutral pH. CCMB1 displayed wild-type growth rates on the set of electron acceptors that are boxed.

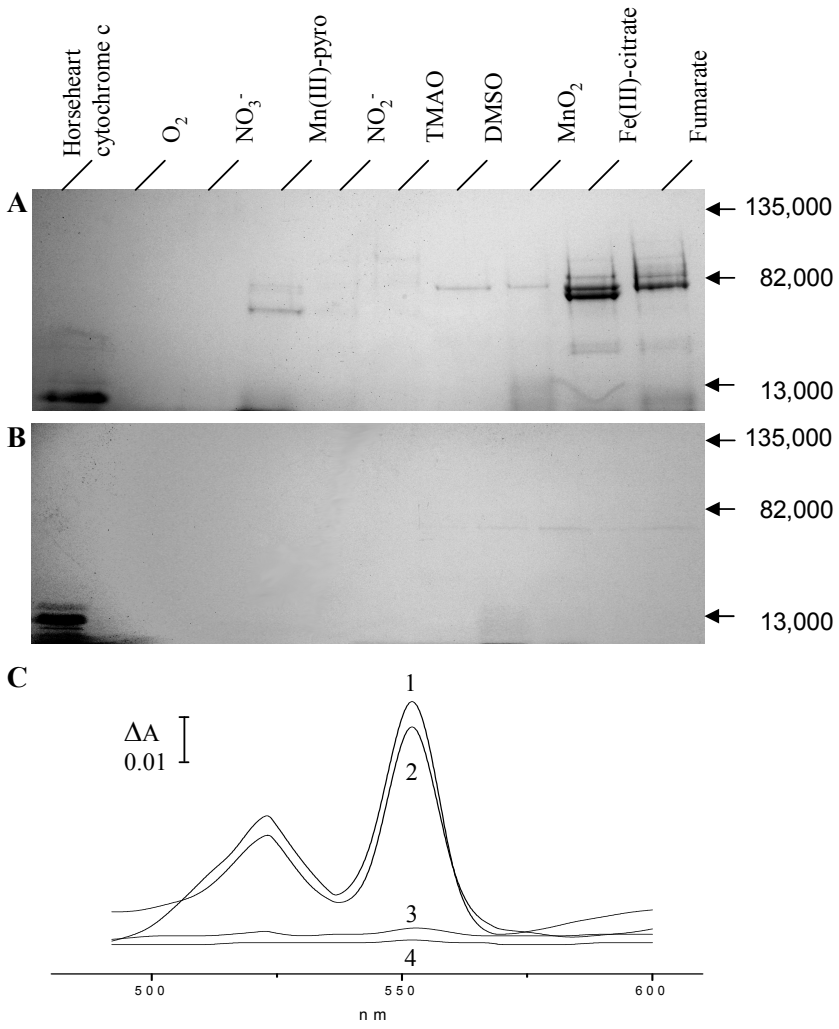


Figure 3.9. Analysis of cytochrome content of wild-type *S. putrefaciens* and respiratory mutant CCMB1. SDS-PAGE heme stains of periplasmic fractions of *S. putrefaciens* (A) and CCMB1 (B) cells grown on the indicated electron acceptors. Horse heart cytochrome *c* was included as a positive control. Molecular mass markers (Daltons) are indicated with arrows. Reduced-minus-oxidized difference spectra (C) of cell extracts from *S. putrefaciens* (1, 2) and CCMB1 (3, 4) grown on Fe(III)-citrate (1, 3) and fumarate (2, 4). Scale bar corresponds to absorbance units.

Table 3.4. Mean specific cytochrome content[a] in *S. putrefaciens* and CCMB1

Electron acceptor added to the growth medium	***S. putrefaciens***	**CCMB1**
O_2	0.04 ± 0.01	< 0.01[b]
MnO_2	0.09 ± 0.02	< 0.01[b]
Mn(III)-pyrophosphate	0.18 ± 0.02	< 0.01[b]
NO_3^-	0.12 ± 0.02	< 0.01[b]
Fe(III)-citrate	0.43 ± 0.07	0.04 ± 0.01
NO_2^-	0.11 ± 0.02	ND
DMSO	0.29 ± 0.03	ND
TMAO	0.14 ± 0.01	ND
Fumarate	0.37 ± 0.05	ND
U(VI)	0.05 ± 0.01	ND
SO_3^{2-}	0.09 ± 0.02	ND
γ-FeOOH	0.07 ± 0.02	ND
$S_2O_3^{2-}$	0.13 ± 0.02	ND

[a] Mean values from periplasmic fractions expressed as the difference between the absorbances at the peak and trough of the Soret region from reduced-minus-oxidized difference spectra per mg protein ml^{-1}. ± standard deviation for three independent fractions is indicated
[b] Value was below detection limit
ND, not determined

(Figure 3.9). Wild-type levels of mature cytochrome *c* therefore, do not appear essential for anaerobic growth on electron acceptors with high E'_0.

Thiol content of the CCMB1 periplasm is greater than the wild-type strain. Previous studies with a variety of other proteobacteria containing *ccm* mutations indicated that periplasmic redox homeostasis was disrupted (2, 9). To determine if the periplasm of CCMB1 was more oxidizing or reducing than the wild-type strain, the thiol (-SH) content in periplasmic extracts of CCMB1 and wild-type *S. putrefaciens* cells was determined after growth on 13 alternate electron acceptors. The thiol content of periplasmic extracts from *S. putrefaciens* after growth on O_2, NO_3^-, NO_2^-, TMAO, DMSO, Mn(III)-pyrophosphate and MnO_2 ranged from 40-238 pmol μg protein^{-1} (Figure 3.10). Although CCMB1 grew on NO_2^-, TMAO and DMSO at rates less than 25% of the wild-type strain, the periplasmic thiol concentration of CCMB1 grown on the identical set of electron acceptors was slightly greater than the wild-type strain (Figure 3.10). Periplasmic fractions could not be recovered from CCMB1 cultures grown on U(VI), SO_3^{2-}, γ-FeOOH, or $S_2O_3^{2-}$ as electron acceptor due to low growth rates (and correspondingly low cell yields) under these growth conditions (data not shown). Periplasmic extracts from the wild-type strain contained the highest thiol content after growth on γ-FeOOH, SO_3^{2-}, or $S_2O_3^{2-}$ as electron acceptor, ranging from 682-1130 pmol μg protein^{-1} (approximately 5-10X greater than the other electron acceptors). Overall, the periplasmic extracts recovered from CCMB1 contained a slightly greater concentration of thiol groups than the wild-type strain.

CcmB deletion mutant Δ*ccmB* is incapable of anaerobic growth on any electron acceptor. An in-frame deletion mutant of *ccmB* (Δ*ccmB*) was constructed and subsequently tested

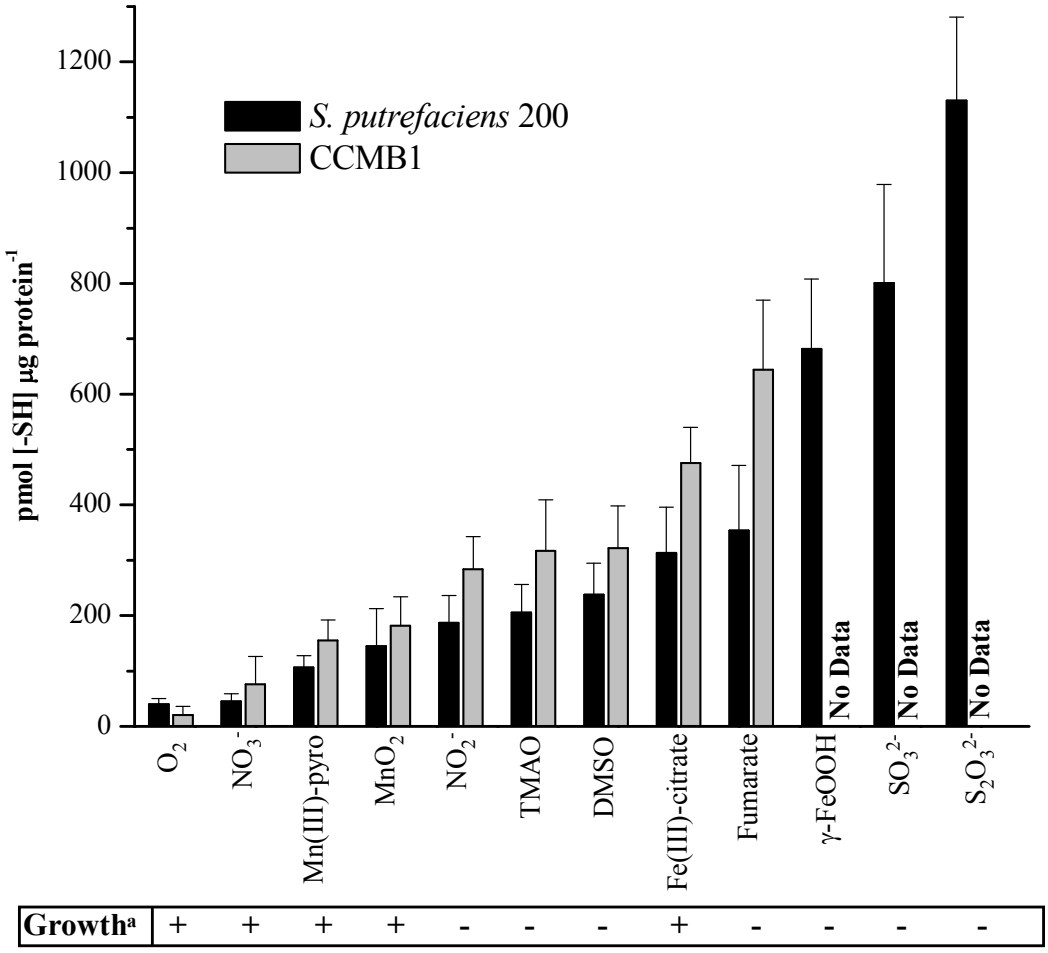


Figure 3.10. Free thiol content in periplasmic fractions of *S. putrefaciens* and CCMB1 cells grown on the indicated electron acceptors. Box below the bar graph indicates CCMB1 growth ability on the electron acceptor ([a]). +, growth rate > 80% wild-type; -, growth rate < 25% wild-type.

for growth on the entire suite of 13 electron acceptors respired by the wild-type strain. Δ*ccmB* was unable to grow anaerobically on any electron acceptor, yet retained the ability to grow aerobically (Figure 3.11). Transconjugates of Δ*ccmB*, containing a wild-type copy of *ccmB* in trans, were restored for the ability to respire at wild-type rates on all electron acceptors.

Discussion

The main objective of the present study was to identify the gene mutated in CCMB1, a *S. putrefaciens* respiratory mutant originally isolated for its inability to respire U(VI) as anaerobic electron acceptor. Subcloning and nucleotide sequence analyses indicate that CCMB1 contains a H108Y mutation in a protein similar to the CcmB permease subunit of an ABC transporter required for cytochrome c maturation in *E. coli* (System I). CcmABC works in concert with membrane protein CcmD and heme chaperone CcmE to transfer heme to apocytochrome *c* (60).

S. putrefaciens respiratory mutant CCMB1 grows on electron acceptors with E'_0 values greater than a threshold level of approximately 0.36 V [O_2, Fe(III)-citrate, MnO_2, Mn(III)-pyrophosphate and NO_3^-]. This finding is unexpected for several reasons. First, anaerobic respiration by *S. oneidensis* on Fe(III)-citrate, MnO_2 and NO_3^- as electron acceptor involves several *c*-type cytochromes including CymA, MtrA, MtrC, and OmcA (3, 43, 46, 52). Second, *ccmC* mutants of *S. oneidensis* are unable to produce mature cytochrome *c* or grow anaerobically on U(VI), fumarate, TMAO, DMSO, Fe(III)-citrate, MnO_2, NO_3^- or NO_2^- as electron acceptor (4, 41). Several possibilities may explain these differences in the anaerobic growth capability of the *Shewanella ccm* mutants: 1) *S. putrefaciens ccmB* and *S. oneidensis ccmC* mutant genotypes display different respiratory

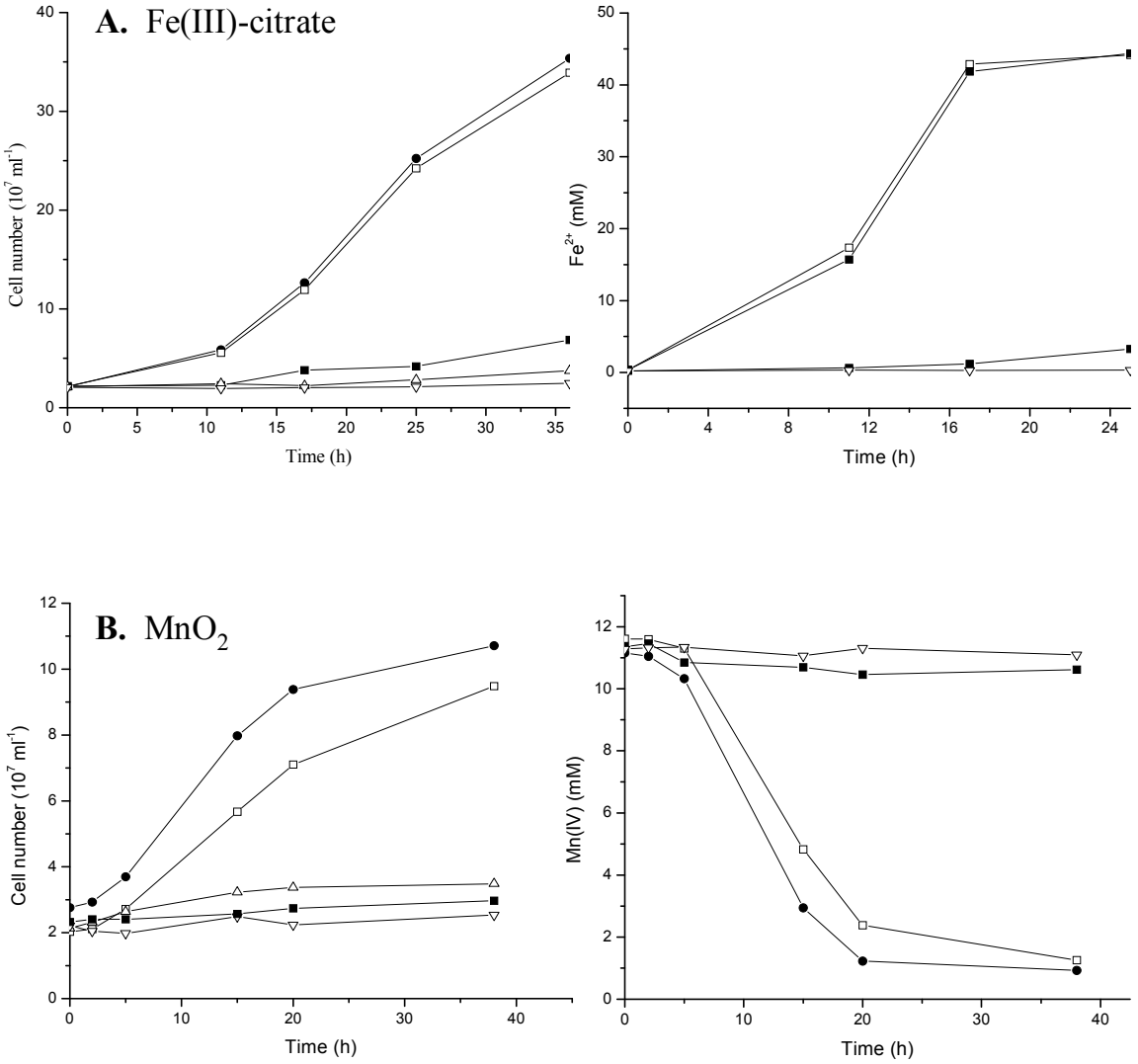


Figure 3.11. Anaerobic growth and corresponding electron acceptor depletion or end-product production of wild-type and mutant Δ*ccmB* strains. A (Fe(III)-citrate), B (MnO_2), C [Mn(III)-pyrophosphate)], D (NO_3^-), E (γ-FeOOH), F [U(VI)], G (O_2), H (DMSO), I (TMAO), J (fumarate), K ($S_2O_3^{2-}$) and L (SO_3^{2-}). *S. putrefaciens* (pBBR1MCS) (-●-), mutant strain Δ*ccmB* (pBBR1MCS) (-■-), mutant Δ*ccmB* (pDC9) (-□-). *S. putrefaciens* with electron acceptor omitted (-△-) and heat-killed cells (inoculum held at 80°C for 30 min prior to inoculation) (-▽-) were included as negative controls.

