NOVEL PATHWAY FOR MICROBIAL FE(III) REDUCTION:

ELECTRON SHUTTLING THROUGH NATURALLY OCCURRING THIOLS

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NOVEL PATHWAY FOR MICROBIAL FE(III) REDUCTION:
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LIST OF SYMBOLS AND ABBREVIATIONS

ADP	adenosine diphosphate
AOM	anaerobic oxidation of methane
ATP	adenosine triphosphate
AQDS	anthraquinone-2,6-disulfonate
CSH	cysteine
CSSC	cystine
DMDS	dimethyldisulfide
DMRB	Dissimilatory metal-reducing bacteria
DMSP	dimethylsulfoniopropionate
DNA	deoxyribonucleic acid
DTDG	dithiodiglycolate
DTDP	dithiodipropionate
DTNB	
	5-(3-Carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid
FAD	flavin adenine dinucleotide
FbFP	FMN_binding fluorescent protein
Fe	iron
FMN	flavin mononucleotide
GFP	green fluorescent protein
GSH	glutathione
GSSG	oxidized glutathione
HFO	hydrous Fe(III) oxide

IM	inner membrane
LB	Luria-Bertani medium
LOV domain	light, oxygen, voltage domain
Mn	manganese
OM	Outer membrane
PCR	polymerase chain reaction
PMF	proton motive force
RNA	ribonucleic acid
SRB	sulfate-reducing bacteria
T2SS	type II secretion syctem
Vmax	maximum reaction rate

SUMMARY

Dissimilatory metal-reducing bacteria (DMRB) play an integral role in the
biogeochemical cycling of metals in a broad range of environments including redox-stratified
water and sediments. DMRB are also involved in the cycling of other elements such as carbon,
nitrogen, and sulfur. The molecular mechanism of bacterial metal respiration, however, is not
fully understood. Understanding the mechanism by which DMRB mediate metal reduction will
contribute to a better understanding of their roles in the environment and to the development of
applications such as the bioremediation of metal- and radionuclide contaminated sites and
generation of electricity in microbial fuel cells. Reduced organic sulfur compounds such as
thiols are widespread in natural environments where DMRB are found. These naturally occurring
thiols are redox reactive and abiotically reduce Fe(III) oxides at high rates. The readily available
pool of thiol compounds may thus provide DMRB with a suite of external electron shuttles for
Fe(III) reduction and provide them with a competitive advantage in metal-rich anaerobic
environments.

The main objectives of the thesis research were to i) determine if naturally occurring
thiols function as electron shuttles to deliver electrons to external Fe(III) oxides during microbial
Fe(III) oxide respiration (Chapter 2), ii) identify the genes involved in the thiol-based electron
shuttling pathway of Fe(III)-respiring *S. oneidensis* (Chapter 3), iii) determine if the CXXC
motif of the outer membrane beta-barrel protein MtrB is required for thiol-based electron
shuttling to external Fe(III) oxides by *S. oneidensis* (Chapter 4), and iv) design a flavin
mononucleotide (FMN)-based flourescent protein (FbFP) reporter system to monitor FMN

concentrations in vivo in individual *S. oneidensis* cells during flavin-based electron shuttling to external Fe(III) oxides (Chapter 5).

S. oneidensis reduced a suite of naturally occurring disulfide compounds commonly found in marine and freswater environments, including cystine, oxidized glutathione, dithiodiglycolate, dithiodipropionate, cystamine, and dimethyldisulfide to their corresponding thiol forms. Addition of the disulfide compounds to anaerobic *S. oneidensis* cultures greatly accelerated the rate and extent of Fe(III) oxide reduction by *S. oneidensis*. The results of Chapter 2 indicate that thiol-based electron shuttling pathways provide *S. oneidensis* with a more efficient pathway for electron transfer to external Fe(III) oxides during anaerobic Fe(III) oxide respiration.

Application of a newly developed disulfide reduction mutant screening technique to random chemical mutants resulted in identification of two respiratory mutants that were unable to grow on Fe(III), Mn(III), and Mn(IV), but retained wild type reduction activity on all non-metal electron acceptors. Subsequent genetic complementation and nucleotide sequencing analyses indicated that both mutants contained a point mutation in the gene encoding the outer membrane beta-barrel protein MtrB, which is a central component in the extracellular electron pathway terminating with the reduction of Fe(III), Mn(III), and Mn(IV). The disulfide reduction deficiencies displayed by the disulfide reduction-deficient mutants correlated with their Fe(III) reduction deficiencies. The results of Chapter 3 indicate that disulfide reduction by *S. oneidensis* is catalyzed by the Fe(III)-, Mn(III)-, and Mn(IV)-reducing Mtr pathway.

MtrB plays a central role in Fe(III), Mn(III), Mn(IV), and disulfide reduction by *S. oneidensis*, yet MtrB homologs are also found in non-Fe(III)-respiring bacteria. Nucleotide sequence analysis revealed that MtrB homologs from metal-reducing *Gammaproteobacteria*

contained a unique N-terminal CXXC motif that was missing from MtrB homologs of non-metal-reducing *Gammaproteobacteria* and metal- and non-metal-reducing bacteria outside the *Gammaproteobacteria*. The pathogen *Vibrio parahaemolyticus*, a *Gammaproteobacterium* containing an MtrB homolog with a CXXC motif, was subsequently tested for metal respiration capability. The results of Chapter 4 indicate that MtrB homologs containing a N-terminal CXXC motif represent a molecular signature unique to metal-reducing members of the *Gammaproteobacteria*.

Applications of Green Fluorescent Protein (GFP) to examine molecular events in single microbial cells are limited by the oxygen-dependent autocatalytic maturation of the GFP chromophore. GFP applications are thus restricted to aerobic microorganisms and are not suitable for in vivo studies of molecular events in anaerobic microorganisms. A novel group of flavin mononucleotide (FMN)-based fluorescent proteins (FbFPs), have been developed as replacements for GFP. FbFPs do not require oxygen for chromophore maturation, and can thus be applied under both aerobic and anaerobic conditions to monitor molecular events in single microbial cells. FbFPs require FMN as cofactor, which also suggests that FbFP fluorescence may be used as an in vivo reporter of internal FMN concentrations. The FbFP reporter system constructed in Chapter 5 provided a novel technology for in vivo monitoring of internal FMN concentrations in single *S. oneidensis* cells during anaerobic growth on an array of terminal electron acceptors, including O_2, fumarate, and Fe(III).

CHAPTER 1

INTRODUCTION

Microbial (dissimilatory) metal respiration is a central component of a variety of environmentally important processes, including the biogeochemical cycling of metals and other elements including carbon, nitrogen, and sulfur [1-3]. In anaerobic marine and freshwater systems, dissimilatory metal-reducing bacteria (DMRB) generate energy by coupling the oxidation of organic compounds or molecular hydrogen (H_2) to the reduction of alternative electron acceptors, including soluble and insoluble forms of transition metals such as Fe(III), Mn(III), and Mn(IV) [4]. DMRB also play an important role in the degradation of toxic hazardous pollutants and the bioremediation of radionuclide-contaminated water and sediments [5]. In addition, microbial metal respiration has recently received attention for its potential applications in the generation of electricity in microbial fuel cells [6, 7].

Phylogeny of the *Gammaproteobacteria*

The phylum *Proteobacteria* contains gram-negative bacteria that display a diversity of physiological attributes [8, 9]. The vast majority of gram-negative *Proteobacteria* include phototrophs, heterotrophs, and lithotrophs [10]. Based on comparative analysis of 16S rRNA sequences, *Proteobacteria* are phylogenetically divided into *Alpha, Beta, Gamma, Delta, Epsilon,* and the newly reported *Zeta* classes [8, 10-13]. Among the classes within *Proteobacteria* phylum, *Gammaproteobacteria* are more closely related to *Betaproteobacteria* than the other classes [10, 14, 15]. The *Gammaproteobacteria* class includes many of the most intensively studied model organisms, including *Escherichia coli, Salmonella, Vibrio, Pseudomonas, Yersinia,* and *Shewanella* [9, 10, 14, 16, 17]. In total, the *Gammaproteobacteria* are composed of approximately 250 genera, which is one of the highest numbers of genera within all bacterial phyla [16, 18].

Phylogenetic diversity of DMRB

Shewanella oneidensis MR-1 and *Geobacter sulfurreducens* were among the first bacterial strains reported to conserve energy through dissimilatory metal reduction [19, 20]. DMRB have been subsequently identified throughout the domains *Archaea* and *Bacteria* [21, 22]. DMRB have been isolated from all major classes in the *Proteobacteria*, including the classes *Alpha* (e.g., *Acidiphilium acidophilum*), *Beta* (e.g., *Rhodoferax ferrireducens* and *Ferribacterium limneticum*), *Gamma* (e.g., *Shewanella oneidensis, Ferrimonas balearica, Aeromonas hydrophila,* and *Pantoea agglomerans* SP1), *Delta* (*Geobacter sulfurreducens, Desulfovibrio profundus, Pelobacter carbinolicus, Desulfuromonas acetoxidans*, and *Geothermobacter ehrlichii*) and *Epsilon* (*Sulfurospirillum barnesii*) (Figure 1.1). Facultative anaerobes in the genus *Shewanella* and obligate anaerobes in the genus *Geobacter,* which belong to the *Gammaproteobacteria* and *Deltaproteobacteria* classes, respectively, represent the most comprehensively studied DMRB. Recent advances on *S. oneidensis* MR-1 and *G. sulfurreducens,* as well as some related strains, have provided insight into the mechanism of microbial metal respiration.

Metal-Reducing Members of the Genus *Shewanella*

S. oneidensis MR-1 is a gram-negative facultative anaerobe formerly known as *Alteromonas putrefaciens* MR-1. *S. oneidensis* MR-1 was isolated from the metal-rich, freshwater anaerobic sediments of Oneida Lake (NY) and was one of the first microorganisms found to generate energy by electron transport chain-linked metal reduction [19]. Most of the other known *Shewanella* species were isolated from marine environments [23], including the tissues of rotting fish and squid [23]. *S. oneidensis* MR-1 displays remarkable respiratory versatility and respires a variety of terminal electron acceptors including O_2, fumarate, nitrate, nitrite, trimethylamine N-oxide, dimethyl sulfoxide, sulfite, thiolsulfate, elemental sulfur, and soluble and insoluble transition metals such

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Figure 1.1 Phylogenetic affiliation of microorganisms contributing to iron cycling. Fe(III)-reducing bactera are designated in red text, while Fe(II)-oxidizing bacteria are designated in black text. Adapted from Weber et al[21].

as Fe(III) citrate, Fe(III) oxide, goethite, hematite, Mn(IV) oxide, and Mn(III) [19, 24-29]**.** The respiratory versatility of *Shewanella* is thought to facilitate survival in fluctuating redox conditions. In the food industry, *Shewanella* species have been identified as the main spoilage bacteria during low temperature storage of fish [30-32]. Although human infections by *Shewanella* species are rare, several infections have been attributed to *S. algae* and *S. putrefaciens* [33].

3

Molecular mechanism of microbial metal respiration

Respiration in gram-negative bacteria is based on the generation of a proton-motive force (PMF) across the inner membrane (IM). Electrons originating from the oxidation of electron donors are transported down the redox gradient of an electron transport chain to terminal reductases, while protons are translocated across the IM to generate PMF. PMF drives ATP synthesis as protons are translocated back into the cytoplasm through IM-localized ATPases, catalyzing the phosporylation of ADP to ATP [34]. Bacterial terminal reductases for soluble electron acceptors such as O_2, nitrate, and fumarate are located on the IM or in the periplasmic space. DMRB, however, are presented with a unique physiological problem: they are required to respire anaerobically on terminal electron acceptors found largely in solid forms that are presumably unable to contact IM-localized electron transport systems [5]. To overcome this problem, *S. oneidensis* is postulated to employ a variety of novel respiratory strategies including i) direct enzymatic reduction of solid electron acceptors via outer membrane (OM)-localized metal reductases [5, 35-37], ii) electron shuttling pathways using exogenous or endogenous electron shuttling compounds [38-42], iii) chelation (solubilization) pathways in which the solid electron acceptors are first nonreductively dissolved by endogenously synthesized organic ligands prior to reduction [28, 43, 44], and iv) nanowire pathways in which electrically conductive pili (nanowires) transfer electrons to external metal oxides [45, 46].

1. Direct enzymatic reduction of Fe(III) oxides. Direct enzymatic reduction of Fe(III) oxides requires that the Fe(III) terminal reductases be localized at the OM (Fig. 1.2). *S. oneidensis* has evolved extracellular electron transfer strategies requiring multiheme *c*-type cytochromes [47]. The *S. oneidensis* OM proteins involved in the terminal steps of electron transfer to insoluble electron acceptors include several *c*-type cytochromes [36, 47-49] that are a

subset of the 42 predicted *c*-type cytochromes in the *S. oneidensis* genome [25]. One of the proposed pathways of electron transfer to insoluble Fe(III) and Mn(IV) oxides is through the OM-localized, extracellular electron conduit encoded by the *omcA-mtrCAB* gene cluster [19, 50-52]. Disruption of the *mtrC* or *omcA* does not affect the ability of *S. oneidensis* MR-1 to reduce soluble electrons acceptors such as fumarate or nitrate. Deletion of *mtrC*, however, decreases the capacity of the mutant to reduce solid Fe(III) oxide to approximately 33% of the wild type rate. While deletion of *omcA* alone does not affect solid Fe(III) oxide reduction, an *mtrC/omcA* double deletion mutant displays a severe deficiency in Fe(III) oxide reduction activity. MtrC is therefore postulated to transfer electrons to extracellular Fe(III) oxides [53, 54], or to extracellular electron shuttles which in turn reduce the Fe(III) oxides [38, 55]. MtrC and OmcA are translocated across the periplasm to the OM through the type II protein secretion system (T2SS)(Fig. 1)[56]. MtrC is also postulated to bind and transfer electrons to flavins secreted by *S. oneidensis* as electron shuttles to transport electrons to external Fe(III) oxides [57] .

MtrA is a soluble decaheme *c*-type cytochrome located in the *S. oneidensis* periplasm (Fig. 1.2)[35]. Amino acid sequence comparisons reveal that MtrA displays a high degree of sequence similarity to NrfB, a *c*-type cytochrome involved in formate-dependent nitrite reduction in *E. coli* [35]. MtrA associates in the OM as part of the extracellular electron conduit consisting of MtrCAB in a 1:1:1 stoichiometry [53]. MtrB is a central component in the metal reduction pathway, and deletion of the *mtrB* gene results in a severe loss of capability to respire on both soluble and insoluble metals [35]. MtrB does not contain hemes and is postulated to function as an OM anchor that facilitates electron transport from MtrA in the periplasm to MtrC or OmcA at
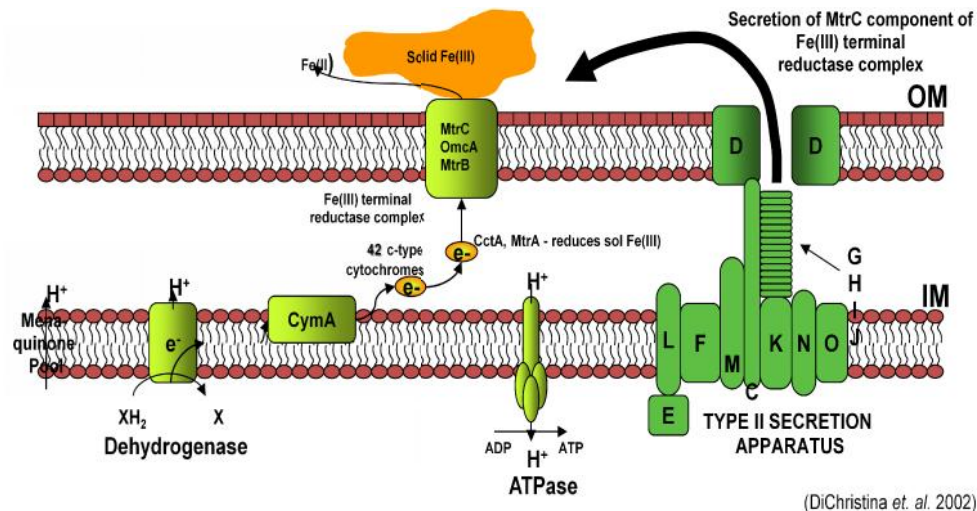
Fig 1.2. Working model of the *S. oneidensis* electron transport chain terminating with reduction of external Fe(III) oxides[5].

the cell surface [47]. The β-barrel structure of MtrB may serve as a sheath for embedding MtrA and MtrC at the inner and outer faces of the membrane, thereby facilitating electron transfer across the OM to external metal oxides [58]. MtrA and MtrB homologs have also been reported in Fe(II)-oxidizing bacteria such as *Rhodopseudomonas palustris*, *Sideroxydans lithotropicus,* and *Dechloromonas aromatica* [59-61]. The presence of MtrAB homologs in both Fe(III)-reducing and Fe(II)-oxidizing bacteria indicates that electron transfer across the OM through the Mtr pathway may be bidirectional [62].