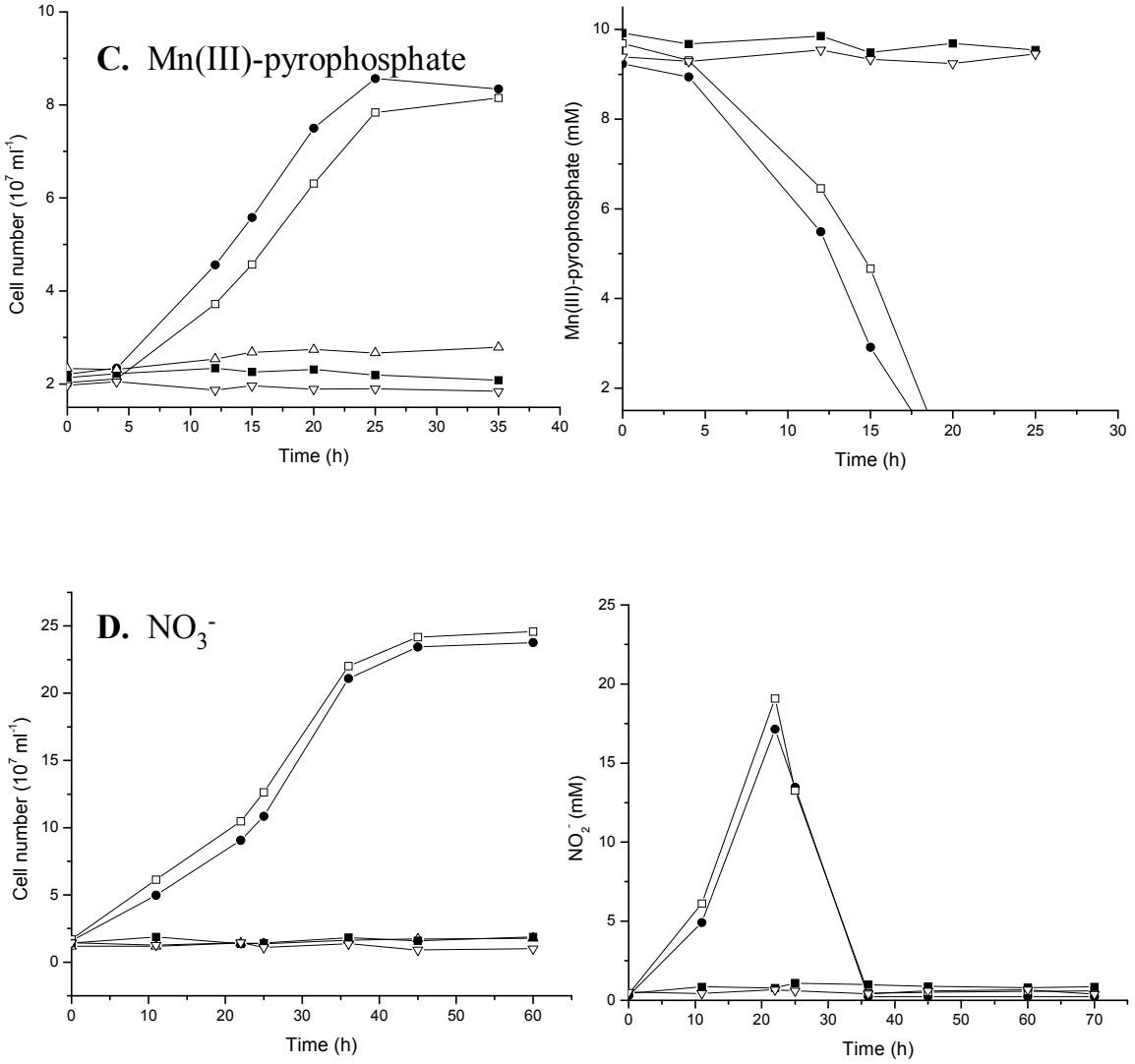
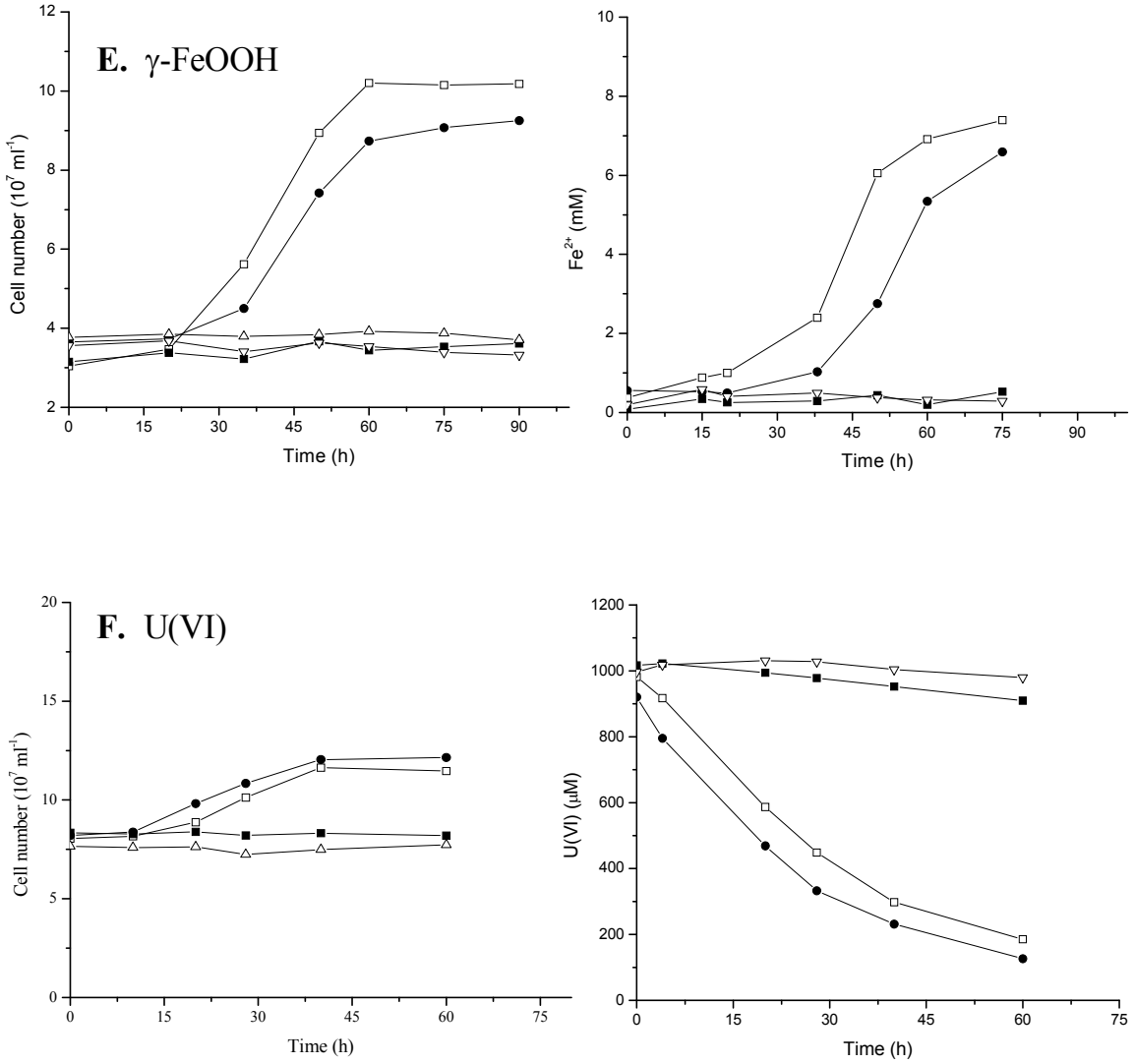


Figure 3.11 continued.



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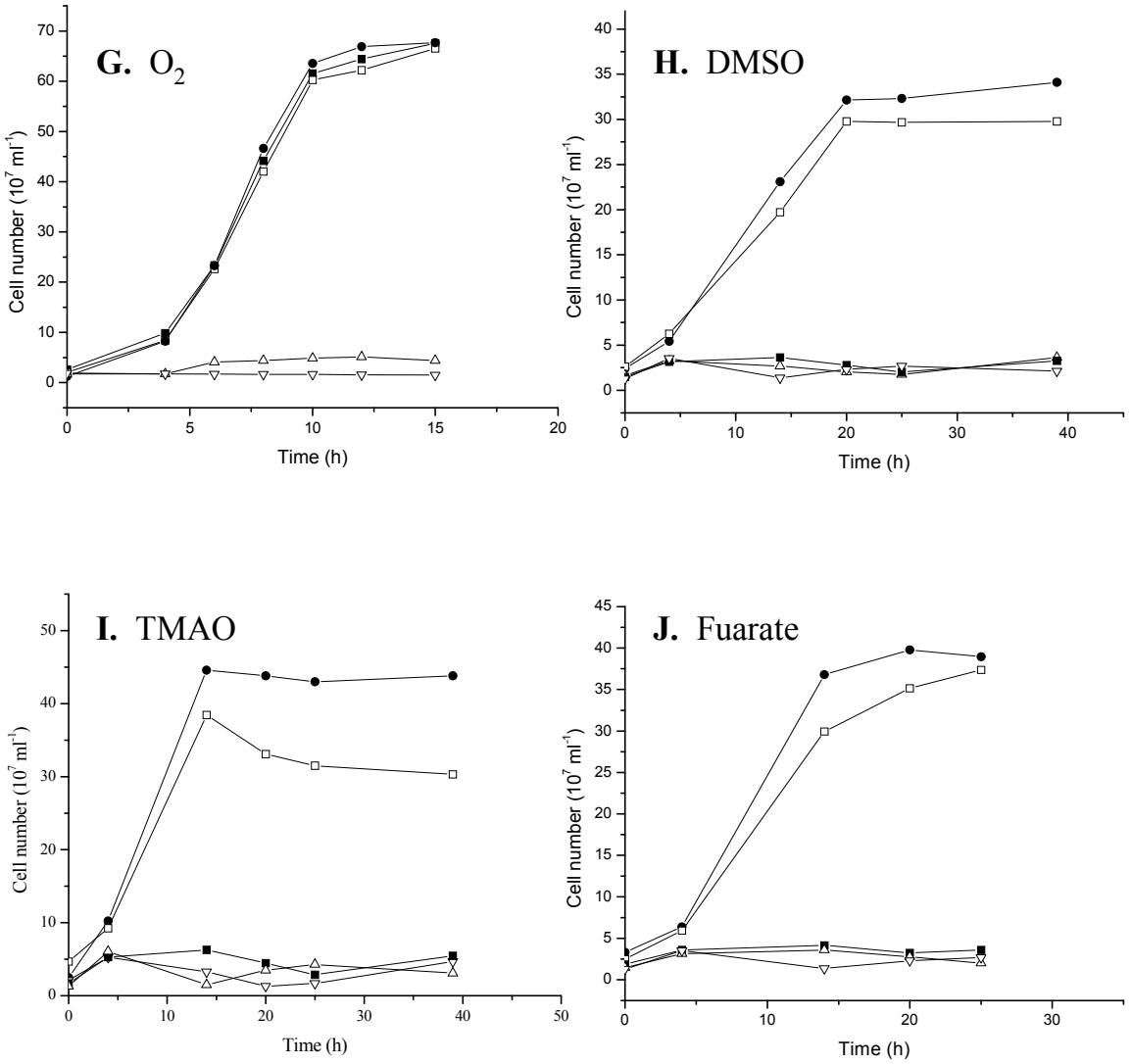

Figure 3.11 continued.

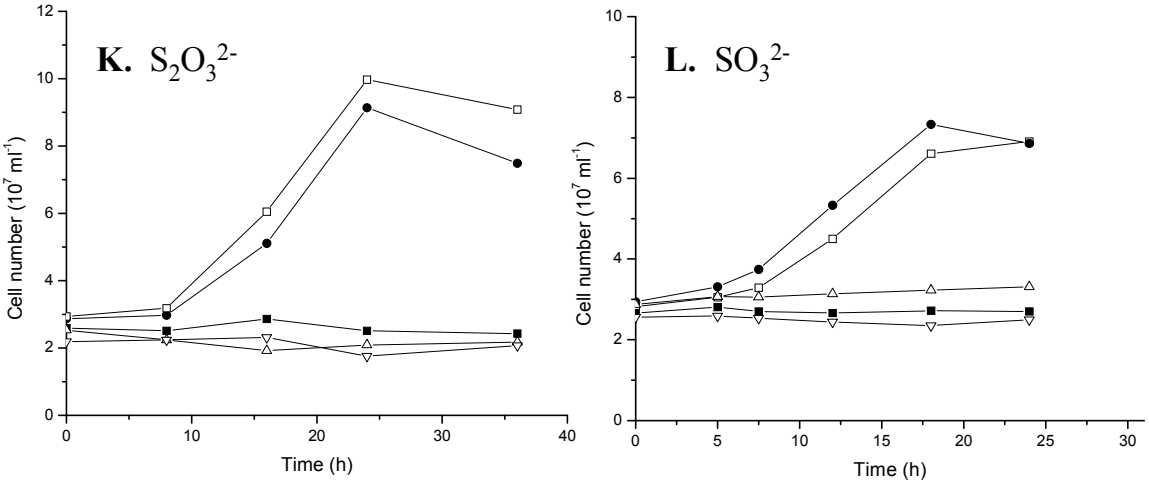

Figure 3.11 continued.

deficiencies, 2) *S. putrefaciens* contains cytochrome *c*-independent respiratory pathways, or 3) replacement of the histidine residue at position 108 with tyrosine permits limited, but sufficient, CcmB activity to sustain growth of CCMB1 on electron acceptors with high E'_0. Results from the present study favor the third possibility. Deletion mutant Δ*ccmB* does not respire anaerobically on any electron acceptor, indicating that anaerobic electron transport pathways in *S. putrefaciens* require mature *c*-type cytochromes. Point mutant CCMB1, on the other hand, retains growth on anaerobic electron acceptors with $E'_0 > 0.36$ V, despite lacking the ability to produce *c*-type cytochromes at detectable levels. These findings suggest that the H108Y CcmB mutant may retain partial Ccm activity, and produce low levels of mature cytochrome *c* that are adequate to sustain wild-type growth rates on electron acceptors above a threshold E'_0.

Mutations in *ccmA*, *ccmB* or *ccmC* in other bacteria often result in mutant phenotypes not associated with cytochrome *c* activity. *Legionella pneumophilia* CcmB and CcmC mutants, for example, display defective iron acquisition capability and aberrant virulence phenotypes (9, 47, 61). *Pseudomonas fluorescens* CcmC mutants are unable to complete synthesis of the siderophore pyoverdine (2). CcmC mutants of *Pantoea citrea*, *Gluconacetobacter diazotrophicus* and *Pseudomonas putida* are unable to oxidize gluconate and 2-ketogluconate, synthesize indole-3-acetic acid or isomerize (cis-trans) unsaturated fatty acids, respectively (30, 38, 53). The seemingly unrelated Ccm mutant phenotypes are postulated to reflect improper redox homeostasis in the periplasm (2, 9).

To determine if CcmB plays a similar role in maintaining periplasmic redox homeostasis in *S. putrefaciens*, CCMB1 and wild-type *S. putrefaciens* were grown on the

suite of 13 electron acceptors and free thiol content was determined in the corresponding periplasmic fractions. The wild-type strain contained approximately 100 pmol μg protein^{-1} free thiol after growth on O_2, NO_3^-, Mn(III)-pyrophosphate and Mn(IV). Free thiol content was approximately 2-3X greater after growth on NO_2^-, TMAO, DMSO, Fe(III)-citrate and fumarate, and approximately 10X greater after growth on γ-FeOOH, SO_3^{2-} and $S_2O_3^{2-}$. The pattern of free thiol content in the wild-type strain reflects the ability of CCMB1 to grow on electron acceptors with $E'_0 > 0.36$ V (NO_3^-/NO_2^- threshold). Periplasmic redox conditions corresponding to a free thiol content of > 300 pmol μg protein^{-1} require wild-type CcmB activity to sustain growth. These results parallel those obtained with *P. fluorescens ccmC* mutants that are unable to complete pyoverdine biosynthesis (2). The periplasm of the *P. fluorescens* mutant strain was abnormally reducing and contained high free thiol content.

Increased free thiol in the periplasm of CCMB1 after growth on electron acceptors with low E'_0 may result from several factors. First, the low redox poise associated with growth of CCMB1 on electron acceptors below a threshold E'_0 may prevent the oxidation of thiol by an CcmB transported allocrite such as an oxidant or free heme. In contrast, electron acceptors with high E'_0 may oxidize excess -SH in the periplasm of CCMB1 and thereby prevent deleterious accumulation of -SH. Second, increased periplasmic free thiol content in CCMB1 may reflect an overabundance of periplasmic apocytochrome *c*. The thioredoxin branch of Ccm may continue to reduce the thiols of the CXXCH motif in apocytochromes despite the inability of CCMB1 to deliver heme. Finally, CcmABC may mediate periplasmic redox homeostasis via an as yet unknown mechanism (2, 9).

The present study is the first to identify an amino acid residue critical for CcmB function. Residue H108 is conserved in > 88% of the 185 bacterial and land-plant mitochondrial CcmB orthologs currently available in the non-redundant database. Residue H108 may therefore play a pivotal role in CcmB activity. Membrane protein topology prediction in *S. putrefaciens* indicates that residue H108 is found at the interface of a hydrophobic transmembrane helix and a hydrophilic cytoplasmic loop, well situated for controlling CcmB complex formation with other Ccm subunits or for allocrite recognition and subsequent transport across the cytoplasmic membrane. The identity of the CcmB allocrite in *E. coli*, however, remains controversial. Initial studies in *E. coli* indicate that heme is the CcmB allocrite and that CcmABC functions as a high-affinity heme transporter (23, 24). More recent results suggest that *E. coli* CcmABC drives the release of holo-CcmE (CcmE with covalently attached heme) from CcmC by coupling the release with ATP hydrolysis (24). Current work is aimed at identifying the CcmABC allocrite and determining the role that H108 plays in cytochrome *c* maturation in *S. putrefaciens*.

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CHAPTER 4

Methionine functionally replaces conserved histidine H108 of cytochrome *c* maturation permease CcmB in *Shewanella putrefaciens* strain 200

Abstract

Shewanella putrefaciens strain 200 requires cytochrome *c* for anaerobic growth on a wide range of electron acceptors, including metals and radionuclides. *S. putrefaciens* employs the System I cytochrome *c* maturation pathway (Ccm) to attach the heme cofactor to apocytochrome *c*. Recent studies on uranium (U(VI)) respiration by *S. putrefaciens* demonstrated that a conserved histidine residue (H108) in the ABC-transporter permease CcmB was required for both Ccm and anaerobic growth on electron acceptors with mid-point redox potentials (E'_0) below a threshold value of 0.36V (15). In the present study, addition of cystine to the growth medium restored anaerobic growth, but not Ccm activity, to the previously generated mutant CCMB1 (H108Y random point mutation in CcmB). H108 was the first residue identified in a CcmB homolog that is required for function. To test the hypothesis that H108 confers the ability to transport heme across the cytoplasmic membrane to S. putrefaciens CcmB, H108 was replaced with the hydrophobic residues alanine (H108A) and leucine (H108L), charged and polar residues lysine (H108K) and tyrosine (H108Y) or methionine (H108M), a hydrophobic residue that coordinates heme iron in cytochrome *c* via site-directed mutagenesis. The site-directed mutants were grouped into three classes based on anaerobic growth

capability, cytochrome *c* content and periplasmic redox condition (measured by free thiol (-SH) content): (i) H108Y and H108K were unable to grow anaerobically on electron acceptors with $E'_0 < 0.36V$, produced periplasmic cytochrome *c* at levels < 10% of the wild-type strain and contained periplasmic (–SH) content at levels two-fold greater than the wild-type strain, (ii) H108A and H108L retained the ability to grow on electron acceptors with $E'_0 < 0.36V$, produced periplasmic cytochrome *c* at levels < 20% of the wild-type strain and contained periplasmic -SH content at levels 50% greater than the wild-type strain, and (iii) H108M retained the ability to grow on electron acceptors with $E'_0 < 0.36V$, produced periplasmic cytochrome *c* at near wild-type levels and contained wild-type levels of periplasmic SH. –SH concentrations correlated inversely with the anaerobic growth rates and periplasmic cytochrome *c* content of the site-directed mutants. Addition of cystine to the growth medium restored anaerobic growth capability to the H108Y and H108K mutants. These results demonstrate that *S. putrefaciens* CcmB requires either histidine or a hydrophobic residue capable of coordinating heme iron in a low spin state (i.e., methionine) at amino acid position 108 for maintenance of periplasmic redox homeostasis and production of mature cytochrome *c*.

Introduction

Cytochrome *c* is a key electron transport chain component for bacterial respiration and photosynthesis (20, 36, 72). Cytochrome *c* is distinguished from other cytochromes by the presence of covalent thioether bonds between vinyl groups in heme and cysteine residues in the characteristic CXXCH heme attachment motif of the apocytochrome. Bacteria, archaea and the mitochondria and chloroplasts of eukarya employ three known cytochrome *c* maturation (Ccm) systems for stereospecific covalent attachment of heme

to apocytochrome *c* (for reviews, see references (72), and (73)). System III Ccm is restricted to fungal and animal mitochondria and consists of a single heme lyase (CCHL). System II Ccm is found in gram-positive bacteria, β- and δ- proteobacteria, chloroplasts and cyanobacteria, and consists of four proteins (ResABC and CcdA) (39, 67, 72). System I Ccm is found in α- and γ- proteobacteria, land plant and protozoan mitochondria and many archaea, and consists of eight dedicated proteins (CcmA-H).

In *Escherichia coli*, System I Ccm is organized into two branches that converge at heme lyase CcmF (34, 58). The thioredoxin branch consists of CcmG, CcmH and DsbD which transfers thiol (-SH)-reducing equivalents to the periplasm to ensure that cysteines of the apocytochrome *c* CXXCH motif remain reduced for heme attachment (22, 68-70). The heme delivery branch consists of CcmABCDE which transfers heme synthesized in the cytoplasm to apocytochrome *c* located in the periplasmic space in an ATP-dependent manner. CcmA, a subunit of a cytoplasmic membrane-spanning ABC-type transporter, is located on the inner aspect of the cytoplasmic membrane and contains an ATP-binding (Walker A) domain. CcmB and CcmC are cytoplasmic membrane proteins that contain six putative cytoplasmic membrane-spanning α-helices. CcmC contains two highly conserved histidine residues that transiently coordinate heme iron at the periplasmic face of the cytoplasmic membrane and transfer heme to heme chaperone CcmE (34, 58). CcmE binds heme covalently at a conserved histidine residue and transfers heme to CcmF for incorporation into apocytochrome *c* (16, 24, 58, 63). Integral membrane protein CcmD plays a role in formation of a CcmC and CcmE complex (1).

The function of the CcmAB transporter is a subject of controversy. CcmAB was initially postulated to transport heme (45, 56, 72), although this hypothesis has been

brought into question by several recent findings. First, heme-containing *b*-type cytochromes are produced at normal levels in the periplasmic space of *ccm* mutants of *E. coli* (72, 74). Second, extracellular addition of heme does not restore cytochrome *c* maturation in *ccmA* and *ccmB* mutants of *Parococcus denitrificans* (56). On the other hand, System I delivers heme to apocytochrome *c* with greater affinity than System II (59). System I Ccm ABC transporter-like complexes are strikingly absent from System II, an indication that CcmAB may function as a heme transporter. In addition, CcmC is unable to transfer heme to CcmE, and holo-CcmE is unable to transfer heme to apocytochrome *c* in *ccmA* and *ccmB* mutants of *E. coli* (11, 27, 28). These results suggest that CcmA and CcmB are required for heme transport across the cytoplasmic membrane and participate in subsequent stepwise delivery of heme to CcmC, CcmE and ultimately CcmF for incorporation into the apocytochrome *c* located in the periplasm (11, 28, 63).

The aliphatic heme prosthetic group associates with hemoproteins via hydrophobic interactions and heme iron coordination (36, 41). Ring nitrogen atoms of the heme tetrapyrole occupy four of the six coordination sites of iron. Nitrogen, sulfur or oxygen atoms of amino acid side chains, O_2, OH^- or H_2O occupy the remaining coordination sites of iron in hemoproteins. The most common heme ligand in prokaryotic proteins is the imidazole moiety of histidine. Histidine coordinates heme iron in cytochromes *a*, *b*, *c*, and *d* (3, 26, 31, 72), microperoxidases (55) and signal transduction sensors such as CooA of *Rhodospirillum rubrum* and FixL of *Bradyrhizobium japonicum* (54, 75, 76). Methionine is an axial heme ligand in cytochrome *c*, cytochrome *bd* oxidase and bacterioferritin (10, 26, 61, 72, 75). Tyrosine

is a heme ligand in CcmE, cytochrome cd_1 and catalase (17, 29, 31, 33, 77). Lysine is a heme ligand in the nitrite reductase subunits NrfA and NrfH (6, 37) and the octaheme tetrathionate reductase (OTR) in *S. oneidensis* (51). Heme iron ligands are generally flanked by hydrophobic patches that stabilize heme-protein interaction in cytochromes *b*, *c* and *f*, CcmE and CcmC (14, 24, 34, 35, 80).

Recent studies on U(VI) respiration by *S. putrefaciens* demonstrated that conserved histidine residue H108 in the ABC-transporter permease CcmB was required for cytochrome *c* maturation and anaerobic growth (15, 79). Mutant strain CCMB1 (H108Y random point mutation in CcmB) was impaired in its ability to grow anaerobically on electron acceptors with mid-point redox potentials (E'_0) below a threshold value of 0.36V, contained cytochrome *c* at levels < 10% of the wild-type strain and contained concentrations of periplasmic -SH at levels 2-fold greater than the wild-type strain. H108 is predicted to reside at the interface of a transmembrane helix and a cytoplasmic loop containing a hydrophobic patch, a position suitable for heme iron coordination. The main objective of the present study was to test the hypothesis that *S. putrefaciens* CcmB regulates periplasmic redox homeostasis during anaerobic growth at low redox potential by transporting heme from the cytoplasm to the periplasm. To examine the possibility that CcmB regulates redox homeostasis in *S. putrefaciens*, CcmB mutant CCMB1 was tested for growth on electron acceptors with low E'_0 in medium amended with the disulfide-containing compounds cystine and oxidized glutathione. To determine if *S. putrefaciens* CcmB residue H108 may bind heme, a set of site-directed mutants were constructed with amino acid residues that are or are not capable of heme iron coordination. H108 was replaced by hydrophobic residues that are not able to

coordinate heme iron (i.e., alanine, leucine), a hydrophobic residue that coordinates heme iron in a low spin state (i.e., methionine) and hydrophilic (polar and basic) residues that coordinate heme iron in a high spin state (i.e., tyrosine, lysine). The resulting mutant strains were tested for anaerobic growth ability on a set of electron acceptors with E'_0 ranging from highly oxidizing (O_2) to highly reducing (thiosulfate, $S_2O_3^{2-}$). Cytochrome *c* is essential for anaerobic growth and U(VI) reduction by *Shewanella* (15, 49), and the results of this study demonstrate that the process of cytochrome *c* maturation provides both electron transport components (i.e., holocytochrome *c*) and a favorable periplasmic redox poise that sustain anaerobic activity.

Materials and methods

Bacterial strains, growth media and cultivation conditions. A previously isolated rifamycin resistant strain of *S. putrefaciens* strain 200 was cultured in the presence of 50 μg ml^{-1} rifamycin in either Luria Broth or a defined salts growth medium (SM, pH 7.5) (19, 21, 52). *S. putrefaciens* and *E. coli* strains harboring cloning vectors were grown in media amended with appropriate antibiotics at the following concentrations: chloramphenicol (25 μg ml^{-1}) and kanamycin (10 μg ml^{-1}) (see Table 4.1 for strains and plasmids used in the present study). Anaerobic growth experiments were carried out in N_2-purged Erlenmeyer flasks containing 50 ml SM medium supplemented with sodium lactate (30 mM). Electron acceptor stocks were added at the following final concentrations (electron acceptor stock solutions were prepared as previously described (19, 79), except where indicated): NO_3^-, (20 mM); NO_2^-, (2.0 mM); U(VI)-carbonate, (1.0 mM); Fe(III) citrate, (50 mM); γ-FeOOH(64), (40 mM); MnO_2 (as colloidal MnO_2), (10 mM) (57); Mn(III)-pyrophosphate, (10 mM) (43); TMAO, (30 mM); SO_3^{2-}, (10 mM);

Table 4.1. Bacterial strains and plasmids used in the present study

Strain or plasmid	Description	Reference
Strains		
Shewanella putrefaciens		
200R	Spontaneous Rif^r derivative of *S. putrefaciens* (NCIB 12577)	(19)
CCMB1	Randomly generated point mutant of 200R containing H108Y in CcmB	(79)
Δ*ccmB*	*ccmB* in-frame deletion mutant of 200R	(15)
Escherichia coli		
β2155	*thrB1004 pro thi strA hsdS lacZ*Δ*M15* (F ϕ*lacZ*Δ*M15 lacI*^{q} *traD36 proA*$^+$ *proB*$^+$) Δ*dapA*::*erm pir*::RP4; Erm^r Km^r	(18)
Plasmids		
pBBR1MCS	Broad-host-range cloning vector, 4.7-kb α–*lac*/multiple cloning site; Cm^r	(44)
pBCCMAB	pBBR1MCS with cloned *ccmAB* from 200R	This study
pBH108H	pBCCMAB with substituted *ccmB* H108H codon	This study
pBH108Y	pBCCMAB with substituted *ccmB* H108Y codon	This study
pBH108A	pBCCMAB with substituted *ccmB* H108A codon	This study
pBH108L	pBCCMAB with substituted *ccmB* H108L codon	This study
pBH108K	pBCCMAB with substituted *ccmB* H108K codon	This study
pBH108M	pBCCMAB with substituted *ccmB* H108M codon	This study

$S_2O_3^{2-}$, (10 mM); DMSO, (50 mM); fumarate, (35 mM). Aerobically-grown cells were inoculated in batch cultures vigorously aerated with atmospheric gas. U(VI) growth experiments were carried out in SM medium with a headspace gas consisting of 10% H_2, 5% CO_2 and the balance N_2. Control experiments consisted of incubating wild-type *S. putrefaciens* in SM liquid growth medium with electron acceptor omitted or with heat-killed cells (held at 80°C for 30 minutes) as inoculum.

Site-directed mutagenesis. The conserved histidine residue H108 of CcmB was replaced with alanine (H108A), leucine (H108L), methionine (H108M), lysine (H108K), tyrosine (H108Y, CCMB1 control) or histidine (H108H, wild-type positive control) in *S. putrefaciens* by altering the corresponding *ccmB* nucleotides. *ccmAB* and flanking regions were PCR-amplified from the wild-type strain and cloned into pBBR1MCS generating pBCCMAB (primer set CCMF and CCMR, Table 4.2). Recombinant

expression vectors encoding CcmB-H108A, -H108L, -H108K, or -H108M were generated by annealing mutagenic oligonucleotides (Table 4.2) to pBCCMAB as template following a PCR-based mutagenesis protocol (QuickChange II XL Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA). A wild-type positive control strain CcmB-H108H and a CCMB1 control strain CcmB-H108Y were constructed in a similar manner. Constructs were verified by DNA sequencing and conjugally transferred into strain Δ*ccmB* by bi-parental mating procedures (19).

Analytical techniques. Batch cultures were monitored for growth by simultaneously measuring cell number and electron acceptor depletion or end product production. Acridine orange-stained cells were counted directly via epifluorescence microscopy

Table 4.2. Oligonucleotide sequences used in the present study

Primer	Sequence
Cloning Primers[a]	
CCMF	GACTGTCGACGGGTGTAACCATTTCCACATTTTAGTTAGTAC
CCMR	GACTACTAGTCCTTGGTGGAGGCAGACTCAT
Sequencing Primers	
CCMBSF	GGGTAAAATCAGTAGGCTGAGC
CCMBSR	CCTCCATGCTATCGCTTGAAC
Mutagenic oligonucleotides for site-directed mutagenesis[b]	
H108Hsens	TATTGGCAAAAGTATTGGCG**CAc**TGGATATTGACTGGC
H108Hanti	GCCAGTCAATATCCA**gTG**CGCCAATACTTTTGCCAATA
H108Lsens	GGCAAAAGTATTGGCG**CtT**TGGATATTGACTGGCG
H108Lanti	CGCCAGTCAATATCCA**AaG**CGCCAATACTTTTGCC
H108Asens	TATTGGCAAAAGTATTGGCG**gcT**TGGATATTGACTGGCGTTC
H108Aanti	GAACGCCAGTCAATATCCA**Agc**CGCCAATACTTTTGCCAATA
H108Ysens	TTGGCAAAAGTATTGGCG**tAT**TGGATATTGACTGGCG
H108Yanti	CGCCAGTCAATATCCA**ATa**CGCCAATACTTTTGCCAA
H108Msens	CTTGTATTGGCAAAAGTATTGGCG**atg**TGGATATTGACTGGCGTTCCATTA
H108Manti	TAATGGAACGCCAGTCAATATCCA**cat**CGCCAATACTTTTGCCAATACAAG
H108Ksens	TATTGGCAAAAGTATTGGCG**aAa**TGGATATTGACTGGCGTTCC
H108Kanti	GGAACGCCAGTCAATATCCA**tTt**CGCCAATACTTTTGCCAATA

[a]Endonuclease recognition sequences for cloning *ccmAB* are underlined
[b]In mutagenic oligonucleotides, changed nucleotides (lower case), altered codons (bold).