2. **Fe(III) chelation (solubilization) pathways.** Some Fe(III)-reducing bacteria such as *S. putrefaciens*, *S. oneidensis*, *S. algae*, and *Geobacter fermentans* generate soluble organic-Fe(III) complexes in the absence of exogenous chelating compounds, an indication that such bacteria synthesize and release organic ligands to solubilize Fe(III) prior to reduction [43, 63]. Soluble organic-Fe(III) is detected electrochemically in *S. oneidensis* and *S. putrefaciens* cultures incubated anaerobically with Fe(III) oxides [64]. Detection of soluble organic-Fe(III) prior to

Fe(II) production suggests that soluble organic-Fe(III) is an intermediate in the reduction of solid Fe(III) oxides [43, 64]. Recent genetic and biochemical analyses indicate that the well-known Fe(III)-chelating hydroxamate-type siderophores do not function as Fe(III)-chelating ligands during anaerobic respiration of solid Fe(III) oxides [44]. The identity of the Fe(III)-solubilizing organic ligands and the proteins involved in their biosynthesis have not yet been identified.

   3. Extracellular electron transfer via nanowires. *S. oneidensis* cultures grown in rich growth medium under O_2-limiting conditions produce pilus-like external appendages termed nanowires, which range from 50-to-150 nm in diameter and tens of microns in length [65]. Scanning tunneling microscopy analyses indicated that the nanowires were electrically conductive [46]. The nanowires of *S. oneidensis* may facilitate electron transfer from the cell surface to external Fe(III) oxides without the need for direct cell-Fe(III) oxide contact. Mutants deficient in MtrC and OmcA and those lacking a functional Type II protein secretion system produce poorly conductive nanowires. Further investigations are required to determine the roles of MtrC, OmcA, and Type II protein secretion in nanowire architecture.

   4. Electron shuttling pathways. *S. oneidensis* transfers electrons to Fe(III) oxides located more than 50 µM (i.e., approximately 50 cell diameters) from the cell surface [63, 66]. Since electrons require a carrier to traverse distances of more than 0.01 µM [67-69], electron shuttling compounds may facilitate electron transfer to external Fe(III) oxides. *S. oneidensis* employs a variety of exogenous redox-active compounds such as humic acids, phenazines, and AQDS as electron shuttles to reduce extracellular Fe(III) oxides [4, 39, 70-72]. Potential endogenous electron shuttles for *Shewanella* include flavins (FMN, FAD, riboflavin) [38, 40], menaquinone [42], melanin [41], and organic sulfur (thiol) compounds (the subject of the present thesis; Figure 1.3). *Shewanella* proteins involved in thiol-based electron shuttling have not been identified and

the molecular mechanism of the electron shuttling pathway is the subject of an ongoing

controversy.
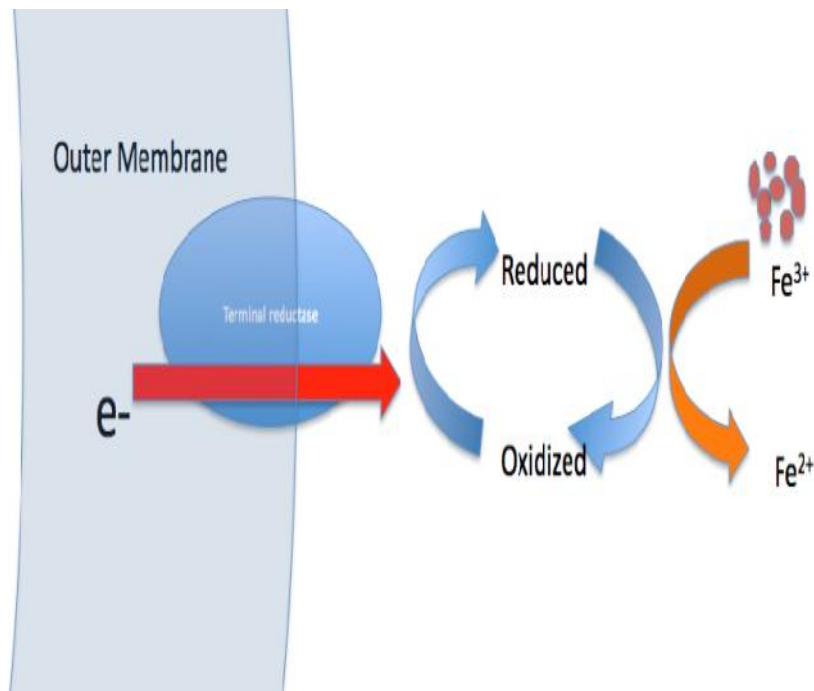
Figure 1.3. Schematic representation of the electron transfer pathway to external Fe(III) oxides reduction via exogenous electron shuttles.

Naturally occurring thiols in the environment

Reduced organic sulfur compounds such as thiols are widespread in marine and

freshwater systems. Thiols have been reported in marine water [73-75], freshwater [76], and

estuarine and salt marsh porewaters [77-80], with concentrations ranging from nanomolar to

millimolar levels [81]. Thiols commonly found in these environments include cysteine,

glutathione, mercaptopropionate, mercaptoacetate, mercaptosuccinic, mercaptoethanol, and methanethiol [75, 76, 78, 81](Figure 1.4).
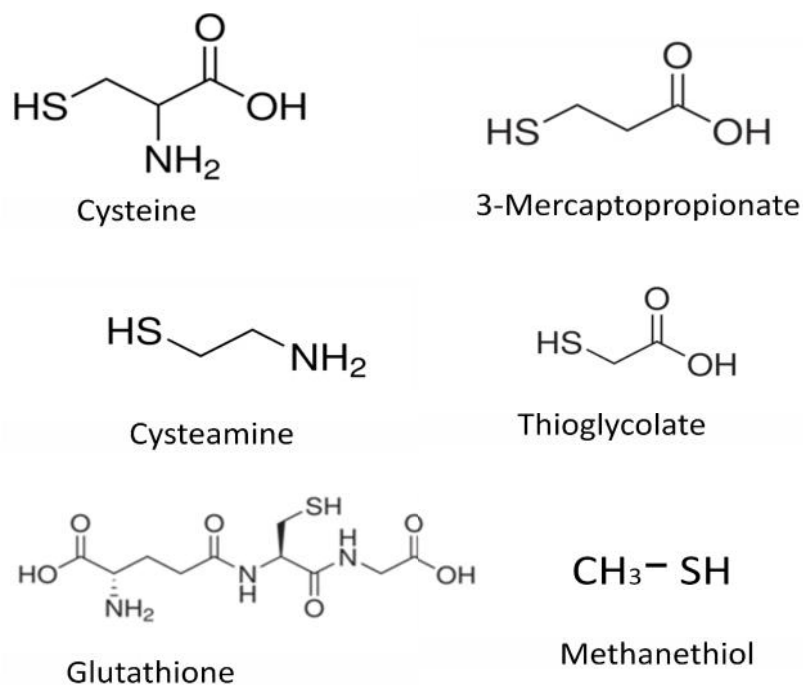
Figure 1.4. Chemical structures of naturally occurring thiols found in marine water, freshwater, and estuarine and salt marsh porewaters.

Thiols play important roles in many biogeochemical processes, and their importance in the environment has gained attention due to the ability of thiols to form complexes with copper [74, 78, 82] and mercury [81]. Thiols in the environment originate from both biological and abiotic sources. Mercaptopropionate and methanethiol are produced by microbial degradation of dimethylsulfoniopropionate (DMSP) [83, 84]. DMSP maintains intracellular osmostic balance in micro and macro algae [85, 86] and halophytic plants [85, 87]. Cysteine and glutathione are major intracellular thiols in many prokaryotic and eukaryotic organisms [88], and their detection in aquatic systems is most likely due to secretion by metabolically active microorganisms or by

release from decaying microorganisms [73, 89]. Mercaptoacetate, mercaptopyruvate, and mercaptoethanol are generally produced by microbial degradation of cysteine and glutathione [84]. Thiols also form abiotically from reactions between sulfide or polysulfide and unsaturated organic compounds [90].

Thiols abiotically reduce Fe(III) oxides to form Fe(II) and their corresponding disulfide [91]. Addition of cysteine to anaerobic cultures of the Fe(III)-reducing bacteria *Geobacter sulfurreducens* and *S. oneidensis* enhances Fe(III) oxide reduction activity [92, 93]. Since thiols are abundant in environments where Fe(III)-reducing bacteria are found, the use of naturally occurring thiols as electron shuttles to Fe(III) oxideds may provide Fe(III)-reducing bacteria with a competitive advantage in metal-rich anaerobic environments.

Detection of single microbial cells by fluorescent protein technology

Phycobiliprotein, a photosynthetic antenna pigment isolated from cyanobacteria, was the first fluorescent protein tested for application as an intracellular fluorescent reporter [94]. The applications of phycobiliprotein, however, were limited due to the requirement of tetrapyrrole as cofactor [95]. Green flouorescent protein (GFP), on the other hand, does not require a cofactor for fluorescence and can be readily expressed in a variety of microorganisms [95-97]. GFP was first recombinantly expressed in both prokaryotic and eukaryotic model organisms (*E. coli* and *C. elegans,* respectively) [98]. GFP facilitates the study and in situ visualization of complex molecular events in single cells and organisms using flow cytometry or fluorescent microscopy [97, 99, 100]. Reflecting the significance of the discovery, the Nobel Prize in Chemistry in 2008 was recently awarded for "the discovery and development of the green fluorescent protein, GFP" [96, 99]. GFP was first isolated from the jellyfish *Aequorea aequorea* [101]. The fluorescent properties of GFP have been enhanced by improvements in fluorescent efficiency, thermostability, photostability, and alteration of emission wavelength [95, 96, 99, 100]. GFP variants now emit a wide range of fluorescent colors that nearly span the entire visible spectrum

[99, 100]. GFP is now widely used to examine molecular events in situ, including gene expression, recombinant protein localization, promoter screening, and monitoring changes in intra- or extracellular conditions [97].

GFP applications, however, are limited by the oxygen-dependent autocatalytic maturation of the GFP chromophore [102]. GFP applications are thus restricted to aerobic systems [103, 104] and are not suitable for in situ studies of molecular events under anaerobic conditions. A novel group of fluorescent proteins, termed flavin mononucleotide (FMN)-based fluorescent proteins (FbFPs), have been developed as replacements for GFP [103]. FbFPs do not require oxygen for chromophore maturation, and can thus be applied under both aerobic and anaerobic conditions [103]. FbFPs originate from LOV (light, oxygen, voltage) domain-containing bacterial photoreceptor proteins that exhibit weak intrinsic autofluorescence when irradiated with blue light [105-107].

Two of the photoreceptor FbFPs, YtvA from *Bacillus subtilis* and SB2 from *Pseudomonas putida,* have been engineered to produce fluorescent quantum yields comparable to GFP variants [103]. Site directed mutagenesis of a photoactive cysteine in the LOV domain in both the truncated YtvA and wild type SB2 proteins, followed by codon optimization, resulted in a 25-fold increase in fluorescent strength [97]. Recently, FbFPs have been used to monitor the presence of single anaerobic bacterial cells with promising results . FbFPs require FMN as cofactor, which also suggests that FbFP fluorescence may be used as an in vivo reporter of internal FMN concentrations. FbFP fluorescence thus provides a novel technology for monitoring internal FMN concentrations in vivo in individual *S. oneidensis* cells during flavin-based electron shuttling to external Fe(III) oxides.
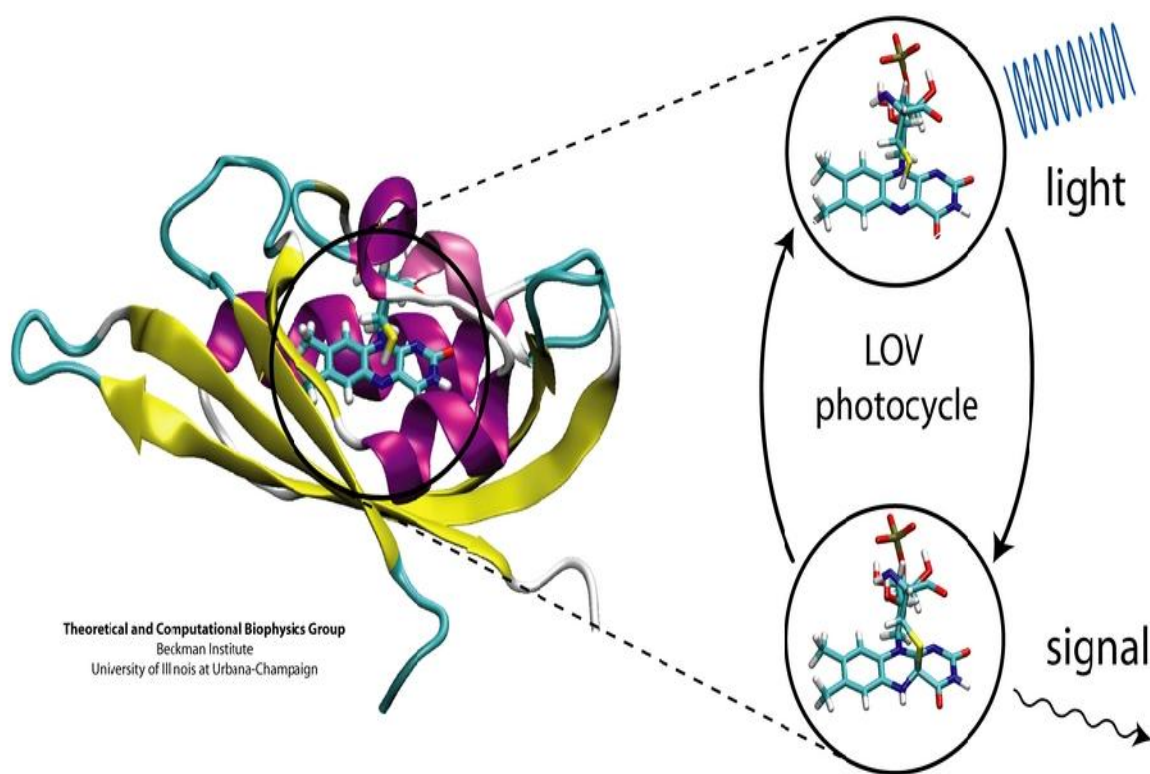
Figure 1.5. LOV domain of *C. reinhardtii* containing the FMN chromophore. The protein is shown in cartoon representation; the chromophore FMN is shown in Licorice representation. A fluorescent signal is emitted upon photoexcitation of the FMN chromophore. Adapted from the Beckman Institute at the Univeristy of Illinois-Chicago (http://www.ks.uiuc.edu/images/ofmonth/2005-07a/lov-diagram.jpg)

The main objectives of the thesis were to i) determine if naturally occurring thiols function as electron shuttles to deliver electrons to external Fe(III) oxides during microbial Fe(III) oxide respiration (Chapter 2), ii) identify the genes involved in the thiol-based electron shuttling pathway of Fe(III)-respiring *S. oneidensis* (Chapter 3), iii) determine if the CXXC motif of the outer membrane beta-barrel protein MtrB is required for thiol-based electron shuttling to external Fe(III) oxides by *S. oneidensis* (Chapter 4), and iv) design a FbFP-based flourescent reporter system to monitor FMN concentrations in vivo in individual *S. oneidensis* cells during FMN-based electron shuttling to external Fe(III) oxides by *S. oneidensis* (Chapter 5).

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CHAPTER 2

Extracellular Fe(III) Oxide Reduction by *Shewanella oneidensis* Proceeds

Through Microbial Reduction of Naturally Occurring Disulfide Compounds

[The abiotic thiol-catalyzed Fe(III) oxide reduction experiments described in Chapter 2 were carried out in collaboration with Eryn Eitel, a PhD student in Dr. Taillefert's research group. Eryn's contributions in Chapter 2 are denoted with an asterisk.]

Summary

The γ-proteobacterium *Shewanella oneidensis* MR-1 reduces a wide range of terminal electron acceptors, including solid Fe(III) oxides. Pathways for Fe(III) oxide reduction by *S. oneidensis* include non-reductive (organic ligand-promoted) solubilization reactions, and either direct enzymatic, or indirect electron shuttling pathways. Results of the present study expand the spectrum of electron acceptors reduced by *S. oneidensis* to include the naturally occurring disulfide compounds cystine, oxidized glutathione, dithiodiglycolate, dithoidiproponiate and cystamine. Subsequent electron shuttling experiments demonstrated that *S. oneidensis* employs the reduced (thiol) form of the disulfide compounds (cysteine, reduced glutathione, mercaptoacetate, mercaptopropionate, and 2-nitro-5-thiobenzoate, cystamine) as electron shuttles to transfer electrons to extracellular Fe(III) oxides. The results of the present study indicate that microbial disulfide reduction may represent an important electron-shuttling pathway for electron transfer to Fe(III) oxides in anaerobic marine and freshwater environments.

Introduction

Fe(III)-reducing bacteria are critical components of a variety of environmentally

important processes, including the biogeochemical cycling of metals, carbon, and sulfur [1, 2],

the reductive immobilization of toxic radionuclides in contaminated subsurface sediments [3],

and the production of electricity in microbial fuel cells [4-6]. Fe(III) oxides exist as sparingly

soluble amorphous or crystalline (oxy)hydroxides at circumneutral pH [7]. Fe(III)-reducing

bacteria such as the γ-proteobacterium *Shewanella oneidensis* MR-1 are therefore required to

transfer electrons to external Fe(III) oxides unable to contact inner membrane (IM)-localized

electron transport chains [8-10]. To overcome this problem, *S. oneidensis* transfers electrons to

external Fe(III) oxides via a variety of novel respiratory strategies including i) direct enzymatic

reduction by Fe(III)-reducing *c*-type cytochromes located on the cell surface or along

extracellular nanowires [11-13], ii) non-reductive Fe(III) oxide solubilization followed by

electron transfer to the resulting soluble organic-Fe(III) complexes [14-17], and iii) indirect

reduction by electron shuttling compounds such as flavins, fulvic acids, humic acids, and

melanin [18-25].

In the electron shuttling pathways, Fe(III)-reducing bacteria reduce the oxidized form of

the electron shuttle to its corresponding reduced form, which subsequently delivers the electrons

to external Fe(III) oxides via abiotic (purely chemical) electron transfer reactions [18, 26, 27].

The resulting oxidized electron shuttle is subsequently re-reduced by the Fe(III)-reducing

bacteria, thus resulting in a catalytic cycle that may be repeated hundreds-to-thousands of times

[21]. Microbial cycling of nanomolar amounts of electron shuttle may thus catalyze the

reduction of millimolar amounts of Fe(III) oxide. Due to their high reactivity with Fe(III) oxides

[28, 29], naturally occurring thiols are predicted to function as electron shuttles to efficiently

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transfer electrons to external Fe(III) oxides during microbial Fe(III) oxide reduction. Microbial Fe(III) oxide reduction activity is enhanced, for example, by adding exogenous cysteine to anaerobic cultures of *Geobacter sulfurreducens* and *S. oneidensis* [30, 31].

In addition to cysteine, a variety of other naturally occurring thiols are detected in marine and freshwater environments, including glutathione, mercaptoacetate, mercaptopropionate, mercaptosuccinate, mercaptoethanol, and methanethiol [32-37]. The sources of these thiols in marine and freshwater environments include microbial deamination of amino acids [38], microbial degradation of dimethylsulfoniopropionate (DMSP) [39], abiotic reactions between sulfide or polysulfide and unsaturated organic compounds [40], and release of intracellular thiols from metabolically active or decaying microorganisms [41]. Intracellular thiols such as glutathione and cysteine, for example, are involved in a number of critical bacterial processes, including maintenance of proper redox homeostasis and providing protection from reactive oxygen (ROS), nitrogen (RNS), and electrophilic (RES) species [42-44]. Depth-dependent profiles of glutathione in coastal marine waters co-vary with chlorophyll concentrations, thus indicating that water column glutathione may be derived from the intracellular pool of glutathione released by phytoplankton, algae, or cyanobacteria [45, 46]. If released into the environment, the suite of intracellular thiols may represent a highly reactive, yet overlooked pool of electron shuttling compounds that Fe(III)- and disulfide-reducing bacteria employ to transfer electrons to Fe(III) oxides. The main objectives of the present study were to determine the ability of *S. oneidensis* to i) reduce the suite of disulfide compounds commonly found in marine and freshwater environments, and ii) employ the corresponding thiols as electron shuttles to catalyze electron transfer to external Fe(III) oxides.

Materials and Methods

Bacterial strain and cultivation conditions. *S. oneidensis* MR-1 was isolated from the metal-rich sediments of Oneida Lake (NY) [47]. All Fe(III) reduction and thiol-based electron shuttling experiments were carried out under anaerobic conditions in defined minimal growth medium (M1, pH 7.0) supplemented with lactate (18 mM), formate (15 mM), or hydrogen (2%) as electron donor. Overnight cultures were inoculated at initial cell densities of 2×10^7 cells ml^{-1}. Anaerobic conditions for incubations with lactate or formate as electron donor were maintained by continuous sparging with N_2 gas. Incubations with H_2 as electron donor were conducted in a Coy anaerobic chamber under an anaerobic atmosphere of 2% H_2, 10% CO_2, and the balance N_2. Poorly crystalline Fe(III) oxides were synthesized by neutralizing a solution of $FeCl_3$ with NaOH to pH 7 [48]. Dimethyldisulfide was purchased from MP Biomedicals. Cystine, cystamine, dithiodiglycolate, dithiodiproprionate, and oxidized glutathione were purchased from Sigma-Aldrich.

Determination of thiol and Fe(II) concentrations. Fe(III) reduction was monitored by measuring HCl-extractable Fe(II) with ferrozine [49]. Disulfide reduction was monitored by measuring thiol production in samples withdrawn and centrifuged under anaerobic conditions for 2 min at 16,000 rpm to pellet cell material and residual Fe(III) oxides. Thiol concentrations were determined by adding 100 µl of a 30 mM solution of 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB; Ellmans Reagent) to 900 µl of culture supernatant and measuring absorbance at 412 nm [50]. Thiol concentrations were calculated from calibration curves with cysteine as the model thiol. Maximum rate of reaction Vmax and Km value for disulfide and Fe(III) oxide reduction were determined from Michaelis-Menten plots.

Fe(III) oxide reduction and electron shuttling experiments. Disulfide shuttling experiments consisted of incubating *S. oneidensis* cell cultures (2×10^7 initial cell densities) in M1 minimal growth medium amended with 40 mM Fe(III) oxide as electron acceptor and lactate (18 mM), formate (10 mM), or H_2 (2%) as electron donor. Disulfides were added at concentrations ranging from 4 µM to 4 mM and incubations were carried out under anaerobic conditions either by vigorous nitrogen sparging (lactate and formate as electron donor) or by incubation in a Coy anaerobic chamber (H_2 as electron donor) with an atmosphere consisting of 2% H_2, 10% CO_2, and the balance N_2. Aliquots were withdrawn for Fe(II) and thiol measurements at select time intervals. Disulfides were omitted in one set of incubations to monitor microbial Fe(III) oxide reduction activity in the absence of thiol-based electron shuttling.

Determination of electron shuttling frequency. To determine the efficiency of each disulfide species as an electron shuttle for Fe(III) oxide reduction, the shuttling frequency (*f*, defined as the number of times a disulfide cycled through successive microbial reduction/Fe(III) oxidation reactions) was calculated by following formula:

$$f = \frac{Extent\ of\ Fe(III)reduction\ with\ disulfide - Extent\ of\ Fe(III)reduction\ without\ disulfide}{Amount\ of\ thiol\ as\ a\ product\ of\ disulfide\ reduction}$$

Results and Discussion

The gram negative, γ-proteobacterium *Shewanella oneidensis* MR-1 respires a variety of terminal electron acceptors, including oxygen, fumarate, nitrate, nitrite, trimethylamine-*N*-oxide, dimethyl sulfoxide, sulfite, thiolsulfate, elemental sulfur, uranyl, pertechnetate, and soluble and insoluble forms of Fe(III), Mn(III), and Mn(IV) [14, 51-56]. Such respiratory versatility is thought to enhance the ability of *S. oneidensis* to survive in redox-stratified environments where terminal electron acceptor identity and abundance fluctuate on small temporal and spatial scales [57-59]. A variety of facultative and strict anaerobic bacteria reduce electron shuttling compounds such as humic acids and flavins, however the microbial reduction of disulfides and the use of the produced thiols as electron shuttles to external Fe(III) oxides is poorly understood.

Involvement of thiols as electron shuttles requires microbially-catalyzed disulfide bond reduction, and subsequent electron transfer from the produced thiol to external Fe(III) oxides. Thiol compounds such as cysteine were orginally described as mediators of interspecies electron transfer between *G. sulfurreducens* and *Wolinella succinogenes* [60]. Electrons derived from acetate oxidation by *G. sulfurreducens* are transferred to exogenous cystine as electron acceptor, while the produced cysteine was reoxidized by *W. succinogenes.* Subsequent studies demonstrated that addition of exogenous cysteine to *S. oneidensis* and *G. sulfurreducens* accelerated the rate of Fe(III) oxide reduction [30, 31]. The ability of Fe(III)-reducing bacteria to employ a suite of naturally occurring thiols detected in the environment as electron shuttles to external Fe(III) oxides, however, has not been explored.

S. oneidensis reduces a variety of naturally occurring disulfides as terminal electron acceptor. *S. oneidensis* was tested in anaerobic minimal growth medium for the ability to reduce the disulfide compounds cystine, oxidized glutathione, dithiodiglycolate,
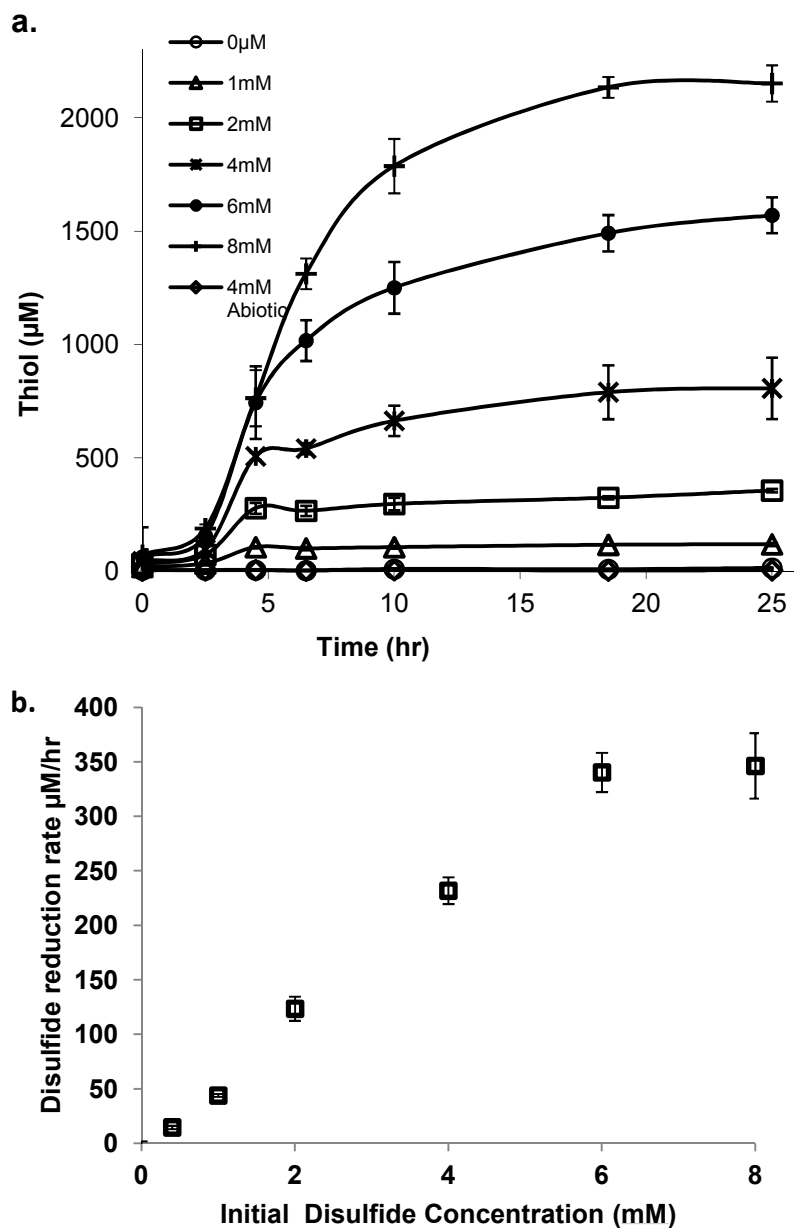
Figure 2.1. (a) Production of cysteine by *S. oneidensis* during cystine reduction with H₂ as electron donor. (b) Michaelis-Menten plot of cystine reduction rates as a function of cystine concentration.

dithiodipropionate, cystamine, and dimethyldisulfide to their corresponding thiol forms cysteine,

reduced glutathione, thioglycolate, mercaptopropionate, cysteamine and methanethiol. *S.*

oneidensis was able to couple the oxidation of lactate, formate or H₂ as electron donor to the

reduction of all disulfides tested. The rate and extent of disulfide reduction by *S. oneidensis* increased as a function of disulfide concentration (Figure 2.1a). Disulfide reduction was not detected in abiotic control experiments. The disulfide reduction rate for each disulfide followed Michaelis-Menten kinetics (Figure 2.1b).

The maximum disulfide reduction rates (Vmax-Di) and Michaelis constant (Km-Di) values of each electron donor-disulfide pair are summarized in Table 2.1. Disulfide reduction rates were electron donor-dependent. With the exception of cystine and dimethyldisulfide, all disulfides were reduced at the highest rate with formate as electron donor, followed by H_2 and lactate. Cystine was reduced at the highest rate with H_2 as electron donor (350 µM/hr), which was 5-fold greater than lactate (70 µM/hr) and 3-fold greater than formate (101 µM/hr) as electron donor. For dimethyldisulfide, which is reduced to the volatile gas methanethiol, the reduction rate was highest with lactate, followed by formate and H_2.

Table 2.1. Maximum rate of disulfide reduction (Vmax-Di) and Michaelis constant (Km-Di) values for disulfide reduction with H_2, lactate, or formate as electron donor. Michaelis-Menten plots used to calculate Vmax-Di and Km-Di for each electron donor/disulfide pair are provided in Appendix A.