(Nikon Diaphot 300 microscope). Fe(III) reduction was monitored by measuring HCl-extractable Fe(II) with the ferrozine technique (71). MnO_2 reduction was monitored by measuring MnO_2 depletion with the benzidine blue colorimetric assay (8). Mn(III)-pyrophosphate depletion was monitored spectrophotometrically at 480 nm (43). U(VI) was measured colorimetrically with Arsenazo III reagent (46). NO_2^- was measured spectrophotometrically with sulfanilic acid-*N*-1-naphthylethylene-diamine dihydrochloride reagent (50). Growth on O_2, TMAO, DMSO, fumarate, $S_2O_3^{2-}$ and SO_3^{2-} was monitored by cell growth only.

Periplasmic protein fractions from *S. putrefaciens* cultures were recovered from cells by osmotic shock treatment (25) after anaerobic growth to late logarithmic phase in SM medium containing Fe(III)-citrate or fumarate as electron acceptor. Cells were harvested by centrifugation, washed in anoxic PBS buffer (200 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4, 4°C) twice and resuspended in hyperosmotic sucrose PBS buffer (20% sucrose w/v). Cells were incubated on ice for 30 min., collected by centrifugation, resuspended in a hypoosmotic solution (5mM $MgCl_2$, pH 7.0, 4°C) and incubated on ice for 20 min. The mixture was centrifuged at 40,000 x *g* for 20 min at 4°C (Beckman Coulter Optima L-100 XP Ultracentrifuge) and the supernatant containing the periplasmic protein fraction was collected. The extent of cytoplasmic protein contamination was determined by measuring shikimate dehydrogenase activity (which remained below 10% in all periplasmic fractions; data not shown).

Free thiol equivalents (-SH free to react with Ellman's reagent) were determined by reaction with 5,5′-dithio-bis-2-nitrobenzoic acid (23), standardized with reduced glutathione and reported on a per μg protein basis. Protein concentration was determined

with the Coomassie Plus Bradford assay (Pierce Biotechnology) standardized with bovine serum albumin.

Cytochrome content in wild-type and *ccmB* mutant extracts was determined by measuring dithionite-reduced-minus-ferricyanide-oxidized difference spectra on a Shimadzu UV-1601 spectrophotometer operated in the split-beam mode. Estimates of total cytochrome content were based on the difference between absorbance values at the peak and trough of the Soret peak standardized per mg total protein (12, 53). Protein fractions were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on a linear gradient gel (4% to 20% resolving) with 200 μg total protein loaded per well (48). Gels were stained for heme peroxidase activity by incubating in a solution consisting of 0.5 M Tris-HCl (pH 7.5), 3,3-dimethoxybenzidine-peroxide (1 mg ml^{-1}) and H_2O_2 (30% stock solution, 0.0025 μl ml^{-1}) (30).

Results

Addition of exogenous cystine or oxidized glutathione to the growth medium restores the ability of *S. putrefaciens* mutant strain CCMB1 to grow anaerobically on electron acceptors with low E'_0. *S. putrefaciens ccmB* mutant CCMB1 was unable to grow on electron acceptors with $E'_0 < 0.36V$ and the periplasm of CCMB1 was found to contain elevated levels of -SH (15). To determine if exogenous disulfide-containing compounds restore growth of *S. putrefaciens* CCMB1 on electron acceptors with low E'_0, oxidized glutathione or cystine were added to anaerobic CCMB1 cultures containing either NO_3^-, DMSO, TMAO, fumarate, U(VI), γ-FeOOH, SO_3^{2-}, or $S_2O_3^{2-}$ as electron acceptor. Growth of CCMB1 on electron acceptors NO_2^-, DMSO, TMAO, fumarate, U(VI) and γ-FeOOH was restored to wild-type rates by the addition of cystine to the growth medium

(Figure 4.1 and Figure 4.2). Addition of oxidized glutathione restored growth of CCMB1 to 50% wild-type rates on NO_2^-, DMSO, TMAO, fumarate, U(VI) and γ-FeOOH. CCMB1 was unable to grow anaerobically on either SO_3^{2-} or $S_2O_3^{2-}$ even after addition of cystine or oxidized glutathione to the growth medium. Increasing the concentration of cystine to 1 mM did not restore anaerobic growth to CCMB1 on SO_3^{2-} or $S_2O_3^{2-}$ (data not shown).

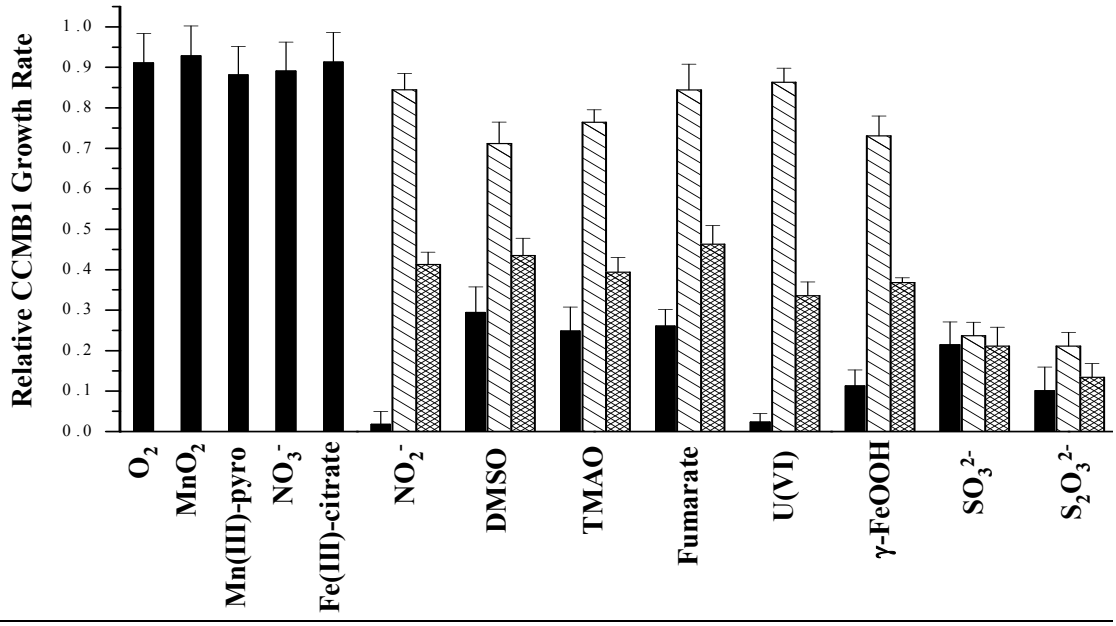


Figure 4.1. Comparison of logarithmic growth rate of CCMB1 and wild-type *S. putrefaciens* on a set of 13 electron acceptors in SM medium (solid bars), in SM medium containing 300 μM cystine (hatched bars) and in SM containing 300 μM oxidized glutathione (shaded bars). Logarithmic growth rates are normalized to *S. putrefaciens* wild-type strain growth on each electron acceptor (h^{-1}): O_2, 1.4; MnO_2, 0.7; Mn(III) pyrophosphate, 0.3; NO_3^-, 0.38; Fe(III) citrate, 0.2; NO_2^-, 0.2; DMSO, 0.9; TMAO, 0.4, fumarate, 0.9; U(VI), 0.05; γ-FeOOH, 0.3; SO_3^{2-}, 0.2; $S_2O_3^{2-}$, 0.1. See Figure 4.2 for individual growth curves. Error bars represent the standard deviations of three parallel yet independent incubations.

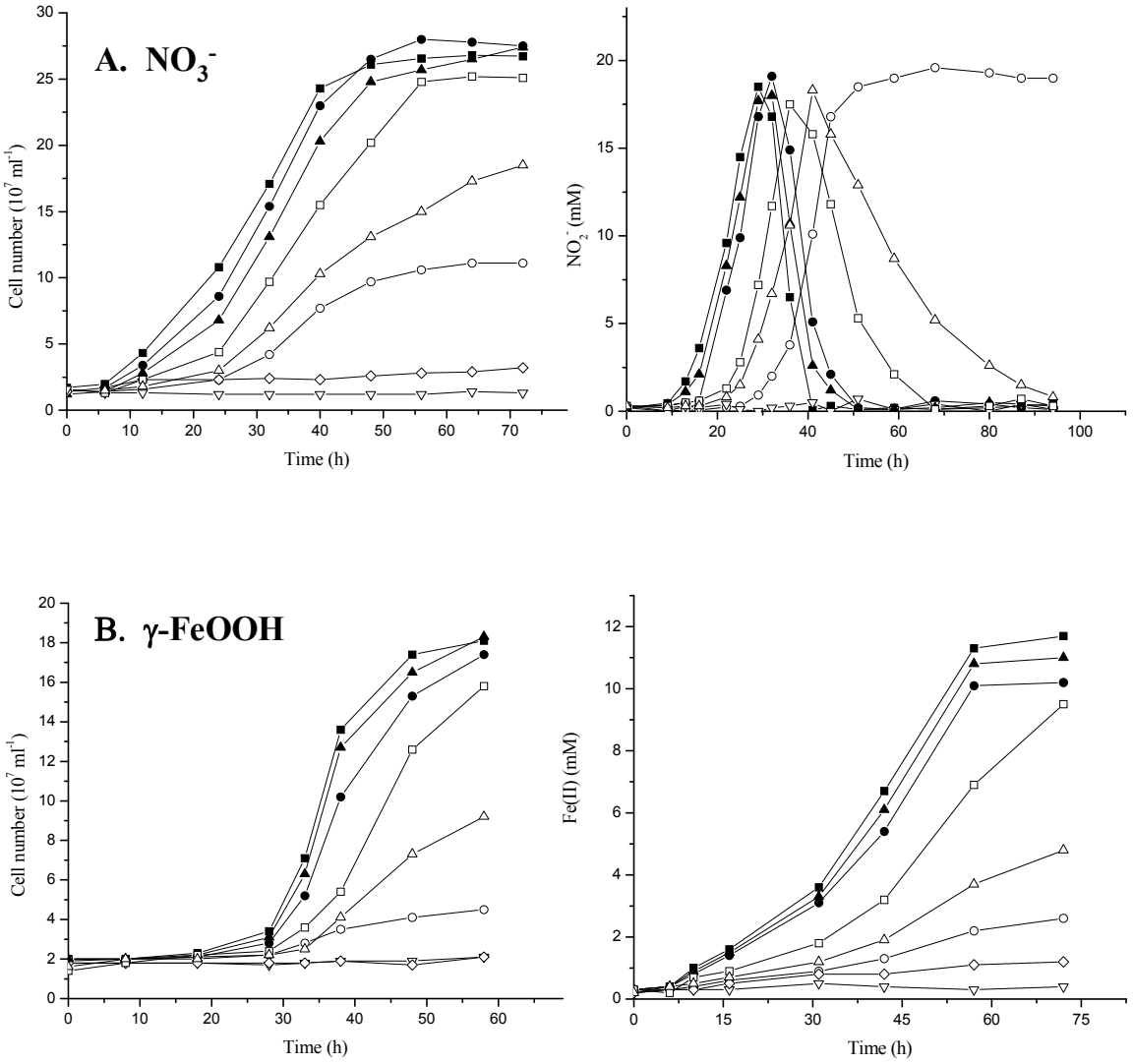


Figure 4.2. Anaerobic growth and corresponding electron acceptor depletion or end-product production of wild-type *S. putrefaciens* and CCMB1 in SM medium containing cystine or oxidized glutathione and the indicated electron acceptors. CCMB1 with cystine added (–□-), CCMB1 with oxidized glutathione added (-Δ-), *S. putrefaciens* with cystine added (-■-), *S. putrefaciens* with oxidized glutathione added (-▲-), CCMB1 with no amendment (-○-), *S. putrefaciens* with no amendment (-●-), *S. putrefaciens* heat-killed control (-▽-).

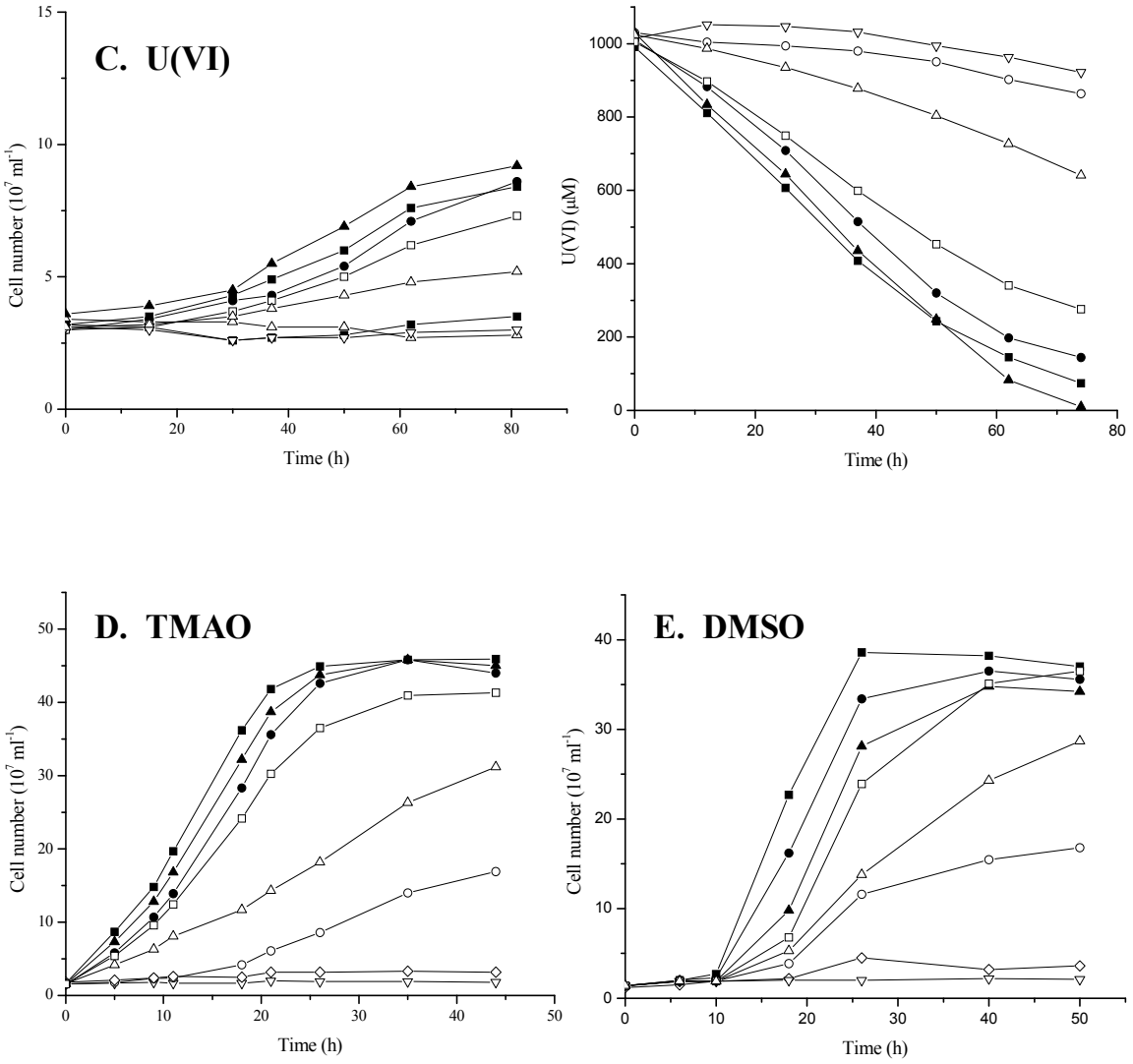


Figure 4.2 continued.