Electron Donor	H_2		Lactate		Formate	
Disulfide	Vmax (µM/hr)	Km (mM)	Vmax (µM/hr)	Km (mM)	Vmax (µM/hr)	Km (mM)
Dithiodipropionate	1.5 +/-0.2	0.6 +/-0.1	1.4 +/-0.1	2.0 +/-0.2	2.5 +/-0.3	0.5 +/-0.05
Dithiodiglycolate	4.3 +/-1.8	1.0 +/-0.2	2.5 +/-0.4	4.8 +/-0.2	7.1 +/-0.2	4.0 +/-0.2
Oxidized Glutathione	2.0 +/-0.3	0.1 +/0.02	0.7 +/-0.1	3.0 +/-0.2	6.4 +/-0.5	0.5 +/-0.05
Dimethyldisulfide	2.0 +/-0.1	1.4 +/-0.2	7.5 +/-3.6	3.0 +/-0.1	5.7 +/-0.3	2.5 +/-0.2
Cystamine	9.3 +/-0.5	5.0 +/-0.2	12.5 +/-0.1	7.0 +/-0.2	22.3 +/-0.6	2.5 +/-0.2
Cystine	350 +/-30	3.0 +/-0.1	70 +/-10	3.0 +/-0.2	101 +/-7	1.5 +/-0.2

Although *S. oneidensis* reduced each of the naturally occurring disulfides, the *V*max-Di varied

over 500-fold, ranging from 0.7 μM/hr for oxidized glutathione with lactate as electron donor to

350 μM/hr for cystine with H_2 as electron donor. Overall, *V*max-Di followed in the order:

cystine> cystamine> dimethyldisulfide> dithiodiglycolate> oxidized glutathione >

dithiodipropionate (Figure 2.2). *S. oneidensis* also reduced the aminothiol disulfides cystine and

cystamine at rates 2-80 fold faster than the mercaptocarboxylate disulfides dithiodipropionate

and dithiodiglycolate.
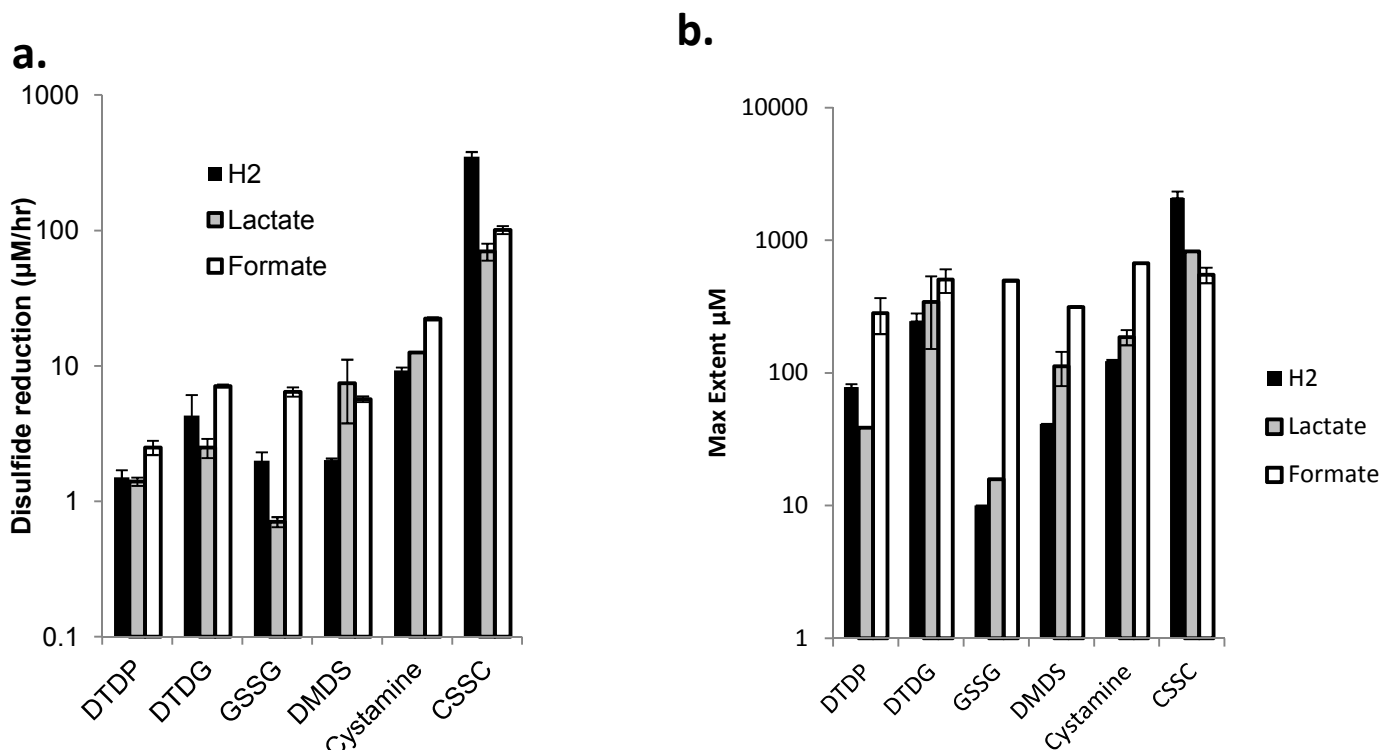
Figure 2.2. Comparison of (a) maximum disulfide reduction rate (*V*max-Di) and (b) maximum extent of disulfide reduction by *S. oneidensis* with H_2, lactate or formate as electron donor. Note disulfide reduction rates and maximum extent of disulfide reduction are plotted in log scale. DTDP: dithiodipropionate; DTDG: dithiodiglycolate; GSSG: oxidized glutathione; DMDS: dimethyl disulfide; CSSC: cystine.

S. oneidensis employs disulfide compounds as electron shuttles to increase the rate and extent of Fe(III) oxide reduction. The ability of *S. oneidensis* to employ disulfides as electron shuttles to increase the rate and extent of Fe(III) oxide reduction was tested by adding disulfides at environmentally relevant concentrations (ranging from 4 μM to 4 mM) to anaerobic minimal growth media containing 40 mM Fe(III) oxide and either lactate, formate, or H_2 as electron donor.

Fe(III) oxide reduction activity by *S. oneidensis* increased as a function of added disulfide concentration, with the Fe(III) reduction rates following Michaelis-Menten kinetics with respect to disulfide concentration (Figure 2.3). The maximum Fe(III) reduction rates (Vmax-Fe) and Michaelis constant (Km-Fe) values of each electron donor-disulfide pair are summarized in Table 2.2. The disulfides accelerated Vmax-Fe approximately 5-50 fold, with cystine accelerating Vmax-Fe the greatest at more than 50-fold with H_2 as electron donor. Correspondingly, the addition of disulfides increased the extent of Fe(III) reduction by approximately 2-20 fold (Figure 2.4).
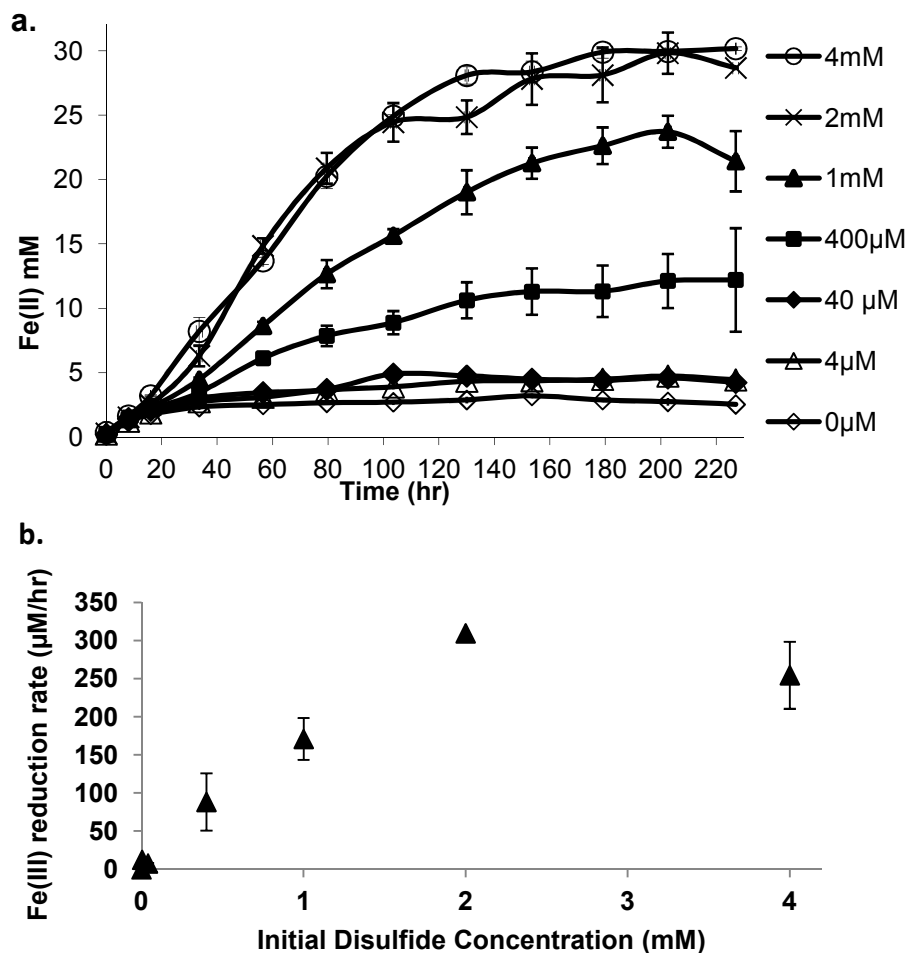
Figure 2.3. **(a) Production of Fe(II) by *S. oneidensis* cultures amended with increasing concentrations of cystine and H$_2$ as electron donor. (b) Michaelis-Menten plot of Fe(III) reduction rates as a function of cystine concentrations amended to *S. oneidensis* cultures with H$_2$ as electron donor.**

 The increased rate and extent of Fe(III) oxide reduction caused by disulfide addition correlated with microbial disulfide reduction activity. To attain the observed extents of Fe(III) reduction, 4 μM cystamine and dimethyldisulfide were cycled nearly 100 times with H$_2$ as electron donor, while 4 μM cystine was cycled 300 times with H$_2$ as electron donor. The shuttling frequencies varied inversely with added disulfide concentrations, with the lowest disulfide concentrations displaying the highest shuttling frequencies for all disulfides (Figure

Table 2.2. Maximum rate of Fe(III) reduction (Vmax-Fe) and Km-Fe values for Fe(III) reduction with disulfides added as electron shuttle and H_2, lactate, or formate as electron donor. Michaelis-Menten plots used to calculate Vmax-Fe and Km-Fe for each electron donor/disulfide pair are provided in Appendix B.

Electron Donor	H_2		Lactate		Formate	
	Vmax	Km	Vmax	Km	Vmax	Km
Disulfide	(µM/hr)	(mM)	(µM/hr)	(mM)	(µM/hr)	(mM)
None	7.1 +/-0.3	-	4.6 +/-0.4	-	4.0 +/-0.3	-
Dithiodipropionate	29.6 +/-5.9	0.6 +/-0.1	26.7 +/-0.1	0.75 +/-0.2	32.7 +/-2.6	0.6 +/-0.2
Dithiodiglycolate	31.4 +/-4.2	0.2 +/-0.05	48.6 +/-10.8	0.4 +/-0.2	26.2 +/-2.0	0.3 +/-0.05
Oxidized Glutathione	31.0 +/-1.0	1.3 +/-0.1	44.0 +/-10.0	1.0 +/-0.3	36.5 +/-6.1	0.1 +/-0.05
Dimethyldisulfide	60.4 +/-1.0	0.2 +/-0.05	47.8 +/-0.6	0.3 +/-0.05	29.6 +/-0.3	0.25 +/-0.05
Cystamine	32.0 +/-1.6	0.02 +/-0.01	42.3 +/-3.3	0.02 +/-0.01	31.5 +/-1.0	0.03 +/-0.01
Cystine	317 +/-5	0.75 +/-0.1	253 +/-4	0.7 +/-0.2	119 +/-23	1.25 +/-0.2

2.6). These results suggest that the higher disulfide concentrations may have converted the disulfide electron shuttling pathway to an anaerobic respiratory pathway with disulfides as terminal electron acceptor, as opposed to an electron shuttling pathway with Fe(III) oxides as terminal electron acceptor. To examine this possibility, future work will compare thiol accumulation rates as a function of increasing disulfide concentrations in the presence and absence of Fe(III) oxides.
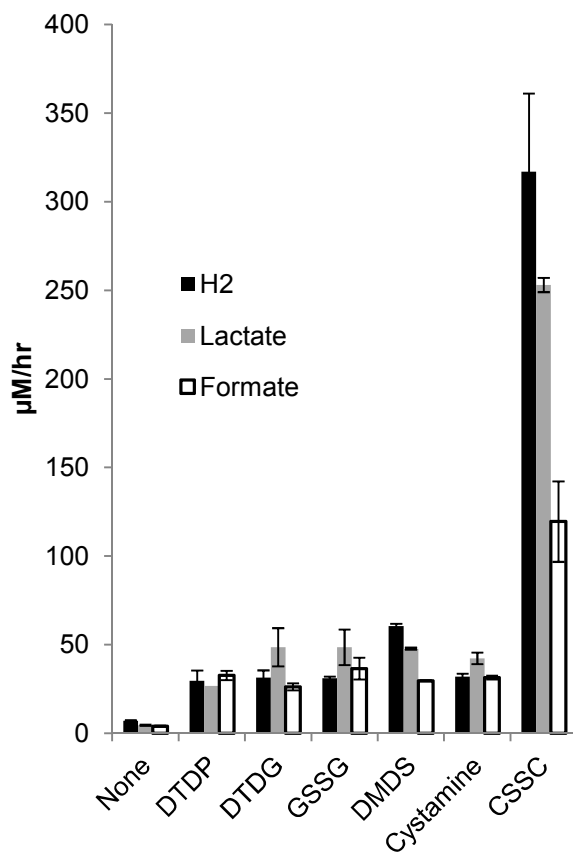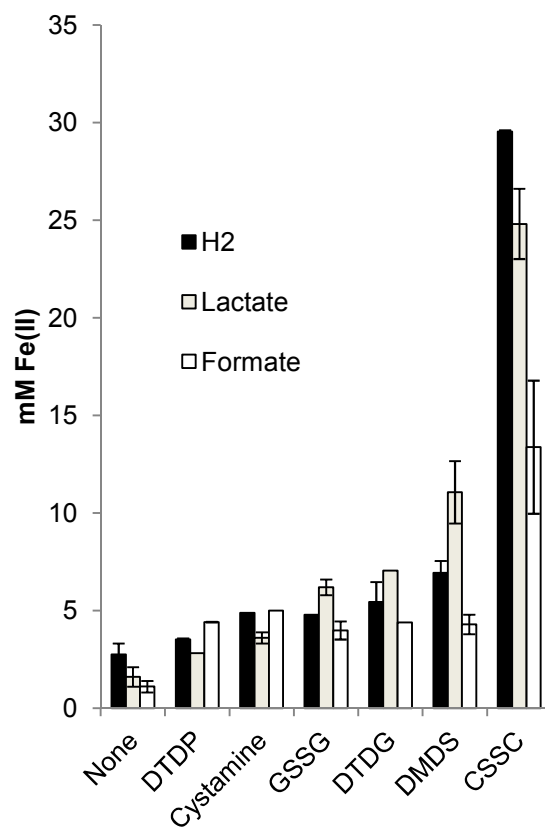
Figure 2.4. Comparison of (a) the maximum rate of Fe(III) reduction with and without addition of disulfides, and (b) the maximum extent of Fe(III) reduction with and without addition of disulfides.
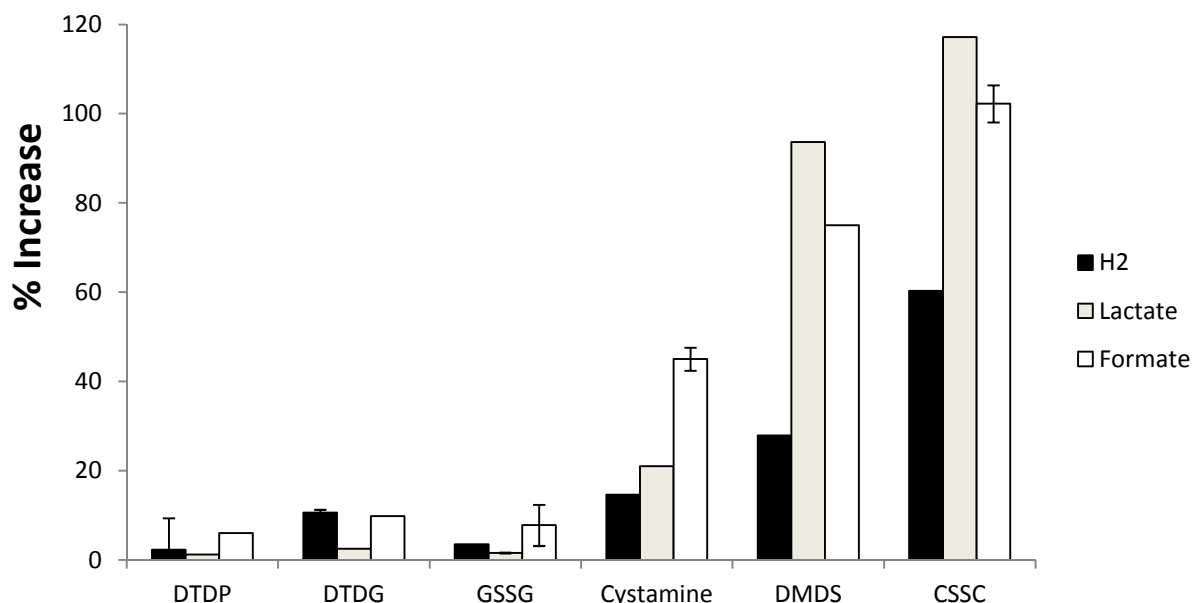
Figure 2.5. Percent increase (compared to the no disulfide control) in the extent of 40 mM Fe(III) oxide reduction by the addition of 4 µM disulfide with H$_2$, lactate, or formate as electron donor.

With the exception of DMDS, the increased rate and extent of Fe(III) oxide reduction by the thiols and shuttling frequency of the disulfides correlated with increases in Vmax-Di (Figures 2.7 and 2.8). These results suggest that microbial disulfide reduction rates, and not the rates of abiotic Fe(III) reduction by thiol, was the limiting reaction in the thiol-based electron shuttling pathway to Fe(III) oxides. To test this possibility, the rates of abiotic Fe(III) reduction by cysteine were measured voltammetrically in M1 growth medium supplemented with 600 µM cysteine and 40 mM Fe(III) oxide (Figure 2.9). The abiotic rate of Fe(III) oxide reduction by cysteine was approximately 10-fold greater than the corresponding rate at which *S. oneidensis* reduced 300 µM cystine, again indicating that microbial disulfide reduction activity is the limiting step in the electron shuttling pathway to Fe(III) oxides. Reasons for the DMDS anomaly are unclear, but may reflect the inability of the highly volatile reduced form of DMDS (methanethiol)[61] to interact and transfer electrons to Fe(III) oxides in the abiotic Fe(III) reduction step. Interestingly, shuttling frequency for all the aminothiols and carboxylic thiols also correlate with microbial disulfide reduction activity (Figure 2.7).

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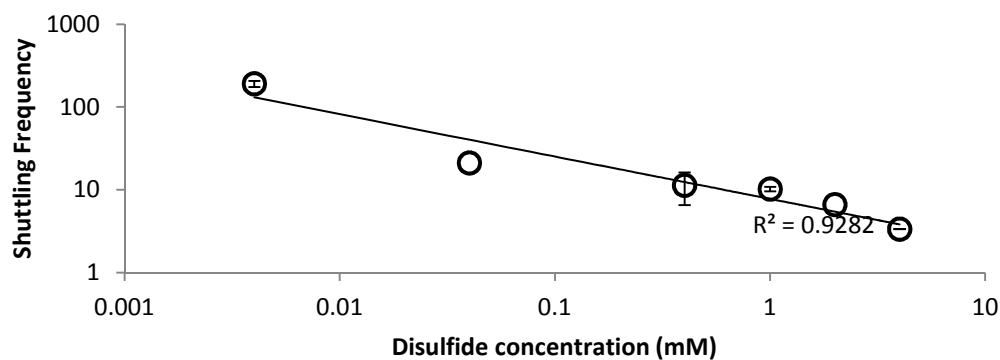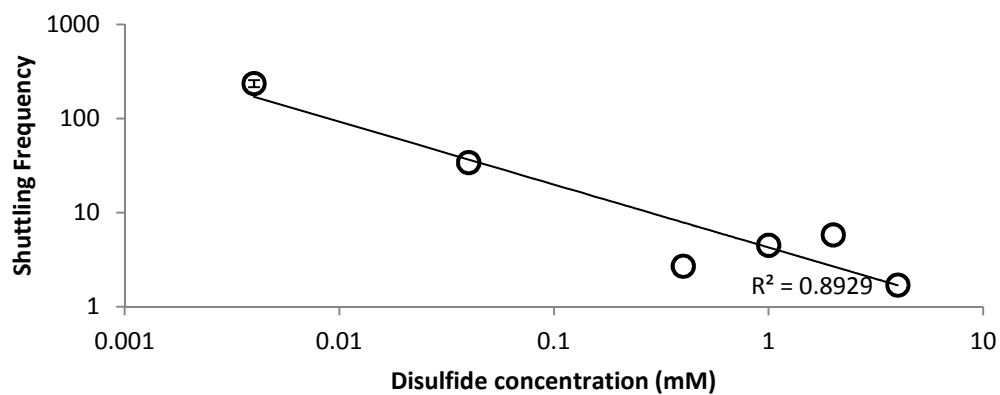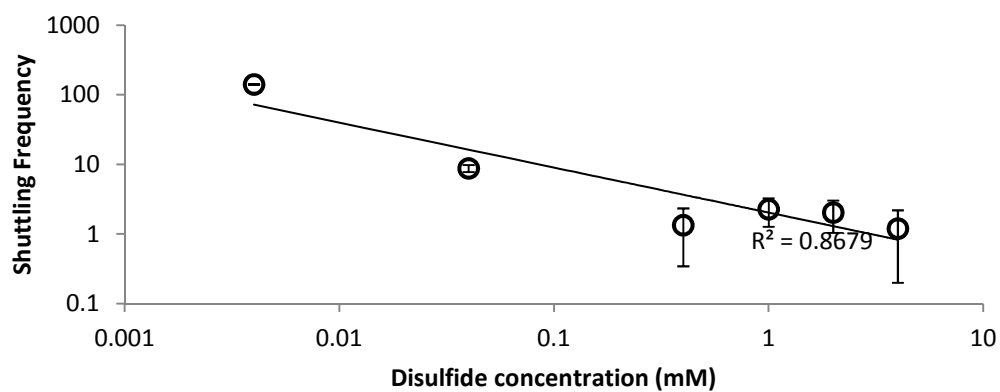
Figure 2.6. **Calculated cystine shuttling frequencies as a function of disulfide concentration with (a) H$_2$, (b) lactate, and (c) formate as electron donor.**
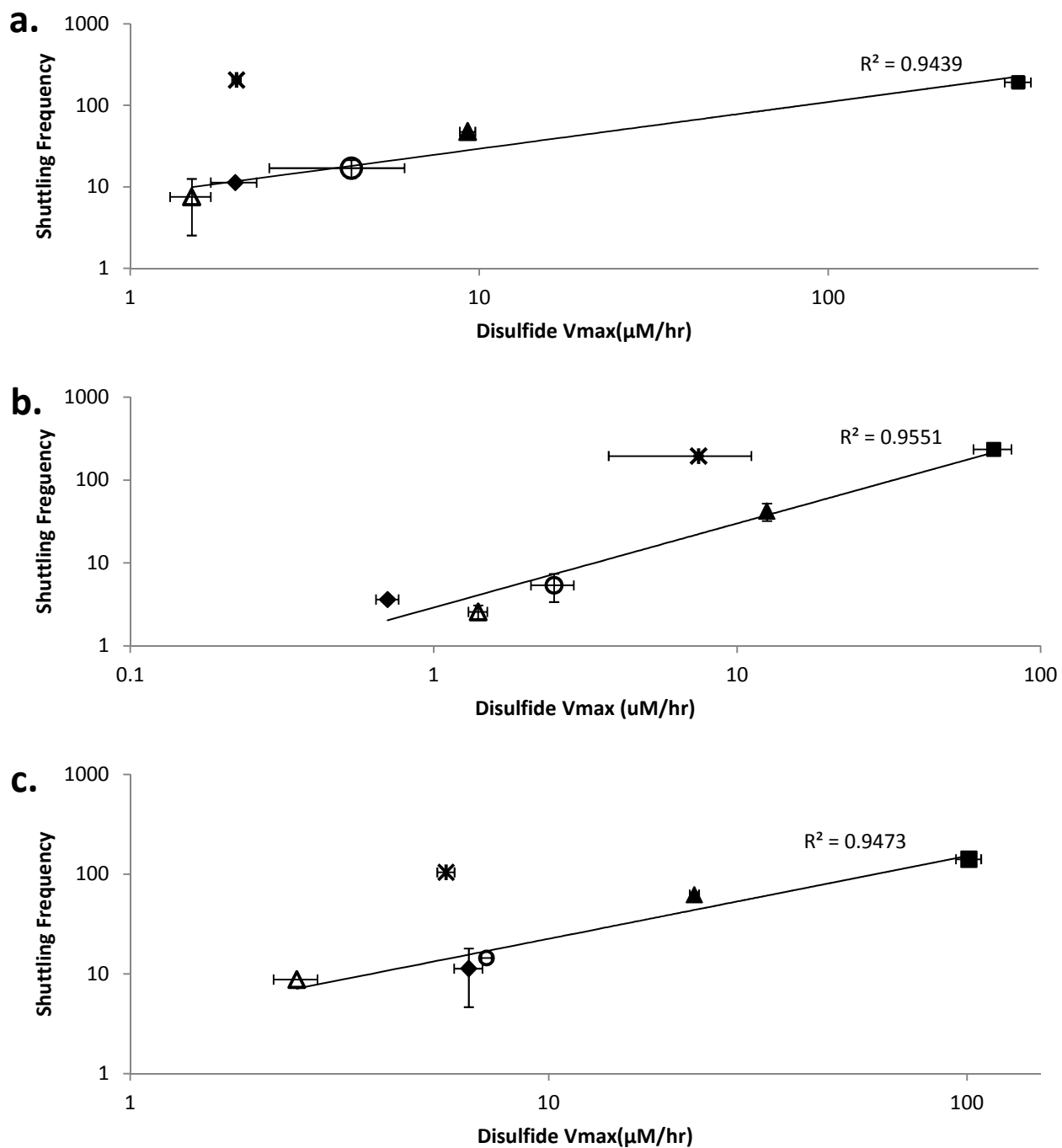
Figure 2.7. Calculated disulfide shuttling frequencies as a function of Vmax-Di with (a) H$_2$, (b) lactate, and (c) formate as electron donor. Filled Square: CSSC; filled triangle: Cystamine; Filled Diamond: GSSG; open circle: DTDG; open triangle: DTDP; cross: DMDS
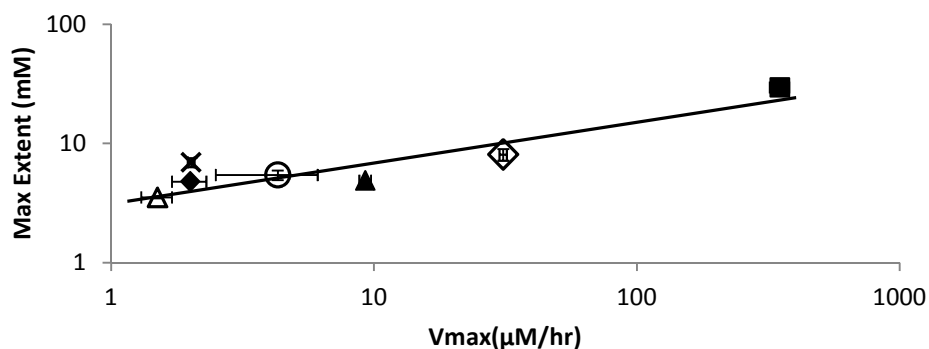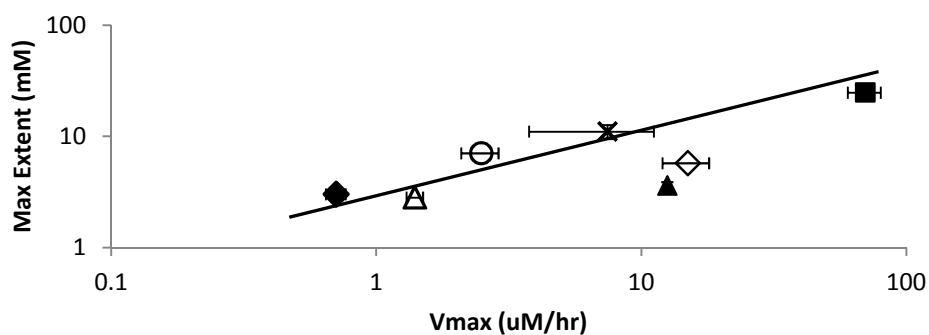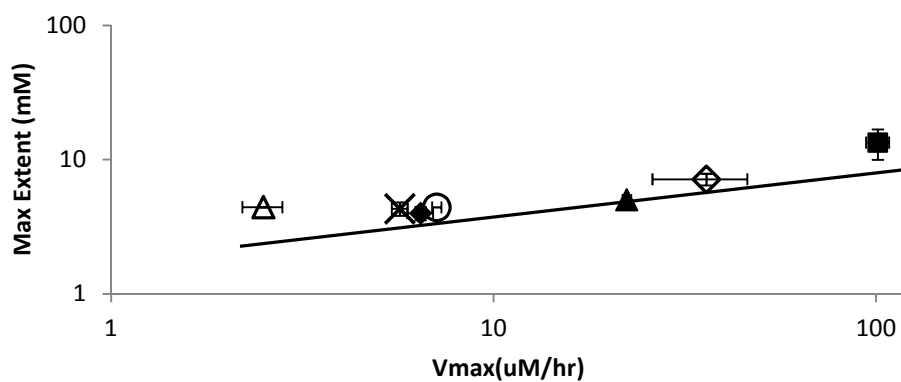
Figure 2.8. Maximum extent of Fe(III) reduction as a function of Vmax-Di with (a) H$_2$, (b) lactate, and (c) formate as electron donor. Filled Square: CSSC; filled triangle: Cystamine; Filled Diamond: GSSG; open circle: DTDG; open triangle: DTDP; cross: DMDS. Filled Square: CSSC; filled triangle: Cystamine; Filled Diamond: GSSG; open diamond: Ellman's; open circle: DTDG; open triangle: DTDP; cross: DMDS
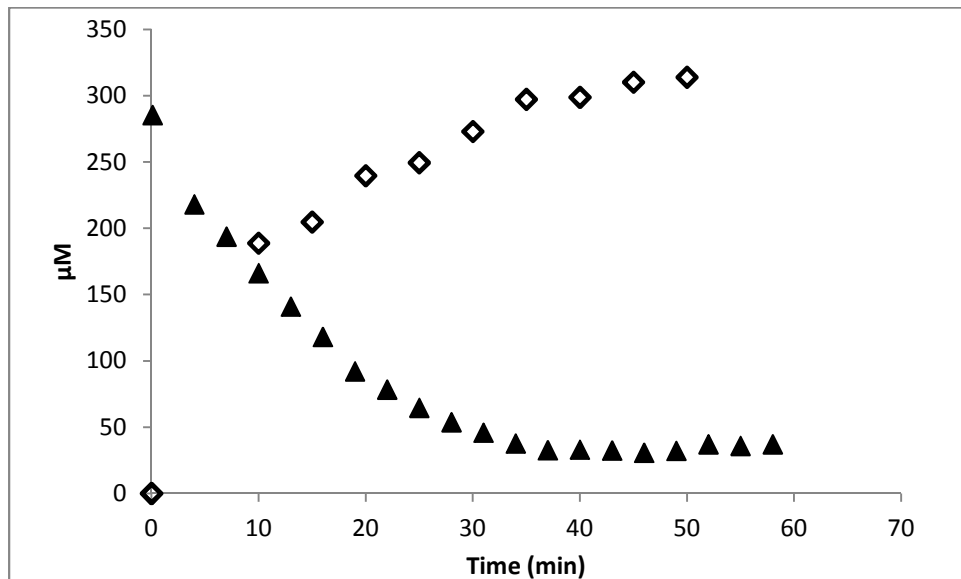
*Figure 2.9 (Experiments carried out by Eryn Eitel of the Taillefert Laboratory). Abiotic reduction of 40 mM Fe(III) oxide by 500 µM cysteine carried out under anaerobic conditions in M1 growth medium. Solid triangles, cysteine; open diamonds, Fe(II) measured by the ferrozine method with HCl extraction.