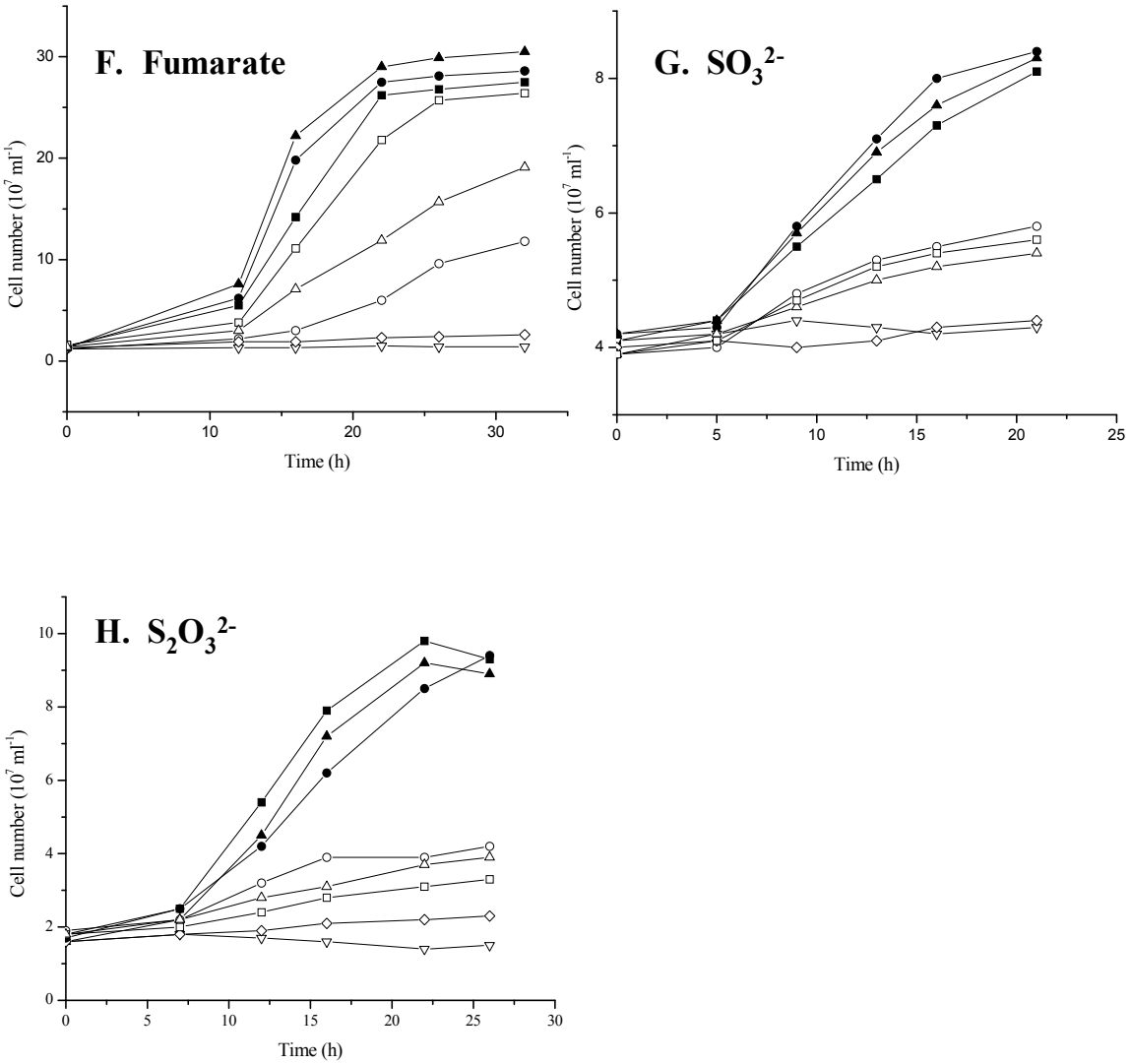


Figure 4.2 continued.

Addition of exogenous cystine to the growth medium does not restore the ability of *S. putrefaciens* mutant strain CCMB1 to produce cytochrome *c*. Anaerobic CCMB1 cultures amended with cystine were analyzed for cytochrome *c* content. CCMB1 and the wild-type strain were grown in SM medium containing fumarate as electron acceptor. Replicate cultures were prepared and cystine was added to the CCMB1 growth medium to restore anaerobic growth on fumarate. The cytochrome *c* content of CCMB1 was at or below detection limits even with cystine added to the growth medium (Figure 4.3). These results demonstrate that *ccmB* mutants of *S. putrefaciens* grow anaerobically at wild-type rates in growth medium amended with exogenous cystine, while the cytochrome *c* content remains at CCMB1 mutant levels (< 10% of wild-type).

S. putrefaciens site-directed mutants H108M, H108A and H108L retain the ability to grow anaerobically on all electron acceptors. H108 of *S. putrefaciens* CcmB is predicted to be located at the inner aspect of the cytoplasmic membrane and is flanked by a transmembrane α-helix (TM III) and a hydrophobic patch in an otherwise hydrophilic cytoplasmic loop (Figure 4.4). To determine if H108 amino acid substitutions affect cytochrome c maturation, periplasmic redox condition, and anaerobic growth ability, H108 was replaced by hydrophobic residues that are not able to coordinate heme iron (i.e., alanine, leucine), a hydrophobic residue that coordinates heme iron (i.e., methionine), and hydrophilic (polar and basic) residues that coordinate heme iron (i.e., tyrosine, lysine). The resulting site-directed mutants were tested for growth on O_2, Fe(III)-citrate, NO_3^-, fumarate, γ-FeOOH, or $S_2O_3^{2-}$ as electron acceptor. Each mutant

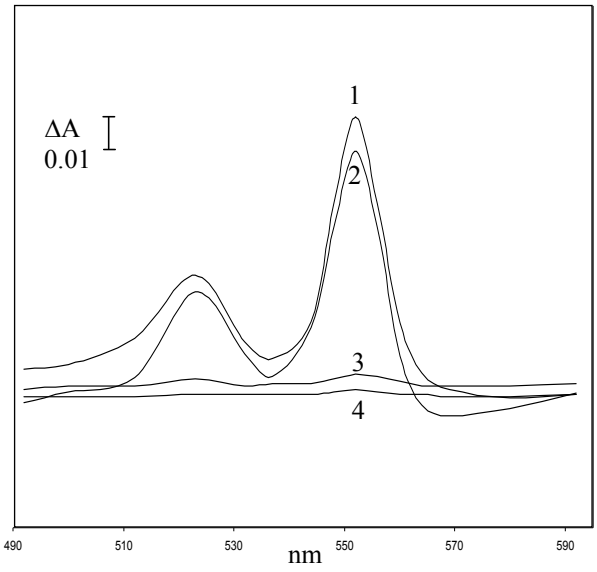


Figure 4.3. Reduced-minus-oxidized difference spectra of periplasmic protein extracts from CCMB1 and *S. putrefaciens* after anaerobic growth in SM medium containing fumarate as electron acceptor and amended with 300 μM cystine. *S. putrefaciens* wild-type with cystine added (1); *S. putrefaciens* wild-type without cystine added (2); point-mutant CCMB1 without cystine added (3); point-mutant CCMB1 with cystine added (4).

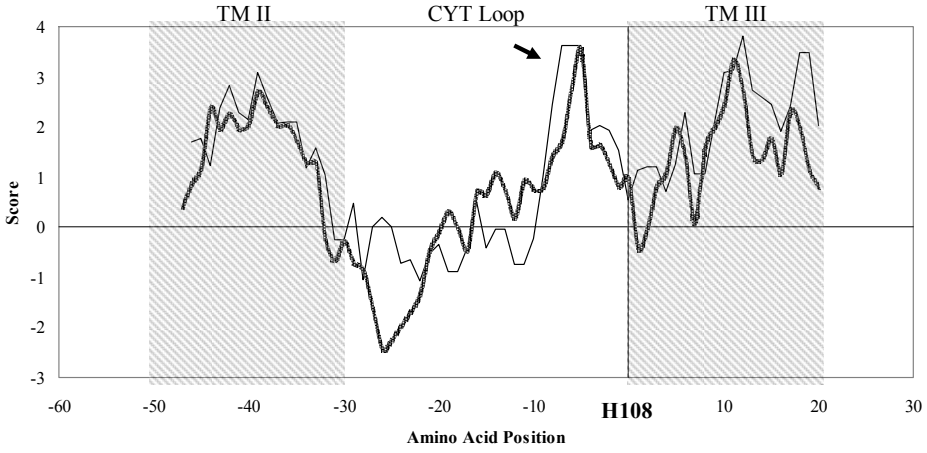


Figure 4.4. Hydrophobicity plot of region flanking H108 in CcmB. *S. putrefaciens* (solid line) and average values from *S. putrefaciens*, *E. coli*, *Tricicum aestivum*, *Arabidopsis thaliana* and *Vibrio cholerae* (shaded line). Transmembrane helices (TM II and TM III) are shaded. Position of H108 in *S. putrefaciens* is indicated. Bold arrow indicates putative hydrophobic platform. Hydrophobicity was determined according to the Kyte-Doolittle scale (47).

strain was capable of aerobic growth at near wild-type rates (Figure 4.5). H108M, H108L and H108A grew anaerobically on all electron acceptors at near wild-type rates, while H108K was severely impaired in its ability to grow anaerobically on electron acceptors with $E'_0 < 0.36V$ (i.e., NO_2^-, fumarate, γ-FeOOH, $S_2O_3^{2-}$). The CCMB1 control strain H108Y grew at near wild-type rates on Fe(III)-citrate and NO_3^-, but grew at < 30% of the wild-type rates on NO_2^-, fumarate, γ-FeOOH and $S_2O_3^{2-}$ (Figure 4.5). H108Y displayed a phenotype nearly identical to CCMB1 indicating that CcmB activity of the H108Y site-directed mutant is nearly equal to that of the corresponding random point mutant CCMB1. Control strain H108H grew anaerobically at rates nearly identical to the wild-type strain (Figure 4.5). After cystine was added to the growth medium, H108Y and H108K were restored for anaerobic growth on NO_2^-, fumarate and γ-FeOOH, but not on $S_2O_3^{2-}$ (Figure 4.6). These results demonstrate that CcmB constructs with histidine, methionine or a hydrophobic residue (i.e., alanine, leucine) at position 108 retain the ability to grow anaerobically on all electron acceptors, while CcmB constructs with hydrophilic (polar or basic) residues (i.e., tyrosine, lysine) at position 108 are unable to grow on electron acceptors with low E'_0.

CcmB mutant H108M retains the ability to produce cytochrome *c* at wild-type levels. CcmB mutants H108M, H108L and H108A retain the ability to grow on all electron acceptors. To determine if H108M, H108L or H108A contained wild-type levels of cytochrome *c*, periplasmic extracts from each mutant were collected after anaerobic growth on fumarate to late logarithmic phase. Based on reduced-minus-oxidized difference spectra and heme stain analysis, H108K, H108Y, H108A and H108L each

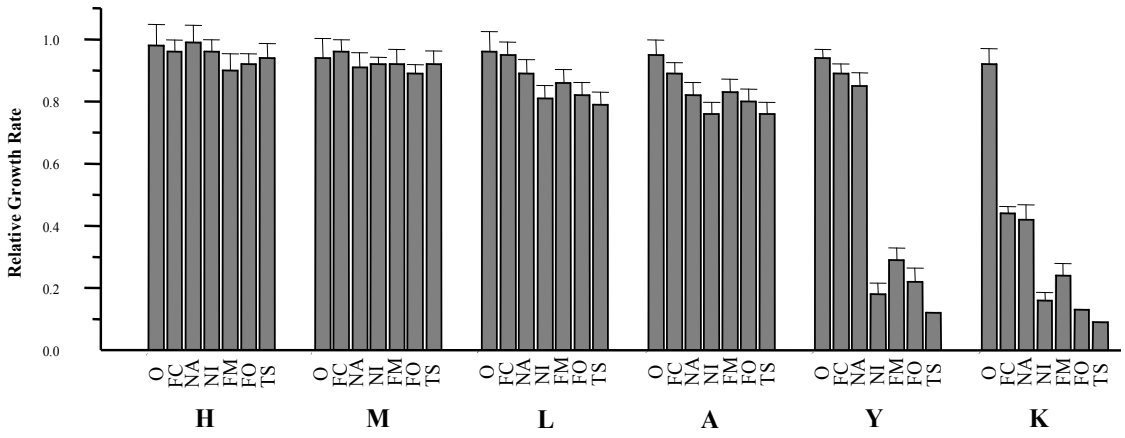


Figure 4.5. Growth rates of *S. putrefaciens* CcmB site-directed mutant strains on a set of 7 electron acceptors (rates normalized to the wild-type strain *S. putrefaciens*). *S. putrefaciens* Δ*ccmB* deletion mutants containing H108 site-directed mutants *in trans* on plasmid pBH108H (positive control) (H), pBH108M (M), pBH108L (L), pBH108A (A), pBH108Y (CCMB1 control) (Y) or pBH108K (K) grown on O_2 (O), Fe(III)-citrate (FC), NO_3^- (NA), NO_2^- (NI), fumarate (FM), γ-FeOOH (FO) or $S_2O_3^{2-}$ (TS) as electron acceptor. Logarithmic growth rates are normalized to *S. putrefaciens* wild-type grown on each electron acceptor (See Figure 4.1). Error bars represent the standard deviation of three parallel yet independent incubations.

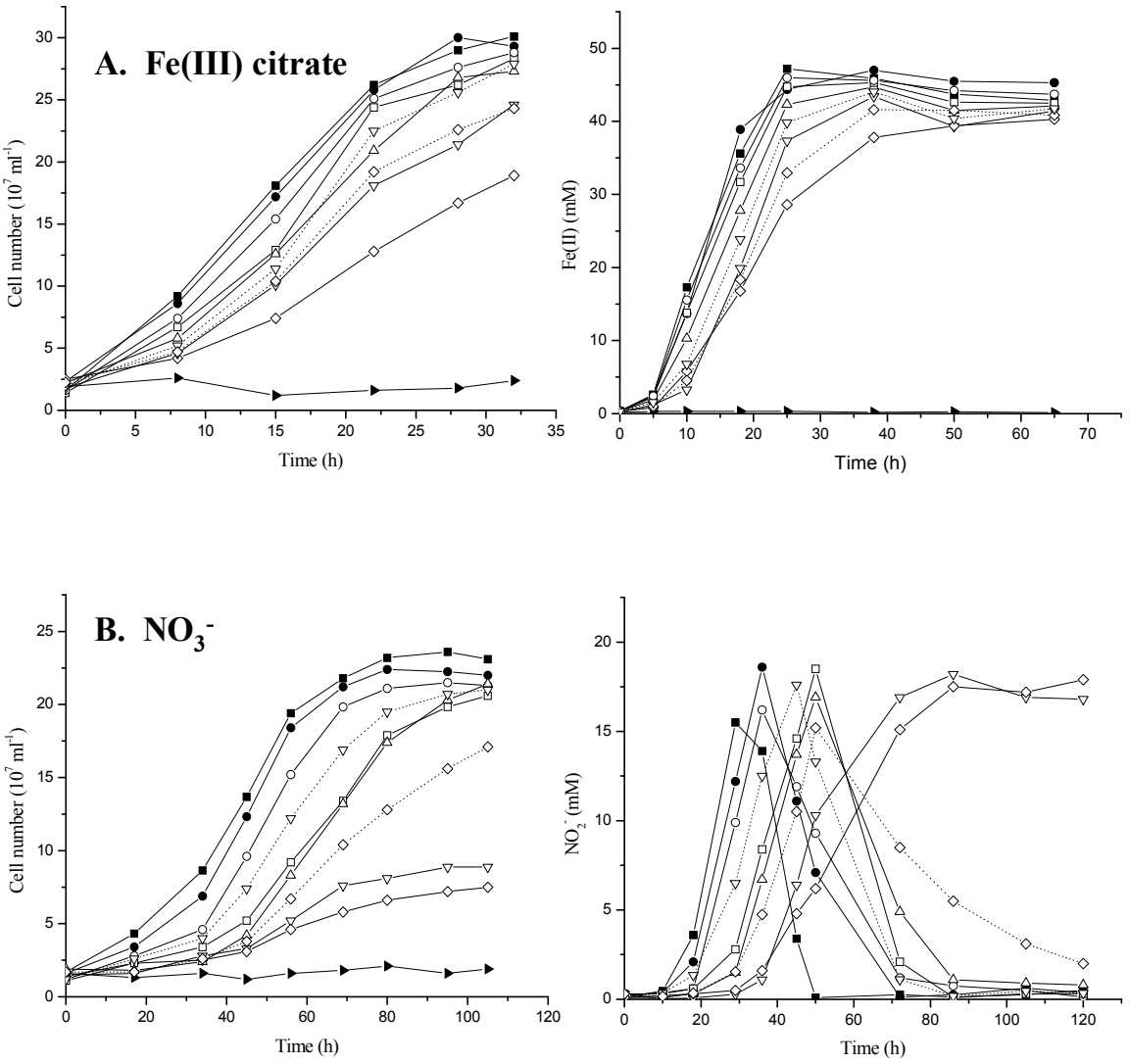
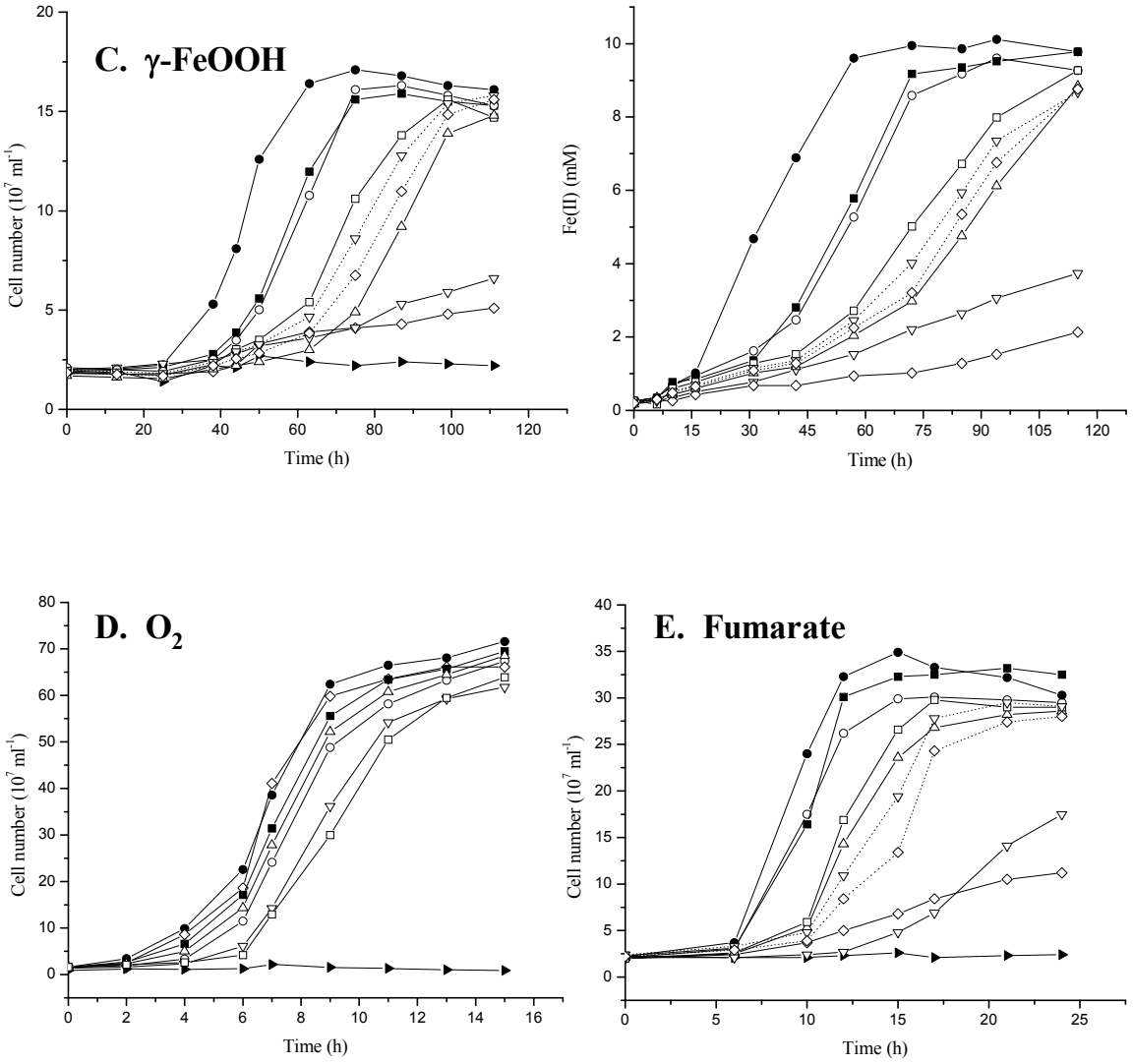