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CHAPTER 3

Shewanella oneidensis respiratory mutants selected for
the inability to respire disulfides as anaerobic electron acceptor
contain mutations in outer membrane β-barrel protein MtrB

Summary

The γ-proteobacterium *Shewanella oneidensis* MR-1 reduces a wide range of anaerobic electron acceptors, including transition metals Fe(III), Mn(III), and Mn(IV). Results of the present study expand the spectrum of electron acceptors reduced by *S. oneidensis* to include both naturally-occurring (cystine) and synthetic (5,5'-dithiobis-(2-nitrobenzoic acid; DTNB)) disulfide compounds. Chemical mutagenesis procedures were combined with a newly developed mutant screening technique to identify *S. oneidensis* (Rsh) mutants unable to reduce disulfides to their corresponding thiol forms. The Rsh mutants were tested for anaerobic growth on a battery of eight metal- and non-metal electron acceptors with either lactate or H_2 as electron donor. A broad spectrum of respiratory mutants were identified, including a subset that failed to reduce Fe(III), Mn(III), and Mn(IV), but retained the ability to reduce all non-metal electron acceptors. Genetic complementation and nucleotide sequence analyses of the metal reduction-deficient Rsh mutants indicated that each contained a mutation in *mtrB*, which encodes the β-barrel component of the MtrCAB extracellular electron conduit. Subsequent tests of the anaerobic respiratory phenotypes of an *mtrB* deletion mutant confirmed the disulfide- and metal reduction-deficent phenotypes of the Rsh and *mtrB* mutants. *S. oneidensis* mutants lacking the MtrC and MtrA components of the extracellular electron conduit also displayed disulfide reduction-deficient phenotypes. These

results indicate that the disulfide and metal reduction pathways of *S. oneidensis* share the

extracellular electron conduit MtrCAB as a common electron transport chain component.

Introduction

The gram negative, facultative anaerobe *Shewanella oneidensis* MR-1 respires a variety

of terminal electron acceptors, including oxygen, fumarate, nitrate, nitrite, trimethylamine N-

oxide, dimethyl sulfoxide, sulfite, thiolsulfate, elemental sulfur, and the transition metals Fe(III),

Mn(III), and Mn(IV) [1-7]. Fe(III) precipitates as sparingly soluble amorphous or crystalline

(oxy)hydroxides at circumneutral pH [8]. *S. oneidensis* is therefore required to transfer electrons

to external Fe(III) oxides unable to contact inner membrane (IM)-localized electron transport

chains [9-11]. To overcome this problem, *S. oneidensis* transfers electrons to external Fe(III)

oxides via a variety of novel respiratory strategies including i) direct enzymatic reduction by

Fe(III)-reducing *c*-type cytochromes located on the cell surface or along extracellular nanowires

[12-14], ii) non-reductive Fe(III) oxide solubilization followed by electron transfer to the

resulting soluble organic-Fe(III) complexes [6, 15-17], and iii) indirect reduction by electron

shuttling compounds such as flavins, fulvic acids, humic acids, melanin [18-25], and naturally

occurring disulfide compounds (see Chapter 2). The molecular mechanism of microbial

disulfide reduction has yet to be elucidated.

Intracellular disulfides (and their reduced thiol forms) are critical for numerous cellular

processes, including the maintenance of intracellular redox conditions and protection from

deleterious reactive oxygen, nitrogen, and electrophilic chemical species [26]. In most

eukaryotes and many gram-negative bacteria, glutathione is the dominant low molecular weight

thiol [27]. The cellular function of gluthathione in gram-positive bacteria is often replaced by

alternative thiols such as mycothiol in *Actinobacteria* [28, 29] and bacilithiol in *Bacillus* [30].

Other intracellular thiols include cysteine, homocysteine, trypanothione, ergothioneine,

coenzyme M, and coenzyme B [31]. Intracellular disulfide-thiol conversions are catalyzed by

two different types of intracellular disulfide reductases. The first consists of pyridine-nucleotide

disulfide oxidoreductases [32] that contain a redox center formed by a disulfide bridge coupled

to a flavin ring and catalyze a simultaneous two-electron transfer to disulfide substrates [33, 34].

Examples of pyridine-nucleotide disulfide oxidoreductases include dihydrolipoamide

dehydrogenase, coenzyme A disulfide reductases, glutathione reductases, mycothione reductases,

thioredoxin reductases, and trypanothione reductases. The second type of intracellular disulfide

reductase includes iron-sulfur proteins (e.g., heterodisulfide reductases) that catalyze disulfide

bond reduction via two successive one-electron transfer steps [35].

As compared to the molecular mechanism of intracellular disulfide reduction, the

mechanistic details of extracellular disulfide reduction by microorganisms has yet to be

elucidated. The main objectives of the present study were to i) develop a rapid mutant screening

to identify respiratory (designated Rsh) mutants of *S. oneidensis* that are unable to reduce

extracellular disulfides as terminal electron acceptor, ii) determine the overall respiratory

capability of the newly isolated bank of Rsh mutants, and iii) identify the genes required for

extracellular disulfide reduction via genetic complementation and nucleotide sequence analyses

of the Rsh mutants.

Materials and methods

     Growth media and cultivation conditions. All bacterial strains and plasmids used in this study are listed in Table 1. For genetic manipulations, *S. oneidensis* MR-1 was cultured at 30°C in Luria-Bertani (LB) medium (10 g liter^{-1} NaCl, 5 g liter^{-1} yeast extract, 10 g liter^{-1} tryptone). For anaerobic-growth experiments, cells were cultured in M1 minimal media supplemented with lactate (18 mM) or hydrogen (2%) as electron donor under a nitrogen atmosphere. When required, antibiotics were added at the following final concentrations: for gentamicin (Gm), 15 μg ml^{-1}, chloramphenicol, 25 μg ml^{-1} and for tetracycline 10 μg ml^{-1}. For growth of *Escherichia coli* β2155 λ *pir*, diaminopimelate (DAP) was added at a final concentration of 100 μg ml^{-1}. Aerobic growth was monitored spectrophotometrically by measuring changes in optical density at 600 nm.

     Development of a rapid mutant screening technique to identify disulfide reduction-deficient (Rsh) mutants. Chemical mutagenesis procedures were combined with a newly developed Rsh mutant screening technique to isolate Rsh mutants based on their inability to reduce extracellular disulfides. Random point mutants were generated via chemical (ethylmethanesulfonate; EMS) mutagenesis and screened for impaired disulfide reduction activity. Rsh mutants were detected after 48 hour anaerobic incubations in M1 minimal medium

Table 3.1. Strains and plasmids used in the present study

Strains	Features	Source
Shewanella oneidensis		
MR-1	Wild-type strain	
Δ*mtrB*	In-frame deletion mutant	J. Burns
Δ*mtrA*	In-frame deletion mutant	J. Burns
Δ*mtrC*	In-frame deletion mutant	J. Burns
Δ*omcA*	In-frame deletion mutant	J. Burns
Δ*omcA*-Δ*mtrC*	In-frame deletion mutant	J. Burns

Δ*gspD*	In-frame deletion mutant	J. Burns
Rsh03	Random point mutant	This study
Rsh13	Random point mutant	This study
Rsh18	Random point mutant	This study
Rsh35	Random point mutant	This study
Rsh38	Random point mutant	This study
Escherichia coli β2155	*thrB1004 pro thi strA hsdS lacZ_M15*	
λ pir	(F9 *lacZΔM15 lacl*q *traD36 proA*1	[36]
	*proB*1) Δ*dapA::erm pir::*RP4 Km^R	

Plasmids	Features	Source
pKO2.0	4.5 kb γR6K, *mob*RP4 *sacB* Gm^R *lacZ*	[37]
pBBR1MCS	Cm^R lacZ	[38]
pBB+*mtrB*	pBBR1MCS containing wild-type copy of *mtrB*	This study
pBB+*mtrA*	pBBR1MCS containing wild-type copy of *mtrA*	This Study
pBB+*mtrC*	pBBR1MCS containing wild-type copy of *mtrC*	This Study
pBB+*omcA*	pBBR1MCS containing wild-type copy of *omcA*	This Study
pBB+*gspD*	pBBR1MCS containing wild-type copy of *gspD*	This Study

with lactate or hydrogen as electron donor and 320 µM of the disulfide compound 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellmans reagent) as anaerobic electron acceptor and thiol-specific indicator. To eliminate the possibility of cross-feeding of metabolites between colonies, colonies arising from EMS-mutagenized cells were incubated in single wells of 96-well microtiter dishes. DTNB was used as disulfide to identify Rsh mutants based on their inability to reduce DTNB to 2-nitro-5-thiobenzoate (TNB⁻), which ionizes to the yellow colored TNB^{2-} dianion in water at neutral pH [39]. The wild type strain turns yellow-colored after 30 hours of anaerobic incubation, while putative Rsh mutants were chosen for their inability to produce the yellow colored TNB^{2-} dianion after 48 hours of anaerobic incubation. Approximately 7,000 mutagenized colonies were screened and 5 putative Rsh mutants (designated Rsh03, Rsh13, Rsh18, Rsh35, and Rsh38) were identified.

Determination of the overall respiratory capability of the Rsh mutant strains.

The five Rsh mutants with impaired disulfide reduction activity were subsequently tested for reduction of a suite of eight alternate electron acceptors with either lactate or H_2 as electron donor. *S. oneidensis* wild-type and Rsh mutant strains were incubated in minimal M1 medium (initial concentration of $2X10^7$ cells ml^{-1}) amended with either 18 mM lactate or 2% H_2 as electron donor and either O_2, 15 mM nitrate, 50 mM dimethyl sulfoxide (DMSO), 10 mM fumarate, 50 mM Fe(III)-citrate, 10 mM MnO_2, 10 mM Mn(III)-pyrophosphate, or 40 mM hydrous Fe(III)-oxide (HFO) as electron acceptor [4]. Electron acceptors were synthesized as previously described [7, 40-43]. Anaerobic conditions were maintained by continuous sparging with N_2 (g). Growth on O_2, DMSO, and fumarate were monitored by measuring increases in cell density at 600 nm. NO_2^- was measured spectrophotometrically with sulfanilic acid-*N*-1-naphthyl-ethylene-diamine dihydrochloride solution [44]. Mn(III)-pyrophosphate concentration was measured colorimetrically as previously described [45]. Mn(IV) concentration was measured colorimetrically after reaction with benzidine hydrochloride as previously described [1]. Fe(III) reduction was monitored by measuring HCl-extractable Fe(II) production with ferrozine [46]. Disulfide reduction was monitored by measuring thiol production in samples withdrawn and centrifuged under anaerobic conditions for 2 min at 16,000 rpm to pellet cell material. Thiol concentrations were determined by adding 100 μl of a 30 mM solution of 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB; Ellmans Reagent) to 900 μl of culture supernatant and measuring absorbance at 412 nm [39]. Thiol concentrations were calculated from calibration curves with cysteine as the model thiol.

Genetic complementation and nucleotide sequence analyses. A previously constructed clone library of partially digested *HindIII* chromosomal DNA fragments of the *S. oneidensis* MR-1 (harbored in broad-host-range cosmid pVK100 and maintained in *Escherichia coli* strain β-2155) were mobilized into the Rsh mutants via conjugal mating procedures. The resulting tranconjugates were screened for restored disulfide reduction activity. Genes involved in disulfide reduction were identified via subcloning with broad host range cloning vector pBBR1MCS, tests of the Rsh subclones for restore disulfide reduction activity, and nucleotide sequencing of the smallest complementing subcloned DNA fragments. Point mutant phenotypes were confirmed via in-frame gene deletion mutagenesis, and subsequent testing of the in-frame gene deletion mutants for impaired disulfide reduction activity.

In-frame gene deletion mutagenesis and genetic complementation analysis. Targeted genes were deleted via a *Shewanella* in-frame gene deletion system designed in our laboratory [37]. Regions corresponding to ~750 bp upstream and downstream of *mtrB* were independently PCR-amplified and subsequently joined using overlap-extension PCR. Primers for *mtrB* deletion are listed in Table 2. The resulting fragment was cloned into suicide vector pKO2.0, which does not replicate in *S. oneidensis*. This construct (designated pKO-*mtrB*) was mobilized into wild-type MR-1 via conjugal transfer from *E. coli* donor strain β2155 λ pir. *S. oneidensis* strains with the plasmid integrated into the genome were selected on solid LB medium containing gentamycin (15 μg mL^{-1}). Single integrations were verified via PCR with primers flanking the recombination region. Plasmids were resolved from the genomes of single integrants by plating on solid LB medium containing sucrose (10% w/v) with NaCl omitted. In-frame deletions were verified by PCR and direct DNA sequencing (GeneWiz, South Plainfield, NJ). Genetic

complementation of Δ*mtrB* was carried out by cloning wild-type *mtrB* into broad-host-range

cloning vector pBBR1MCS [38] and conjugally transferring the recombinant vector into Δ*mtrB*

via bi-parental mating procedures [47].

Results and Discussion

Overall respiratory capability of the Rsh mutants. Five putative Rsh mutants were selected for their inability to reduce DTNB as terminal electron acceptor, and their overall respiratory capability was determined (Table 3.2). Based on their growth phenotypes, the resulting mutants were placed into three mutant classes: i) two mutants (Rsh03 and Rsh18) were unable to grow with lactate as electron donor, but retained the ability to grow at wild type rates with formate and hydrogen as electron donor on all electron acceptors (putative lactate dehydrogenase mutants); ii) one mutant (Rsh13) only grew on O_2 as electron acceptor and was unable to grow on all other electron acceptors tested (putative anaerobic regulatory mutant), and iii) two mutants (designated Rsh35 and Rsh38) grew at wild type rates on all non-metal electron acceptors but were unable to grow on Fe(III) oxide, Mn(III)-pyrophophate, and Mn(IV) oxide, and displayed a 36 hour lag prior to growth on Fe(III) citrate.
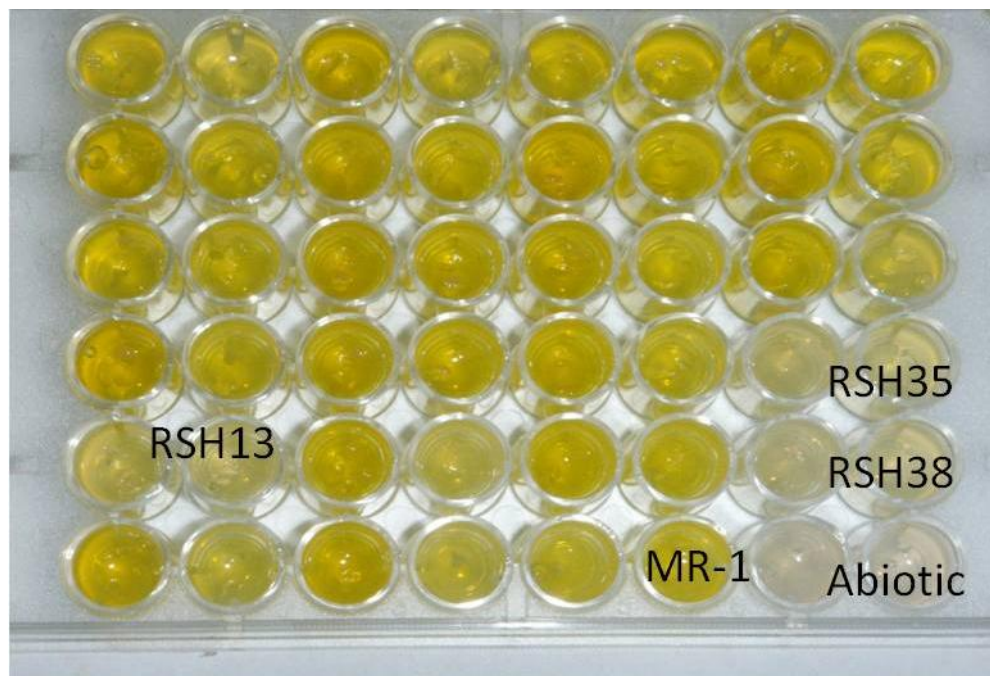
Figure 3.1. Photograph of screening plate during identification of Rsh13,Rsh35 and Rsh38. Production of yellow colored TNB^{2-} dianion is indicator of wild-type disulfide (DTNB) reduction activity.

Table 3.2. Overall respiratory capability of RSH mutants

Electron acceptor	O_2	Fumarate	DMSO	Nitrate		Fe(III) oxide		Fe(III) citrate		Mn(III)		Mn(IV)	
Strain	L	L	L	L	H_2	L	H_2	L	H_2	L	H_2	L	H_2
WT	+	+	+	+	+	+	+	+	+	+	+	+	+
RSH03	-	-	-	-	+	-	+	-	+	-	+	-	+
RSH13	+	-	-	-	-	-	-	-	-	-	-	-	-
RSH18	-	-	-	-	+	-	+	-	+	-	+	-	+
RSH35	+	+	+	+	+	-	-	-	-	-	-	-	-
RSH38	+	+	+	+	+	-	-	-	-	-	-	-	-

L, Lactate as electron donor; H_2, H_2 as electron donor; +, > 70% of WT phenotype.
-, <30% of WT phenotype.

Genetic complementation analysis of Rsh35 mutant. Approximately 1,500 Rsh35 tranconjugates were screened and one (designated Rsh35-40) displayed restored disulfide reduction activity identical to the wild type (Figure 3.3). Subsequent subcloning and anaerobic growth experiments demonstrated that only those subclones containing a wild-type copy of *mtrB* restored anaerobic growth capability to Rsh35 (Fig 3.4). Rsh35 genomic DNA corresponding to the *mtrB* region was PCR-amplified and sequenced. A single nucleotide transition (CAA to TAA, which resulted in a truncated mutant MtrB form) was identified in Rsh mutant *mtrB* at amino acid position 401 (Figure 3.5).

Genetic complementation analysis of mutant Rsh13. Rsh13 only grew with O_2 as electron acceptor and was unable to grow on all anaerobic electron acceptors tested. An enrichment genetic complementation strategy was designed to exploit the anaerobic
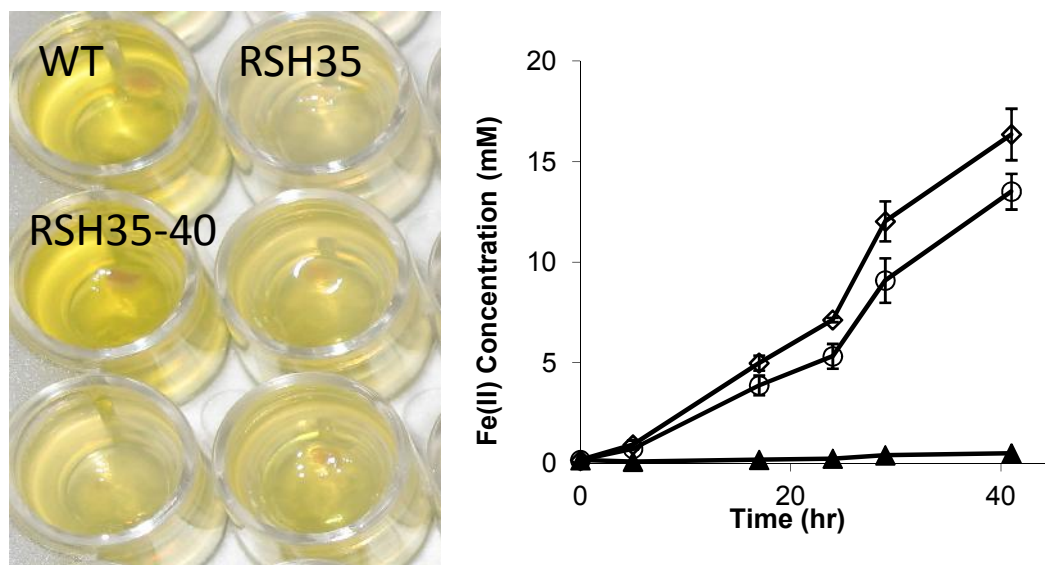
Figure 3.3. Genetic complementation of RSH35 mutant. Left panel : Disulfide reduction screening assay from which complementing transconjugate RSH35-40 was identified. Right panel : Fe(III) citrate reduction plot. MR-1 (open diamond), RSH35-40 (open circle) and WT (closed triangle). RSH-35-40 regained Fe(III) citrate reduction ability.

respiratory mutant phenotype. A previously constructed clone library of partially digested

HindIII chromosomal DNA fragments of the *S. oneidensis* MR-1 was mobilized into Rsh13

mutant by the two-way mating procedures described above. Rsh13 tranconjugates were then

incubated anaerobically with fumarate as electron acceptor until tranconjugates with restored

anaerobic respiratory ability emerged. Tranconjugates with restored fumarate reduction activity

were identified (Figure3.6), and subsequent subcloning and nucleotide sequence analysis

identified a point mutation in the gene coding CRP, the cAMP-responsive regulator of catabolite

repression (SO_0624). The mutation consisted of a single nucleotide transition at amino acid

position 180 (TGC to TAC, with cysteine replaced by tyrosine) (Figure3.7).
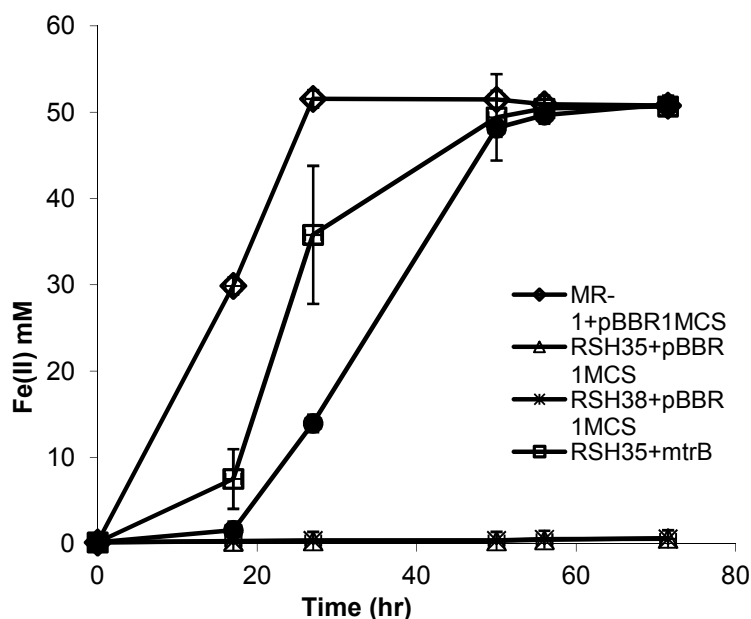
Figure 3.4. Restoration of wild-type Fe(III) citrate reduction activity to Rsh35 and Rsh38 via complementation with recombinant pBBR1MCS containing a wild-type copy of *mtrB*.

```
WT      TGSYDYYDRDNNTQVEEWTQISINNVNGKVAYNTPYDNRTQRFKVAADYRITRDIKLDGG 420
RSH35   TGSYDYYDRDNNTQVEEWTQISINNVNGKVAYNTPYDNRTXRFKVAADYRITRDIKLDGG 420
        ***********************************************  ****************
```

Figure 3.5. Rsh35 mutant contains a point mutation at nucleotide coding amino acid number 401 in β-barrel outer membrane protein MtrB.
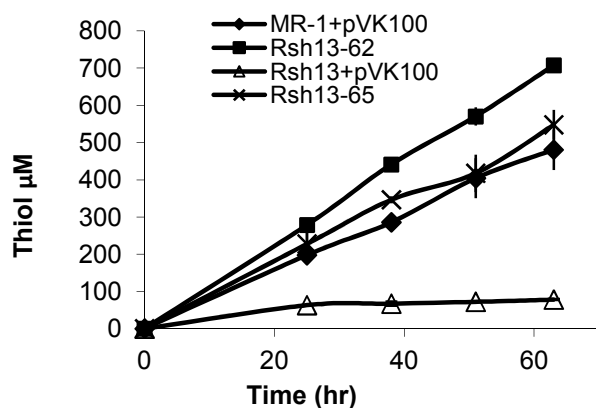
Figure 3.6. Genetic complementation of Rsh13. Two tranconjugates Rsh13-62 (filled square) and Rsh13-65 (X) selected from fumarate enrichment regained disulfide reduction capability. WT (filled diamond); Rsh13 (open triangle).

```
WT      QMAYRLQSTSQKVGNLAFLDVAGRIAQTLLHLAKQPDAMTHPDGMQIKITRQEIGQIVGC 180
RSH13   QMAYRLQSTSQKVGNLAFLDVAGRIAQTLLHLAKQPDAMTHPDGMQIKITRQEIGQIVGY 180
```

Figure 3.7 Identification of point mutation on nucleotide coding *crp* gene of Rsh13

Other *mtr* deletion mutants also display impaired disulfide reduction activities. A previously contructed set of *mtr* deletion mutants (Δ*mtrB*, Δ*mtrA*, Δ*mtrC*, Δ*omcA and* Δ*mtrC-*Δ*omcA double mutant*) were subsequently tested for disulfide and Fe(III) oxide reduction activities. All the *mtr* deletion mutants showed disulfide reduction rate lower than wild type (Figure3.8). Δ*mtrB* and Δ*mtrA* displayed the most severe deficiency with 6-20% of wild type rate, while Δ*mtrC* and Δ*omcA* displayed reduction rate of 50-70% and 80-90% of wild type rate respectively. In addition, Δ*mtrC-*Δ*omcA double mutant* displayed the reduction rate of 20-30% of wild type. Interestingly, the Fe(III) oxide reduction deficiencies displayed by the *mtr* mutants
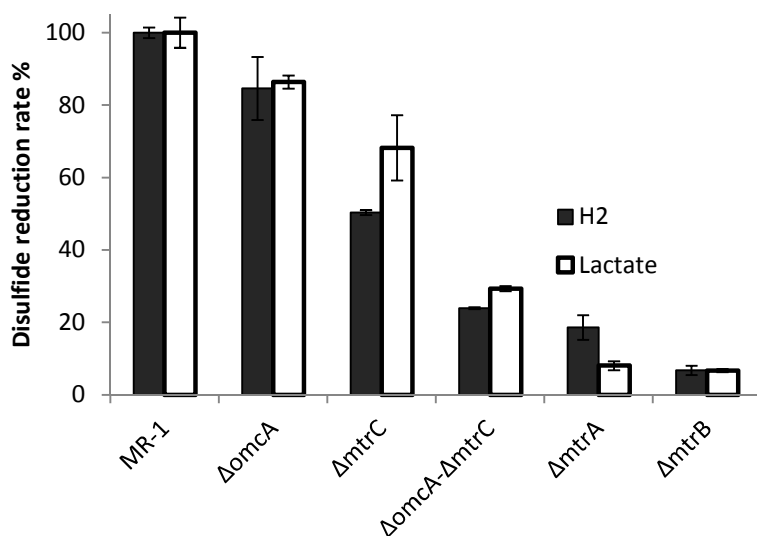
Figure 3.8. Disulfide reduction deficiencies displayed by Mtr mutants with lactate and H$_2$ as electron donor.

correlated with their disulfide reduction deficiencies (Figure 3.9). Furthermore, *S. oneidensis* mutants lacking the MtrC and MtrA components of the extracellular electron conduit also displayed disulfide reduction-deficient phenotypes. These results indicate that the disulfide and metal reduction pathways of *S. oneidensis* share the extracellular electron conduit MtrCAB as a

common electron transport chain component. The extracellular electron conduit MtrCAB thus appears to be multi-functional, displaying the ability to transfer electrons to extracellular Fe(III) citrate, Fe(III) oxide, flavins [9, 48-50], and disulfides (this study). In this manner, *S. oneidensis* may utilize a single terminal reductase complex (MtrCAB) to transfer electrons to multiple external electron acceptors.
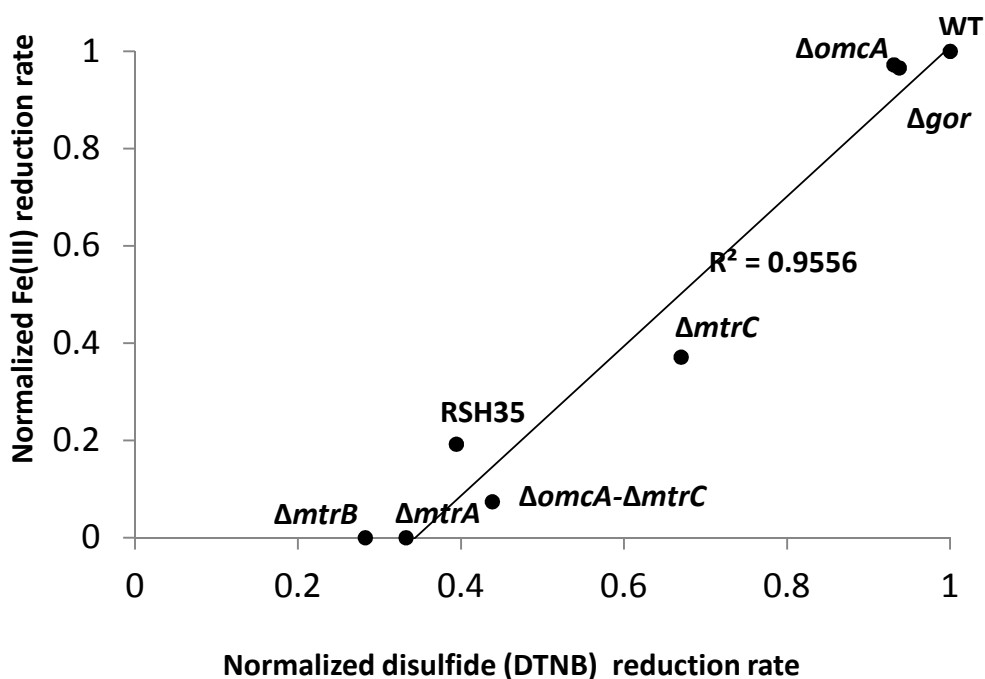
Figure 3.9. Correlation of disulfide (DTNB) and Fe(III) oxide reduction deficiencies of *mtr* mutants.

Alternately, the findings of the present study suggest that Fe(III) reduction is the last step of a multi-step electron shuttling pathway in which *S. oneidensis* secretes disulfides to the cell surface where MtrCAB-catalyzed disulfide reduction reactions produce the corresponding thiols that subsequently reduce oxidized flavin abiotically to their reduced flavin forms, which finally reduce Fe(III) oxide abiotically in the terminal Fe(III) oxide reduction step. Current work is

focused on testing the hypothesis that microbial Fe(III) respiration is catalyzed by the multi-step electron shuttling pathway which begins with enzymatic disulfide reduction at the cell surface and terminates with abiotic Fe(III) oxide reduction outside the cell.

Acknowledgements

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CHAPTER 4

Identification of a Molecular Signature Unique to

Metal-Reducing *Gammaproteobacteria*

[The work described in Chapter 4 was done in collaboration with Dr. Justin Burns, a former PhD student in Dr. DiChristina's research group. Dr. Burn's contributions in this chapter are denoted with an asterisk. The work described in Chapter 4 has also recently been published in *FEMS Microbiology Letters*: Wee, S., J. Burns and T. DiChristina. 2014. Identification of a molecular signature unique to metal-reducing *Gammaproteobacteria*. *FEMS Microbiology Letters*, 350: 90-99.

Summary

Functional genes required for microbial (dissimilatory) metal reduction display high sequence divergence, which limits their utility as molecular biomarkers for tracking the presence and activity of metal-reducing bacteria in natural and engineered systems. In the present study, homologs of the outer membrane beta-barrel protein MtrB of metal-reducing gammaproteobacteria were found to contain a unique N-terminal CXXC motif that was missing from MtrB homologs of non-metal-reducing gammaproteobacteria and metal- and non-metal-reducing bacteria outside the gammaproteobacteria. To determine if the N-terminal CXXC motif of MtrB was required for dissimilatory metal reduction,

each cysteine in the CXXC motif of the representative metal-reducing gamma-proteobacterium *Shewanella oneidensis* was replaced with alanine, and the resulting site-directed mutants were tested for metal reduction activity. Anaerobic growth experiments demonstrated that the first, but not the second, conserved cysteine was required for metal reduction by *S. oneidensis*. The ability to predict metal reduction by gammaproteobacteria with unknown metal reduction capability was confirmed with *Vibrio parahaemolyticus*, a pathogen whose genome encodes an MtrB homolog with an N-terminal CXXC motif. MtrB homologs with an N-terminal CXXC motif may thus represent a molecular signature unique to metal-reducing members of the *Gammaproteobacteria*.

Introduction

Dissimilatory metal-reducing bacteria occupy a central position in a variety of environmentally important processes, including the biogeochemical cycling of carbon and metals, the bioremediation of radionuclides and organohalides, and the generation of electricity in microbial fuel cells [1-3, 104]. Metal-reducing bacteria are scattered and deeply rooted throughout both prokaryotic domains [105, 106]. Functional genes required for microbial metal reduction display high sequence divergence, which limits their use as molecular biomarkers to examine fundamental ecological principles and environmental parameters controlling metal reduction in both natural and engineered systems. A variety of *c*-type cytochromes, for example, are key components of the electron transport systems of many metal-reducing bacteria [11, 107], yet their widespread occurrence in non-metal-reducing bacteria and high sequence divergence limits their utility as molecular biomarkers for tracking the presence and activity of metal-reducing bacteria as a functional group. The gene encoding the eukaryotic-like citrate synthase (*gltA*) in the *Geobacteraceae* family has received attention as a molecular biomarker for tracking the presence and activity of metal-reducing *Geobacteraceae* in subsurface environments [108, 109]. However, *gltA* is found only in members of the *Geobacteraceae* family, thus limiting its application as a molecular biomarker for metal-reducing bacteria outside the *Geobacteraceae* family.