Figure 4.6 Anaerobic growth and corresponding electron acceptor depletion or end-product production of wild-type *S. putrefaciens* and CcmB H108 site-directed mutants. *S. putrefaciens* wild-type (-●-), *S. putrefaciens* heat-killed control (-►-), H108H (positive control) (-■-), H108M (-○-), H108L (-□-), H108A (-Δ-), H108Y (-▽-), H108Y with cystine added (···▽···), H108K (-◊-), H108K with cystine added (···◊···).



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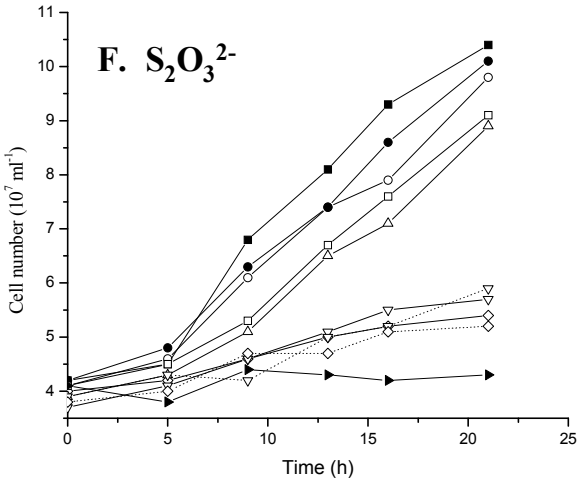

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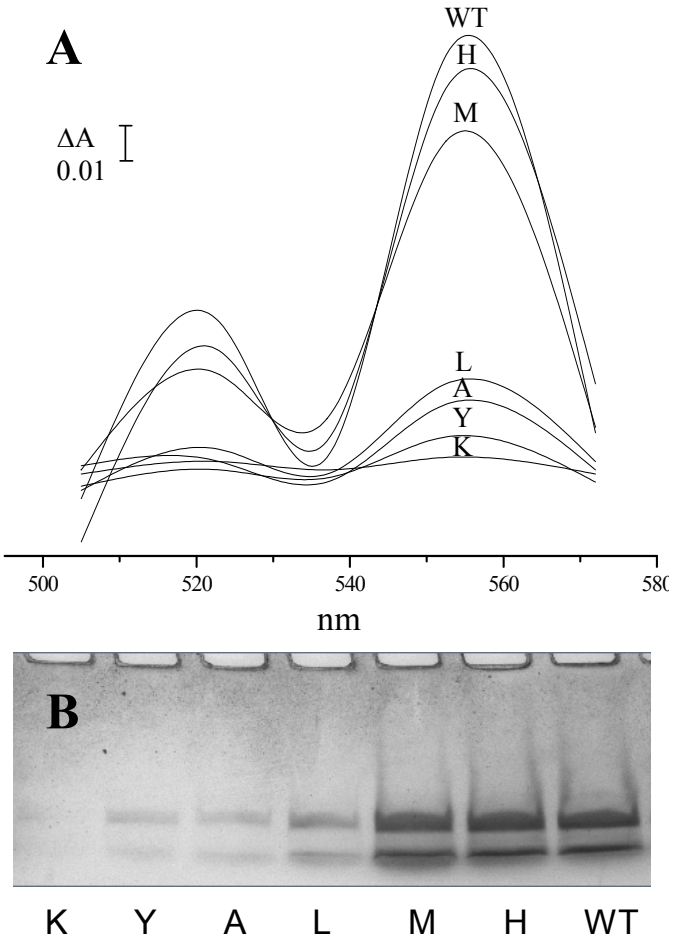


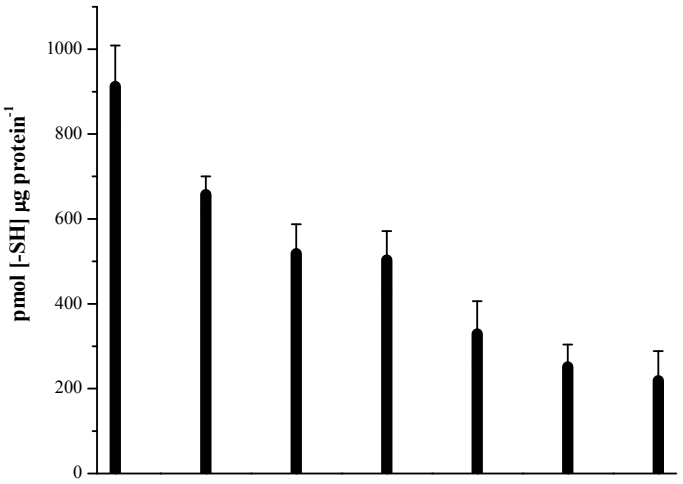
Figure 4.7. Reduced-minus-oxidized difference spectra (A) and SDS-PAGE heme stain (B) of periplasmic protein fractions of wild-type *S. putrefaciens* and CcmB H108 site-directed mutants after growth on Fe(III)-citrate. *S. putrefaciens* (pBBR1MCS) (WT) and Δ*ccmB* deletion mutants containing H108 site-directed mutants *in trans* on plasmid pBH108H (positive control) (H), pBH108A (A), pBH108L (L), pBH108K (K), pBH108Y (Y) and pBH108M (M).

contained < 20% of the wild-type cytochrome *c* content, while H108M contained near wild-type levels of cytochrome *c* (Figure 4.7). H108L and H108A grew at near wild-type rates on all electron acceptors even though the periplasmic cytochrome *c* content was < 20% of the wild-type strain.

-SH content in the periplasmic space is inversely related to the growth rate of H108M, H108L, H108A, H108Y and H108K. The periplasmic -SH content of *S. putrefaciens* CCMB1 is two-fold greater than the wild-type strain after growth on electron acceptors with $E'_0 < 0.36$V (Chapter 3, Figure 3.10). To determine if elevated -SH levels correlate with the anaerobic growth deficiencies of the set of H108 site-directed mutants, the -SH content in the periplasmic fraction of each site-directed mutant was measured after anaerobic growth on fumarate and compared to that measured in the periplasm of the wild-type strain. The wild-type strain, control strain H108H and H108M contained < 400 pmol [-SH] μg protein^{-1} (Figure 4.8). H108Y and H108K contained > 600 pmol [-SH] μg protein^{-1} and H108A and H108L contained > 400 and < 600 pmol [-SH] μg protein^{-1}. -SH measurements correlated inversely with the growth rate and cytochrome *c* content of each strain, indicating that residue H108 plays a key role in both cytochrome *c* maturation and CcmB-driven regulation of periplasmic redox homeostasis in *S. putrefaciens*.

Discussion

Rsults of a previous study demonstrated that *S. putrefaciens ccmB* mutant CCMB1 (H108Y random point mutation in CcmB) grew at near wild-type rates on electron acceptors with $E'_0 > 0.36$ V [i.e., O_2, Fe(III) citrate, MnO_2, NO_2^-] but displayed severe growth deficiencies on electron acceptors with $E'_0 < 0.36$V i.e., NO_2^-, DMSO,



Growth[a]	-	-	+	+	+	+	+
Cyt. *c*[b]	-	-	-	-	+	+	+

Figure 4.8. -SH content in periplasmic fractions of *S. putrefaciens* CcmB H108 site-directed mutants after growth on fumarate. *S. putrefaciens* (pBBR1MCS) (WT) and *S. putrefaciens* Δ*ccmB* deletion mutant containing CcmB H108 site-directed mutants *in trans* on plasmid pBH108K (K), pBH108Y (Y), pBH108L (L), pBH108A (A), pBH108M (M) and positive control pBH108H (H). The box below the bar graph indicates mutant growth rate[a] and cytochrome *c* content[b] in the periplasmic protein fractions of each strain grown to mid log-phase (+, value > 80% of wild-type grown on fumarate; -, value < 30% of wild-type grown on fumarate).

TMAO, fumarate, U(VI), -FeOOH, SO_3^{2-}, $S_2O_3^{2-}$] (15). An in-frame gene deletion mutant (Δ*ccmB*) mutant was unable to grow anaerobically on any electron acceptor suggesting that *S. putrefaciens* requires CcmB to sustain anaerobic growth. Regardless of the electron acceptor used for growth, the periplasm of CCMB1 contained < 10% of the cytochrome *c* content of the periplasm of the wild-type strain and contained abnormally high (two-fold greater than wild-type) levels of –SH in the periplasmic space (Figure

3.10). Abnormally high -SH was also detected in *ccmC* mutants of *Pseudomonas fluorescens* that are unable to complete cytochrome *c* maturation or biosynthesis of the fluorescent siderophore pyoverdine (7). Addition of oxidized glutathione to the growth medium restored pyoverdine biosynthesis to the *P. fluorescens ccmC* mutants. Cytochrome *c* maturation, however, was not restored by the addition of oxidized glutathione. A similar result was obtained in the present study with *S. putrefaciens* mutant CCMB1. Addition of cystine (and to a lesser extent oxidized glutathione) to the growth medium restored anaerobic growth capability to CCMB1 (on all electron acceptors except SO_3^{2-} or $S_2O_3^{2-}$) but not cytochrome *c* maturation activity. Surprisingly, these results indicate that wild-type growth rates of *S. putrefaciens* are achieved in anaerobic cultures with abnormally low cytochrome *c* content. CCMB1 cultures containing SO_3^{2-} or $S_2O_3^{2-}$ as electron acceptor were not restored to wild-type growth rates by addition of cystine to the growth medium. The potent chemical reductant H_2S, the end product of SO_3^{2-} and $S_2O_3^{2-}$ respiration, most likely accumulates to high concentration in the already overly-reducing CCMB1 periplasm and disrupts periplasmic redox homeostasis even in the presence of cystine. These results indicate that maintenance of proper redox poise is a primary function of *S. putrefaciens* CcmB during anaerobic growth on electron acceptors with low E'_0.

The elevated periplasmic –SH content of the *S. putrefaciens ccmB* mutants may be due to a variety of factors including a decrease in periplasmic heme iron content, accumulation of apocytochrome *c* or the inactivation of periplasmic proteases that otherwise degrade immature apocytochrome *c*. Heme iron oxidizes –SH *in vivo* (5, 13, 40, 65). Periplasmic Fe(III) in the form of unincorporated heme or mature cytochrome *c*

may therefore be required for maintenance of periplasmic redox homeostasis. The periplasmic –SH content in *ccmB* mutants of *S. putrefaciens* may also be elevated due to accumulation of apocytochrome *c*. Although the heme delivery branch of the *S. putrefaciens* CcmB mutants is compromised, the Ccm thioredoxin branch (CcmGH) may continue to reduce cysteines in the CXXCH motif of apocytochrome *c* resulting in an increase in the periplasmic –SH content. In *E. coli*, *Rhodobacter sphaeroides* and *Paracoccus denitrificans*, periplasmic proteases (e.g., DegP) that degrade immature apocytochrome *c* require an essential disulfide bridge for protease activity (32, 38, 60, 62, 66). The disulfide bridge does not form in *E. coli dsbA* mutants unable to oxidize the protease –SH groups (66), and apocytochrome *c* is not degraded (32). Current work is focused on determining the stability of apocytochrome *c* in *ccmB* mutants and the effect of apocytochrome *c* accumulation on redox homeostasis during anaerobic growth of *S. putrefaciens* on electron acceptors with low E'_0.

The *S. putrefaciens* site-directed *ccmB* mutants H108A, H108L, H108Y, H108K, and H108M were tested for cytochrome *c* production, periplasmic redox condition and anaerobic growth capability. The site-directed mutants were grouped into three classes: (i) H108Y and H108K were unable to grow anaerobically on electron acceptors with E'_0 < 0.36V, produced periplasmic cytochrome *c* at levels < 10% of the wild-type strain and contained periplasmic (–SH) content at levels two-fold greater than the wild-type strain, (ii) H108A and H108L retained the ability to grow on electron acceptors with E'_0 < 0.36V, produced periplasmic cytochrome *c* at levels < 20% of the wild-type strain and contained periplasmic -SH content at levels 50% greater than the wild-type strain, and (iii) H108M retained the ability to grow on electron acceptors with E'_0 < 0.36V, produced

periplasmic cytochrome *c* at near wild-type levels and contained wild-type levels of periplasmic SH. H108Y and H108K were unable to grow anaerobically on electron acceptors with low E'_0 and contained low cytochrome *c* content. The –SH content in the H108Y and H108K mutants was two-fold greater than the wild-type strain. These results indicate that CcmB activity is nearly abolished by replacing H108 with a hydrophilic (polar or charged) residue (tyrosine or lysine) even if the substituted residues are capable of ligating heme iron. Hydrophobic patches in hemoproteins stabilize heme-protein interactions and modify the electrostatic potential surrounding the heme. For example, *E. coli* CcmC contains a tryptophan-rich heme docking site (34, 58), which facilitates periplasmic heme transfer to CcmE. *E. coli* CcmE also contains a hydrophobic heme docking site consisting of phenylalanine, valine and leucine (24, 33). In addition, cytochrome c_{551}, a physiological redox partner of cytochrome cd_1 nitrite reductase (NIR) in *Pseudomonas aeruginosa*, contains a hydrophobic patch surrounding the heme docking site (4, 78). Mutations in the c_{551} hydrophobic patch result in decreased cytochrome E'_0 and lower electron transfer rates to NIR, possibly due to increased heme solvent exposure (14). In a similar manner, *S. putrefaciens* CcmB (and *E. coli* and CcmB orthologs in land-plant mitochondria; Fig. 3) contains a hydrophobic patch (ranging from amino acid position 94 to 105) flanking the conserved histidine residue H108 in an otherwise hydrophilic cytoplasmic loop (Fig. 3). The hydrophobicity of the cytoplasmic loop in CcmB decreases in CcmB site-directed mutants containing polar or charged amino acids (tyrosine or lysine) at amino acid position 108.

S. putrefaciens CcmB mutants H108A and H108L contain hydrophobic residues in place of histidine at position 108. Although H108A and H108L retained the ability to

grow on electron acceptors with $E'_0 < 0.36V$, periplasmic cytochrome *c* content was less than 20% of the wild-type strain while the periplasmic -SH content was 50% greater than the wild-type strain. In a previous site-directed mutagenesis study (42), the heme axial ligand of cytochrome b_{558} of *E. coli* was replaced with leucine and the resulting cytochrome *bd* oxidase mutant retained activity and aerobic growth capability. The heme iron, however, was converted from low spin to high spin (42). *S. putrefaciens* CcmB mutants H108L and H108A are able to maintain hydrophobicity at the interface between the cytoplasmic loop and TM III in CcmB but may be unable to maintain heme iron in the low spin form for incorporation into apocytochrome *c*. The ability of H108L and H108A to grow anaerobically at wild-type rates suggests that laboratory-grown cultures of *S. putrefaciens* contain cytochrome *c* at a level approximately five-fold greater than that required for anaerobic growth at optimal rates.

CcmB mutant H108M retained wild-type anaerobic growth capability and cytochrome *c* maturation activity. Periplasmic –SH content of anaerobically grown H108M cultures was nearly identical to that of the wild-type strain. Hydrophobic methionine residues coordinate heme iron via a thioether side-chain in many heme-binding proteins (10, 26, 75). For example, methionine occupies the 5^{th} and 6^{th} coordination sites of heme iron in bacterioferritin of *E. coli* and *Pseudomonas aeruginosa* (9, 10, 61, 72). The ligand field strength of methionine is sufficient to coordinate heme iron in the low spin state (10), a possible requirement for *S. putrefaciens* CcmB activity. Results from the present study indicate that *S. putrefaciens* CcmB requires either histidine or a hydrophobic residue capable of coordinating heme iron in a low spin state

(i.e., methionine) at amino acid position 108 for maintenance of periplasmic redox homeostasis and production of mature cytochrome *c*.

H108 of *S. putrefaciens* CcmB is highly conserved among CcmB orthologs in other prokaryotic and eukaryotic organisms and is strictly conserved in organisms that employ the Ccm variant system containing CcmE with a HxxxY heme-binding motif (i.e., the majority of α- and γ-proteobacteria and plant mitochondria) (2). H108 is absent in organisms that employ the Ccm system containing CcmE with either the CxxxY or HxxxHxxxH motifs (i.e., the majority of archaea and *Desulfovibrio* species). *E. coli* CcmC contains two highly conserved histidine residues that transiently coordinate heme iron at the periplasmic face of the cytoplasmic membrane and transfer heme to heme chaperone CcmE (34, 58). *E. coli* CcmE binds heme covalently at a conserved histidine residue and transfers heme to CcmF for incorporation into apocytochrome *c* (16, 24, 58, 63). The essential histidine residues in *E. coli* CcmC and CcmE are conserved in the CcmC and CcmE homologs of *S. putrefaciens*. Current work is focused on determining the ability of CcmB to bind heme iron at H108 and examining the possibility that CcmB functions as the first component of a multi-component, histidine-based heme delivery system that transports heme across the cytoplasmic membrane for subsequent stepwise delivery to CcmC, CcmE and ultimately CcmF for incorporation into apocytochrome *c*.

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CHAPTER 5

CONCLUSIONS

Subsurface uranium contamination is widespread due to anthropogenic activities associated with nuclear energy and weapons development over the past 60 years. Enhancement of microbial uranium reductive precipitation [U(VI) to U(IV)] is an attractive strategy to immobilize uranium *in situ*, however, the molecular mechanisms of U(VI) reduction are poorly understood. Members of the γ-proteobacteria genus *Shewanella* are ubiquitous in the environment and capable of remarkable respiratory versatility. *S. putrefaciens* strain 200 grows anaerobically on a wide variety of electron acceptors including uranium.

In previous studies, a point mutant of *S. putrefaciens* (designated Urr14 and referred to as CCMB1 in this work) was isolated and found to retain anaerobic growth capability on several electron acceptors but was unable to grow anaerobically on NO_2^- or U(VI). A 35 kB wild-type DNA fragment restored anaerobic growth capability to CCMB1 on U(VI) and NO_2^-. In the present study, CCMB1 anaerobic growth rates on a suite of electron acceptors were compared with the wild-type strain. CCMB1 was found to grow at near wild-type rates on electron acceptors with $E'_0 > 0.36V$ [O_2, NO_3^-, Fe(III)-citrate, MnO_2 and Mn(III)-pyrophosphate] but at rates < 30% that of the wild-type on electron acceptors with $E'_0 < 0.36V$ [NO_2^-, U(VI), DMSO, TMAO, fumarate, γ-FeOOH, SO_3^{2-} and $S_2O_3^{2-}$] (1). The gene mutated in CCMB1, *ccmB*, was identified via genetic

complementation and DNA sequence analysis. CcmB is a homolog of an ABC-type transporter permease subunit required for cytochrome *c* maturation in *E. coli*.

An in-frame *S. putrefaciens ccmB* gene deletion mutant (Δ*ccmB*) was constructed and was unable to grow on any anaerobic electron acceptor, suggesting that cytochrome *c* is required for anaerobic growth by *S. putrefaciens*. CCMB1 contained < 10% the cytochrome *c* content of the wild-type strain after anaerobic growth demonstrating that wild-type levels of cytochrome *c* are not required by *S. putrefaciens* to sustain optimal growth rates on electron acceptors with $E'_0 > 0.36V$.

The CCMB1 periplasmic content of the thiol functional group (-SH) was 2-fold greater than the wild-type strain after anaerobic growth, an indication that redox homeostasis was disrupted in CCMB1 (1). Anaerobic growth of CCMB1 on electron acceptors with $E'_0 < 0.36V$ was restored to near wild-type rates in growth medium containing 300 μM cystine, an oxidizing compound containing a disulfide bond (2). Cytochrome *c* content in CCMB1 cultures restored for anaerobic growth remained < 10% that of the wild-type strain, however, after cystine addition. These results suggest that maintenance of redox homeostasis is a primary CcmB function during growth on electron acceptors with $E'_0 < 0.36V$.

The results of the present study suggest that the CcmAB-transported molecule (allocrite) or holocytochrome *c* maintains proper redox poise in the *S. putrefaciens* periplasm during growth at low redox potential, however, the allocrite transported by CcmAB is unknown. In the present study, mutant CCMB1 was found to contain a H108Y mutation in the CcmB permease subunit. H108 is highly conserved in CcmB orthologs in other γ-proteobacteria, α-proteobacteria and the mitochondria of land plants.

Histidine is a common iron axial ligand in heme-binding proteins and is present in the characteristic cytochrome *c* heme-binding motif (CXXCH). To test the hypothesis that H108 confers the ability to transport heme across the cytoplasmic membrane in *S. putrefaciens*, a set of CcmB site-directed mutants was constructed. H108 was replaced with the hydrophobic residues alanine (H108A) and leucine (H108L), charged or polar residues lysine (H108K) and tyrosine (H108Y) or methionine (H108M), a hydrophobic residue that coordinates heme iron in cytochrome *c*. The site-directed mutants were grouped into three classes based on anaerobic growth capability, cytochrome *c* content and periplasmic redox condition (measured by free thiol (-SH) content): (i) H108Y and H108K were unable to grow anaerobically on electron acceptors with $E'_0 < 0.36V$, produced periplasmic cytochrome *c* at levels < 10% of the wild-type strain and contained periplasmic (–SH) content at levels two-fold greater than the wild-type strain, (ii) H108A and H108L retained the ability to grow on electron acceptors with $E'_0 < 0.36V$, produced periplasmic cytochrome *c* at levels < 20% of the wild-type strain and contained periplasmic -SH content at levels 50% greater than the wild-type strain, and (iii) H108M retained the ability to grow on electron acceptors with $E'_0 < 0.36V$, produced periplasmic cytochrome *c* at near wild-type levels and contained wild-type levels of periplasmic SH. –SH concentrations correlated inversely with the anaerobic growth rates and periplasmic cytochrome *c* content of the site-directed mutants. Only a hydrophobic residue capable of coordinating heme iron (i.e., methionine) functionally replaced the conserved histidine in *S. putrefaciens* CcmB. These results are consistent with the hypothesis that CcmB binds and transports heme from the cytoplasm to the periplasm. Current work is focused on determining the ability of CcmB to bind heme iron at H108 and examining the

possibility that CcmB functions as the first component of a multi-component, histidine-based heme delivery system that transports heme across the cytoplasmic membrane for subsequent stepwise delivery to CcmC, CcmE and ultimately CcmF for incorporation into apocytochrome *c*.

The present study was the first to examine Ccm deficiencies during respiration on electron acceptors with a wide range of E'_0. Surprisingly, *S. putrefaciens* requires 10-20% the cytochrome *c* content measured in laboratory-grown cultures to grow anaerobically on electron acceptors with $E'_0 < 0.36V$ at optimal rates. Redox homeostasis, however, is disrupted in *S. putrefaciens* strains that are unable to transport heme from the cytoplasm to the periplasm or incorporate heme into apocytochrome *c*, and the overly reducing redox poise of the periplasm prevents anaerobic growth on electron acceptors with $E'_0 < 0.36V$. Ccm activity, and the associated translocation of heme from the cytoplasm to the periplasm, may therefore be essential to produce electron transport chain components and prevent thiol accumulation under highly reducing conditions such as those likely to develop in uranium-contaminated aquifers engineered for rapid U(VI) reduction or in anoxic environments containing electron acceptors with $E'_0 < 0.36V$.

APPENDIX

CYTOCHROME *C* CONTENT IN *S. PUTREFACIENS* CORRELATES INVERSELY WITH CULTURE AMBIENT REDOX POTENTIAL (EH)

The results of the present study indicated that the requirement for cytochrome *c* maturation subunit CcmB is greater during growth on electron acceptors with $E'_0 < 0.36V$ than during growth on electron acceptors with $E'_0 > 0.36V$. To test the hypothesis that *S. putrefaciens* maintains redox homeostasis by producing cytochrome *c*, culture ambient redox potential (Eh) was monitored with a platinum redox electrode (Corning Incorporated, Corning, NY). Culture Eh decreased concomitantly with cell growth, electron acceptor depletion or end-product accumulation (Figure A.1). Periplasmic protein extracts were isolated from each culture at late-logarithmic growth phase and 200 μg total protein was separated via SDS-PAGE electrophoresis. The SDS-PAGE gel was stained for heme with dimethoxybenzidine (see Materials and methods, Chapter 3). This set of experiments demonstrates that the cytochrome *c* content in *S. putrefaciens* correlates inversely with culture Eh and indicates that cytochrome *c* participates in maintaining periplasmic redox homeostasis during growth at low redox potential.

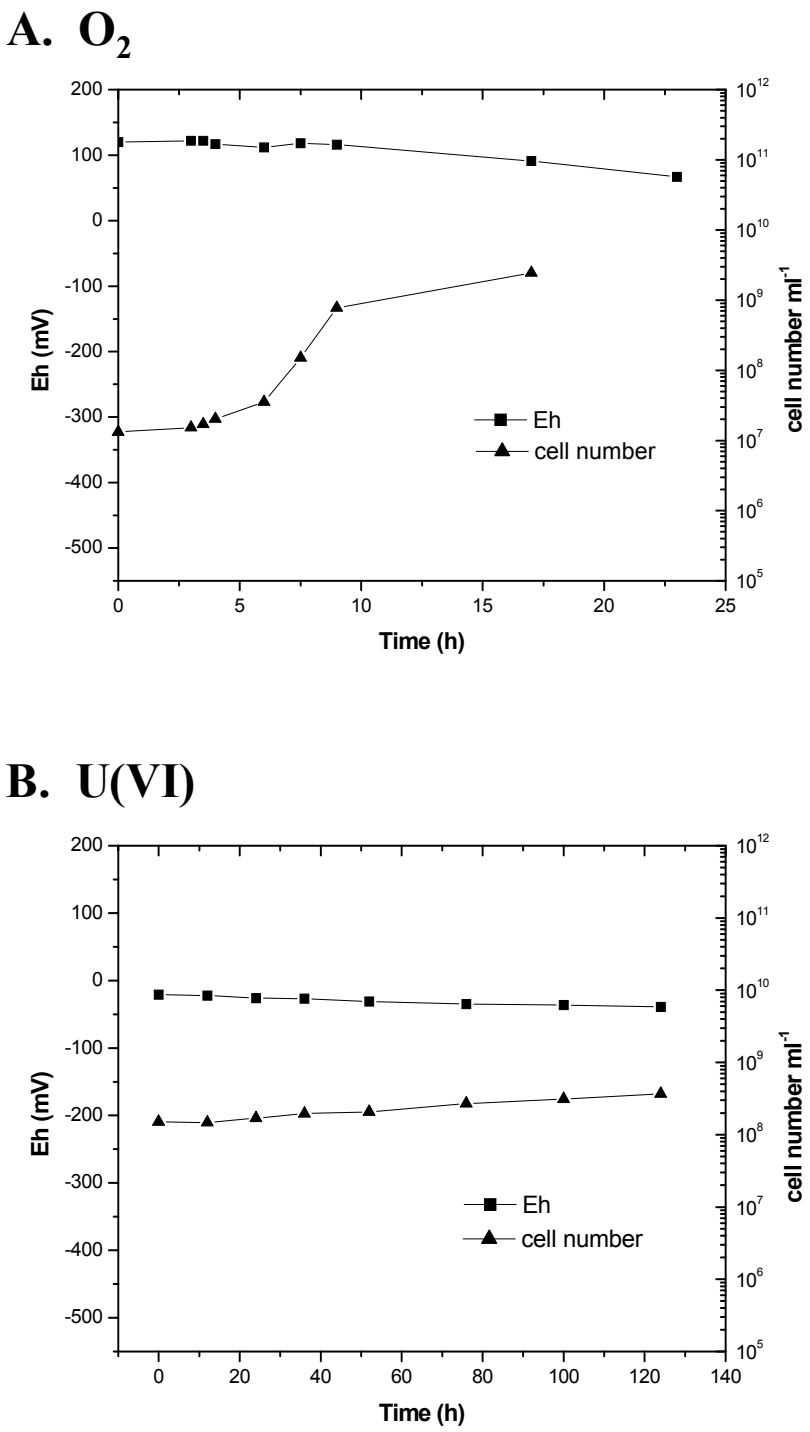
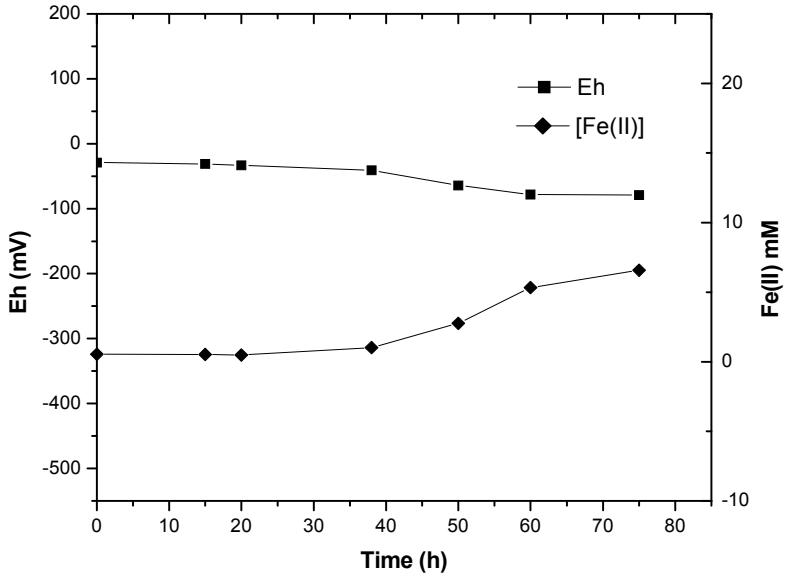


Figure A.1. Culture medium Eh during growth of *S. putrefaciens* on 13 electron acceptors. Cell number, depletion of electron acceptors (i.e., NO_2^-, F; Mn(III), H) or accumulation of end-products (i.e., Fe(II), C and L; NO_2^-, F) were monitored concomitantly with Eh.

C. γ-FeOOH



D. Mn(IV)

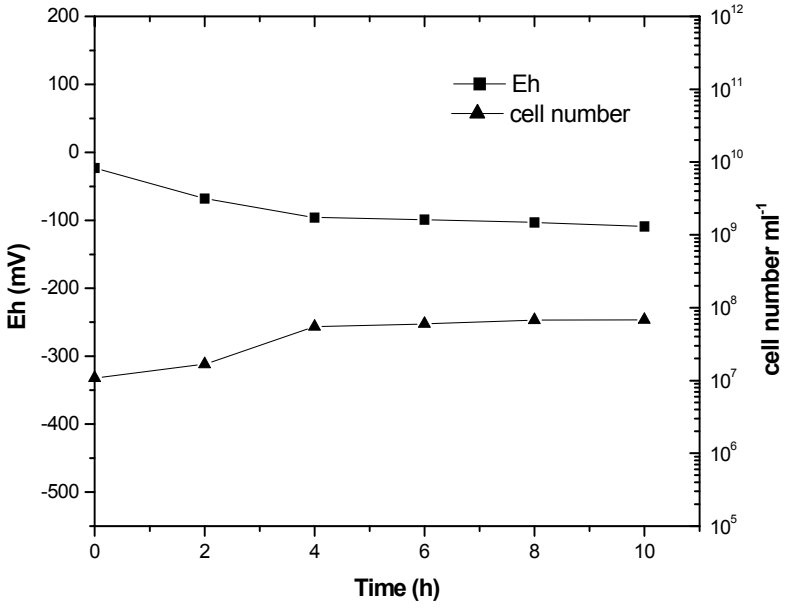
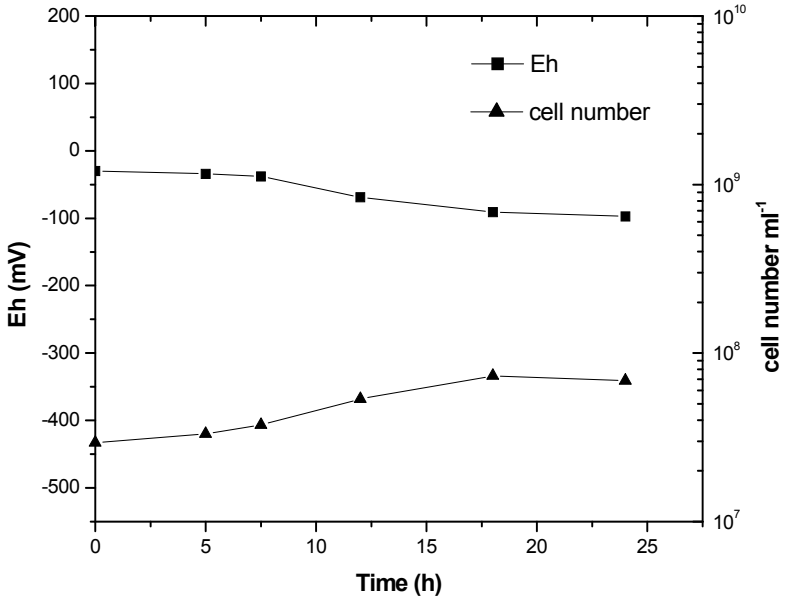


Figure A.1 continued.

E. SO_3^{2-}



F. NO_3^- and NO_2^-

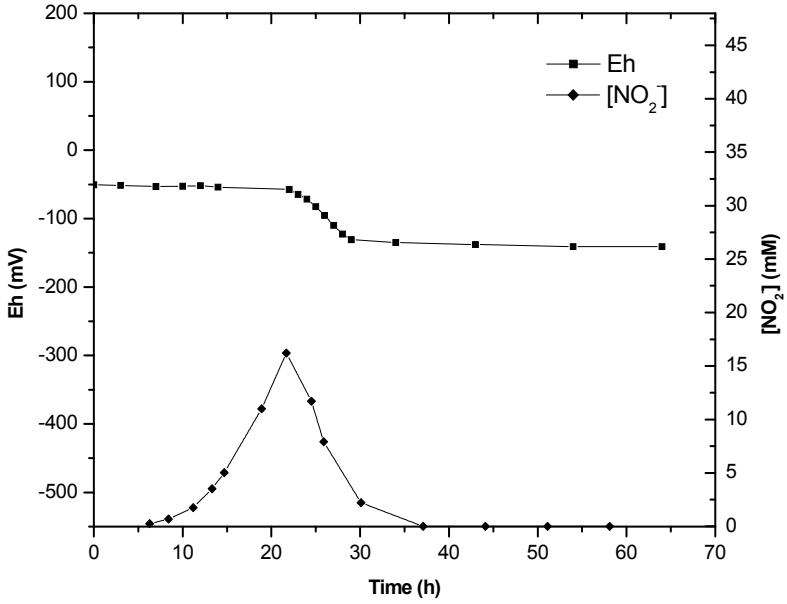
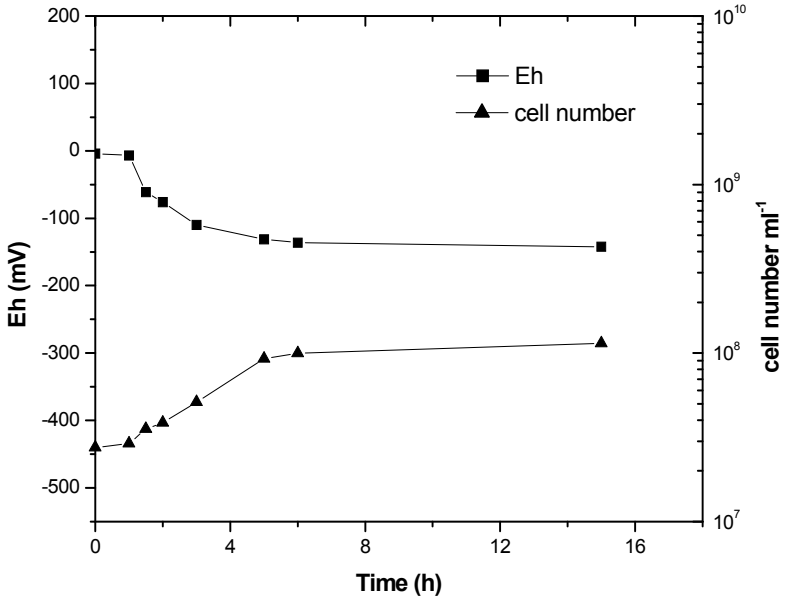


Figure A.1 continued.

G. $S_2O_3^{2-}$



H. Mn(III)-pyrophosphate

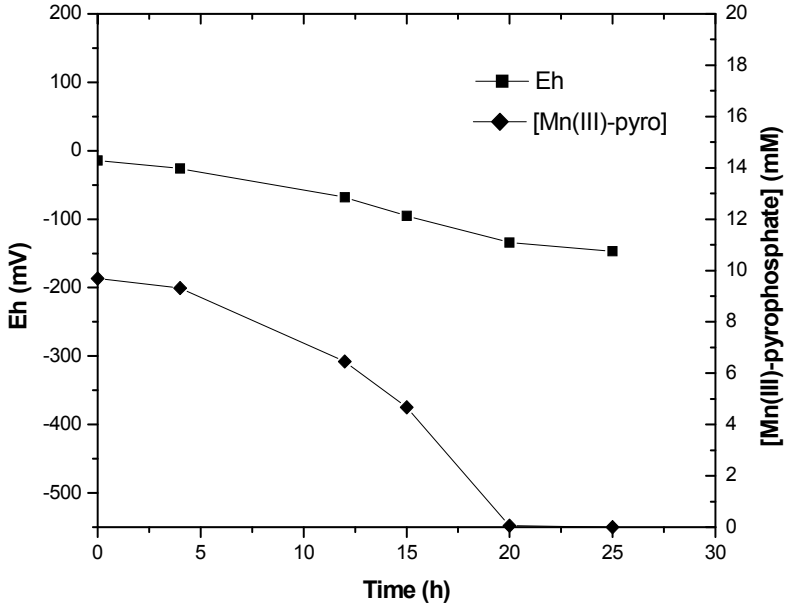
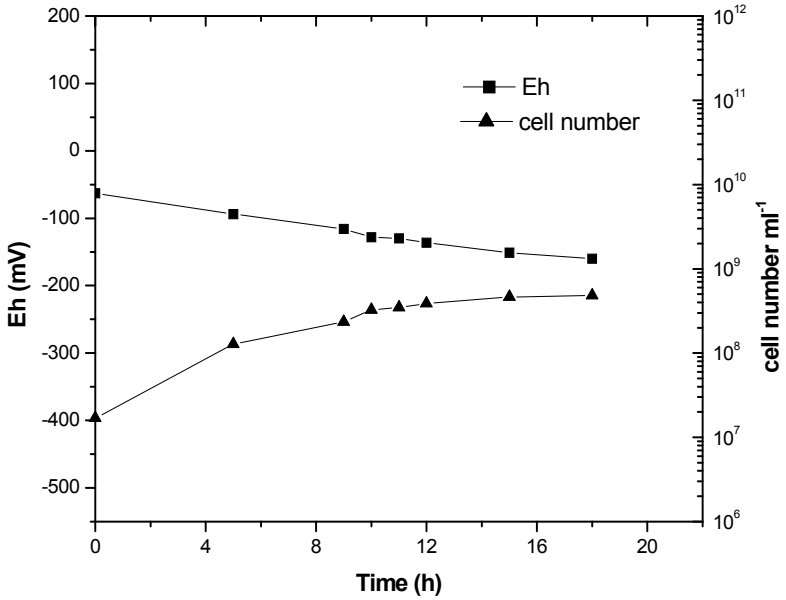


Figure A.1 continued.

I. TMAO



J. DMSO

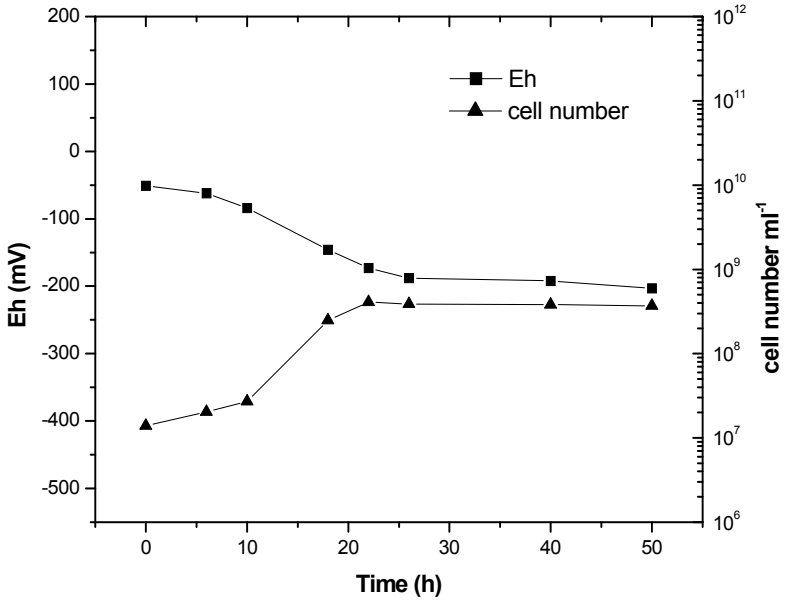
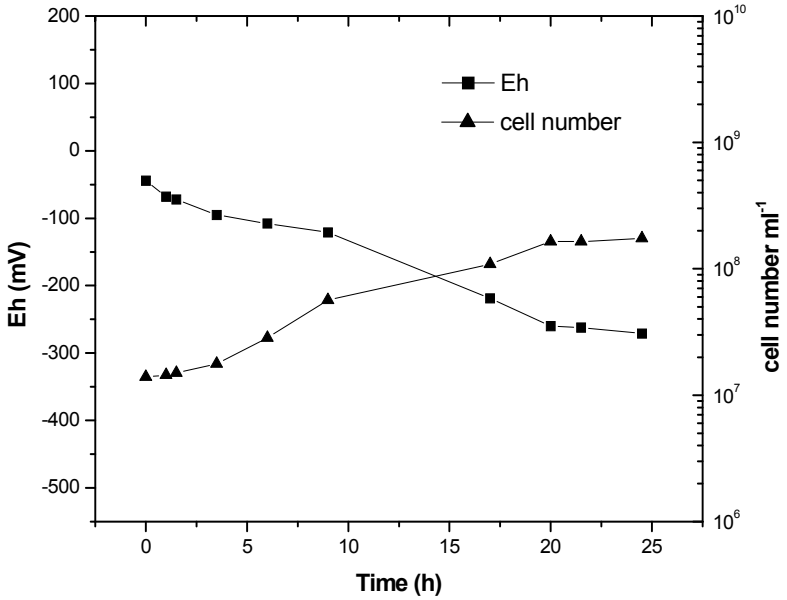


Figure A.1 continued.

K. Fumarate



L. Fe(III)-citrate

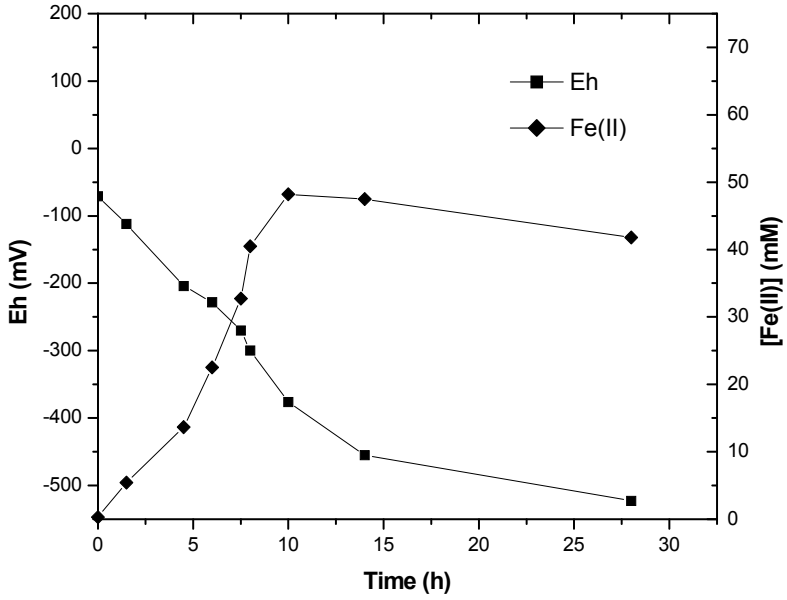


Figure A.1 continued.

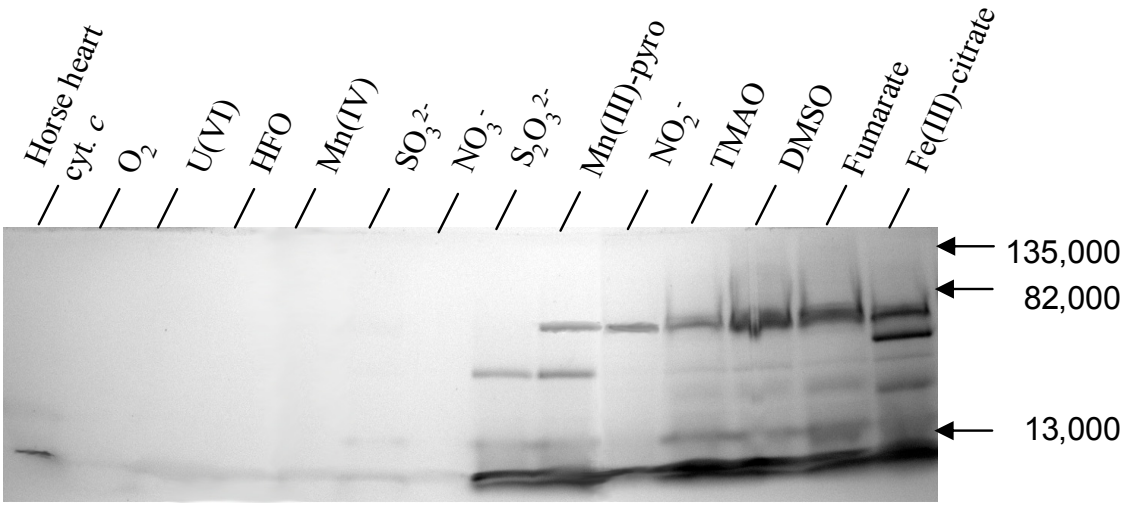


Figure A.2. SDS-PAGE heme stain of *S. putrefaciens* periplasmic fractions after growth on the indicated electron acceptors. Samples are arranged in order of increasing cytochrome *c* content. Horse heart cytochrome *c* was included as a heme-positive control. Molecular mass markers (Daltons) are indicated with arrows.

VITA

JASON ROBERT DALE

DALE was born in Boynton Beach, Florida. The younger of two children, he attended St. Joan of Arc Catholic School and Spanish River Community High School in Boca Raton, Florida. He developed an interest in biotechnology while attending Florida Atlantic University, Boca Raton, Florida and earned a Bachelor of Science degree (microbiology, *cum laude*) from FAU in 2001. After a research internship during the summer of the same year at the National Institutes of Health in Bethesda, Maryland, he joined the laboratory of Thomas J. DiChristina at Georgia Tech to study microbial U(VI) reduction and pursue a Doctor of Philosophy degree in Biology. His interest in discovering molecular mechanisms of microbially-mediated mineral transformations continues.