The large γ-*proteobacteria* class within the phylum *Proteobacteria* [19] was selected as a bacterial group to search for molecular signatures unique to metal-reducing bacteria outside the *Geobacteraceae* family. The large number of genera (over 250) and

complete or nearly complete genomes (over 200) in the γ-*proteobacteria* class [19] facilitates nucleotide sequence comparisons of genes in both metal- and non-metal-reducing bacteria, potentially aiding in the identification of molecular signatures unique to metal-reducing γ-*proteobacteria*. The γ-*proteobacteria* class includes *Shewanella oneidensis*, a gram-negative, facultative anaerobe that reduces a wide range of metals, including Fe(III) and Mn(IV) as terminal electron acceptor [9, 110]. *S. oneidensis* employs a number of novel respiratory strategies for dissimilatory metal reduction, including: i) localization of *c*-type cytochromes on the cell surface (or along extracellular nanowires) where they may deliver electrons to external metals [60, 111, 112]; ii) non-reductive dissolution of metal oxides to form more readily reducible organic-metal complexes [28, 59, 113]; and iii) delivery of electrons to external metals via endogenous or exogenous electron shuttles [35, 114, 115].

S. oneidensis contains an electron transport chain that consists of IM-localized primary dehydrogenases, menaquinone, and CymA, a menaquinol-oxidizing *c*-type cytochrome that functions as a central branch point in electron transport to Fe(III), Mn(IV), nitrate (NO_3^-), nitrite (NO_2^-), dimethylsulfoxide (DMSO) and fumarate [116]. CymA transfers electrons to the periplasmic *c*-type cytochrome MtrA [117], which interacts with outer membrane (OM)-localized protein complexes composed of transmembrane β-barrel protein MtrB [31, 118] and decaheme *c*-type cytochrome MtrC [48, 119]. Purified MtrC reduces Fe(III) [120, 121], and in proteoliposomes, purified MtrB, MtrC, and MtrA form a lipid-embedded "porin-cytochrome" complex [122] that transfers electrons from internal reduced methyl viologen to external Fe(III) substrates [52, 123].

Previous nucleotide sequence analyses indicated that the N-terminus of *S. oneidensis* MtrB contained a unique CXXC motif [31]. The identification of a CXXC motif in *S. oneidensis* MtrB was unusual since CXXC motifs are generally not found in OM β-barrel proteins, most likely to avoid protein folding problems caused by redox-reactive cysteines during passage across the intermembrane space in eukaryotes or the periplasmic space in bacteria [124-126]. The identification of an unusual CXXC motif in the N-terminus of MtrB led us to hypothesize that this motif may represent a molecular signature unique to metal-reducing γ-*proteobacteria*. To test this hypothesis, nucleotide sequence analyses were carried out to correlate dissimilatory metal reduction capability with the presence of MtrB homologs containing an N-terminal CXXC motif. Site-directed mutational analyses were performed to determine if the N-terminal CXXC motif of MtrB was required for metal reduction by the representative metal-reducing γ-*proteobacterium S. oneidensis*. The ability to predict dissimilatory metal reduction by a γ-*proteobacterium* with unknown metal reduction capability was then tested with *Vibrio parahaemolyticus*, a human pathogen whose genome encodes an MtrB homolog with an N-terminal CXXC motif.

Materials and methods

Bacterial strains and cultivation conditions

Bacterial strains and plasmids used in the present study are listed in Table 1. For genetic manipulations, all *E. coli* and *S. oneidensis* strains were cultured at 30°C in Luria Bertani medium (10 g L^{-1} NaCl, 5 g L^{-1} yeast extract, 10 g L^{-1} tryptone). For aerobic and anaerobic growth experiments, all *S. oneidensis* strains were cultured in a defined salts medium (M1) supplemented with 20 mM lactate as carbon/energy source [9]. *V. parahaemolyticus* and *V. harveyi* were tested for anaerobic metal reduction activity in Marine Broth (Difco) growth

Table 4.1. Strains and plasmids used in the present study

Strains	Features	Source
Shewanella oneidensis		
MR-1	Wild-type strain	ATCC
Δ*mtrB*	In-frame deletion mutant	This study
C42A	Site-directed mutant	This study
C45A	Site-directed mutant	This study
C42A plus *mtrB*	C42A complemented with wild-type *mtrB*	This study
Escherichia coli		
β2155 λ pir	*thrB1004 pro thi strA hsdS lacZ_M15* (F9 *lacZΔM15 lacl*q *traD36 proA*1 *proB*1)	[162]
XL10 Gold	Δ*dapA::erm pir::*RP4 Km^R Km^R electrocompetent	Agilent
Vibrio parahaemolyticus	Wild-type strain RIMD 2210633	ATCC

Vibrio harveyi	Wild-type strain BB120	ATCC

Plasmids	Features	Source
pKO2.0	4.5 kb γR6K, *mob*RP4 *sacB* Gm^R *lacZ*	[140]
pBBR1MCS	Cm^R lacZ	[141]
pKO2.0-*mtrB*	pKO2.0 with in-frame deletion of *mtrB*	This study
pKO2.0+*mtrB*	pKO2.0 containing wild-type copy of *mtrB*	This study

medium. Bacterial growth experiments were carried out in a B. Braun Biostat B batch reactor with automatic feedback control of pH, temperature, and dissolved O_2 concentration. Electron acceptors were synthesized as previously described [29, 127-130] and added at the following final concentrations: NO_3^-, 10 mM; NO_2^-, 2 mM; Fe(III) citrate, 50 mM; amorphous MnO_2, 15 mM; trimethylamine-*N*-oxide (TMAO), 25 mM; $S_2O_3^{2-}$, 10 mM; fumarate, 30 mM; and dimethylsulfoxide (DMSO), 25 mM. Gentamycin [131] was supplemented at 15 μg mL^{-1}. For growth of *Escherichia coli* β2155 λ pir, diaminopimelate (DAP) was amended at 100 μg mL^{-1}.

Analytical procedures

Cell growth was monitored by direct cell counts via epifluorescence microscopy and by measuring terminal electron acceptor depletion or end product accumulation. Acridine orange-stained cells were counted (Zeiss AxioImager Z1 Microscope) according to previously described procedures [24]. Cell numbers at each time point were calculated as the average of 10 counts from two parallel yet independent anaerobic incubations. NO_2^- was measured spectrophotometrically with sulfanilic acid-*N*-1-naphthyl-ethylene-

diamine dihydrochloride solution [132]. Fe(III) reduction was monitored by measuring

HCl-extractable Fe(II) production with ferrozine [133]. Mn(IV) concentration was

measured colorimetrically after reaction with benzidine hydrochloride as previously

described [24]. Mn(III)-pyrophosphate concentration was measured colorimetrically as

previously described [134]. $S_2O_3^{2-}$ concentrations were measured by cyanolysis as

previously described [135]. Growth on O_2, TMAO, DMSO, and fumarate were

monitored by measuring increases in cell density at 600 nm. Control experiments

consisted of incubations with cells that were heat-killed at $80^{\circ}C$ for 30 min prior to

inoculation.

Nucleotide and amino acid sequence analyses

Genome sequence data for *S. oneidensis* MR-1, *S. putrefaciens* 200, *S.

putrefaciens* CN32, *S. putrefaciens* W3-18-1, *S. amazonensis* SB2B, *S. denitrificans*

OS217, *S. baltica* OS155, *S. baltica* OS195, *S. baltica* OS185, *S. baltica* OS223, *S.

frigidimarina* NCIMB400, *S. pealeana* ATCC 700345, *S. woodyi* ATCC 51908, *S.* sp.

ANA-3, *S.* sp. MR-4, *S.* sp. MR-7, *S. loihica* PV-4, *S. halifaxens* HAW-EB4, *S.

piezotolerans* WP3, *S. sediminis* HAW-EB3, and *S. benthica* KT99 were obtained from

the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov)

or the Department of Energy Joint Genome Institute (DOE-JGI, http://jgi.doe.gov). MtrB

homologs in the NCBI databases were identified via BLAST analysis [136] using *S.

oneidensis* MtrB as the search query. Multiple alignments of MtrB homologs were

generated with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) [137]. β-

barrel architecture of the MtrB homologs was predicted using the program PRED-TMBB

[138]. LOGO diagrams were generated using the ClustalW alignment files [139].

In-frame gene deletion mutagenesis * and genetic complementation analysis

mtrB was deleted from the *S. oneidensis* genome via application of a *Shewanella*

in-frame gene deletion system [140]. Regions corresponding to ~750 bp upstream and

downstream of *mtrB* were independently PCR-amplified and subsequently joined using

overlap-extension PCR. Primers for *mtrB* deletion are listed in Table 2. The resulting

fragment was cloned into suicide vector pKO2.0, which does not replicate in *S.*

oneidensis. This construct (designated pKO-*mtrB*) was mobilized into wild-type MR-1

via conjugal transfer from *E. coli* donor strain β2155 λ pir.

Table 4.2.　　Primers used for in-frame gene deletion mutagenesis, site-directed
　　　　　mutagenesis, and DNA sequencing

Deletion mutagenesis primers

MtrBD1	GACTGGATCCCTCCTCTAAGAGTCCAATGGCTGGC
MtrBD2	CAGCATCAGCATTTGTGCGGTGTAGCCTGTGTTGGCTAATAACGCTAGAGT
MtrBD3	ACTCTAGCGTTATTAGCCAACACAGGCTACACCGCACAAATGCTGATGCTG
MtrBD4	GACTGTCGACACATTTAGCCAAGCCCTAAGCCGT
MtrBDTF	CAGAGCAAGTCGAAGCCACCTTAG
MtrBDTR	CCATCGGTACTATGGCAAACAGAGC

Site-directed mutagenesis primers

C42A-Sense	GTGAAATTATCCGCATGGAGCGCAAAAGGCTGCGTCGTTGAAACG
C42A-Anti	CGTTTCAACGACGCAGCCTTTTGCGCTCCATGCGGATAATTTCAC
C45A-Sense	GCATGGAGCTGTAAAGGCGCAGTCGTTGAAACGGGCACA
C45A-Anti	TGTGCCCGTTTCAACGACTGCGCCTTTACAGCTCCATGC

Sequencing primers

MtrB-SeqF	GATCACTCTAGCGTTATTAGCCAAC

MtrB-SeqR   GTTGCTTGAACCTGCTGTTATC

MtrB cloning primers
MtrB-CompF   GACTGGATCCGGTTCTAACCATCCAT
MtrB-CompR   GACTGTCGACCAGAGGCGGGCTTTT

S. oneidensis strains with the plasmid integrated into the genome were selected on solid

LB medium containing gentamycin (15 μg mL^{-1}). Single integrations were verified via

PCR with primers flanking the recombination region. Plasmids were resolved from the

genomes of single integrants by plating on solid LB medium containing sucrose (10%

w/v) with NaCl omitted. In-frame deletions were verified by PCR and direct DNA

sequencing (GeneWiz, South Plainfield, NJ). Genetic complementation of Δ*mtrB* was

carried out by cloning wild-type *mtrB* into broad-host-range cloning vector pBBR1MCS

[141] and conjugally transferring the recombinant vector into Δ*mtrB* via bi-parental

mating procedures [111].

Site-directed mutagenesis*

Single amino acid mutations in MtrB (C42A or C45A) were constructed using the

Quickchange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara,

CA). The *mtrB* gene and regions ~750 bp upstream and downstream were PCR-

amplified as a single fragment and subsequently cloned into pBBR1MCS. Mutagenesis

primers C42A-Sense, C42A-Antisense, C45A-Sense, and C45A-Antisense (Table 2)

were used in mutagenesis PCR reactions according to the manufacturer's instructions.

The resulting PCR products were subsequently transformed into XL10 Gold Kan^{R}

competent cells (Agilent Technologies, Santa Clara, CA). Correct amino acid mutations

(C42A or C45A) were verified by direct DNA sequencing using primers MTRB-SeqF

and MTRB-SeqR (Table 2). The mutated *mtrB* constructs were subsequently cloned into

suicide vector pKO2.0, and were "knocked-in" to the native chromosomal position.

Nucleotide sequence changes were verified by PCR and DNA sequencing of *S.

oneidensis* "knock-in" transformants. Genetic complementation of mutant C42A was

carried out by cloning wild-type *mtrB* into broad-host-range cloning vector pBBR1MCS

[141] and conjugally transferring the recombinant vector into mutant C42A via bi-

parental mating procedures [111].

Results and discussion

Identification of N-terminal CXXC motifs in MtrB homologs within the genus
Shewanella

Recent proteoliposome studies indicated that electrons are transferred from internal reduced methyl viologen to external Fe(III) substrates by a porin-cytochrome complex composed of *S. oneidensis* β-barrel protein MtrB and decaheme cytochromes MtrA and MtrC [107, 122, 142]. *S. oneidensis* MtrB was predicted to contain a 55-amino acid N-terminus followed by 28 β-sheets that form a transmembrane β-barrel domain [123]. MtrB homologs with high sequence similarity were identified in the genomes of 22 metal-reducing members of the genus *Shewanella* (Table S1, Fig. S1), but not in the genome of non-metal-reducing *S. denitrificans* [143]. Multiple sequence alignment of the 22 *Shewanella* MtrB homologs indicated that each consisted of a 46-82 amino acid N-terminus followed by a C-terminus with 25-30 β-sheets (Table S1, Fig. S1). The N-terminus of all 22 *Shewanella* MtrB homologs contained a CKXC motif corresponding to amino acid positions 42-45 in *S. oneidensis* MtrB (Fig. 1, Table S1, Fig. S1). The *S. oneidensis* genome also contains three additional MtrB paralogs (MtrE, DmsF, and SO4359)[144] with lower overall amino acid sequence similarity to MtrB (43%-55%, and e-values ranging from 1e-38 to 4e-127). Each of the three additional MtrB paralogs also contained a conserved N-terminal CKXC motif (Table S2, Fig. S2). The identification of N-terminal CXXC motifs in the MtrB homologs of all 22 metal-reducing *Shewanella* strains was unusual since CXXC motifs are generally not found in

transmembrane β-barrel proteins, most likely to avoid protein folding problems caused by the redox reactive cysteines during passage across the intermembrane space or periplasm [124-126]. CXXC motifs are generally found in cytoplasmic and periplasmic proteins where they carry out a diverse array of functions such as catalyzing disulfide bond exchanges, binding transition metals, or acting as the redox-sensing module of transcriptional activators [145-147]. Transmembrane β-barrel proteins found in the mitochondria and chloroplast of higher eukaryotes and the OM of Gram-negative bacteria are generally involved in active ion transport or passive nutrient uptake [148]. *S. oneidensis* MtrB appears to function as a structural sheath facilitating interaction and electron transfer from MtrA to MtrC in a transmembrane porin-cytochrome complex [52, 123, 149, 150]. The N-terminal CXXC motif of the *Shewanella* MtrB homologs may facilitate such electron transfer via as yet unknown molecular interactions.

Identification of N-terminal CXXC motifs in MtrB homologs outside the genus *Shewanella*

Nine MtrB homologs displaying amino acid sequence similarity to *S. oneidensis* MtrB had been previously reported in bacterial genomes outside the genus *Shewanella*, including metal- and non metal-reducing *Acidobacteria* and α-, β-, γ-, and δ-*proteobacteria* [52]. Four additional MtrB homologs were subsequently identified in the MtrAB modules of Fe(II)-oxidizing α- and β-*proteobacteria* [54]. The rapid expansion of sequenced bacterial genomes has resulted in a sharp increase in the number of proteins displaying similarity to *S. oneidensis* MtrB. As of July 2013, the list of MtrB homologs

identified outside the *Shewanella* genus numbered 52 (Table S3, Fig. S3), including one

each from the phyla *Acidobacteria* and NC10 group, and 50 from the α-, β-, γ-, and δ-

proteobacteria. The 52 MtrB homologs facilitated amino acid sequence analysis of MtrB

homologs in bacteria that cross phylogenetic and phenotypic lines, including metal- and

non-metal-reducing strains.
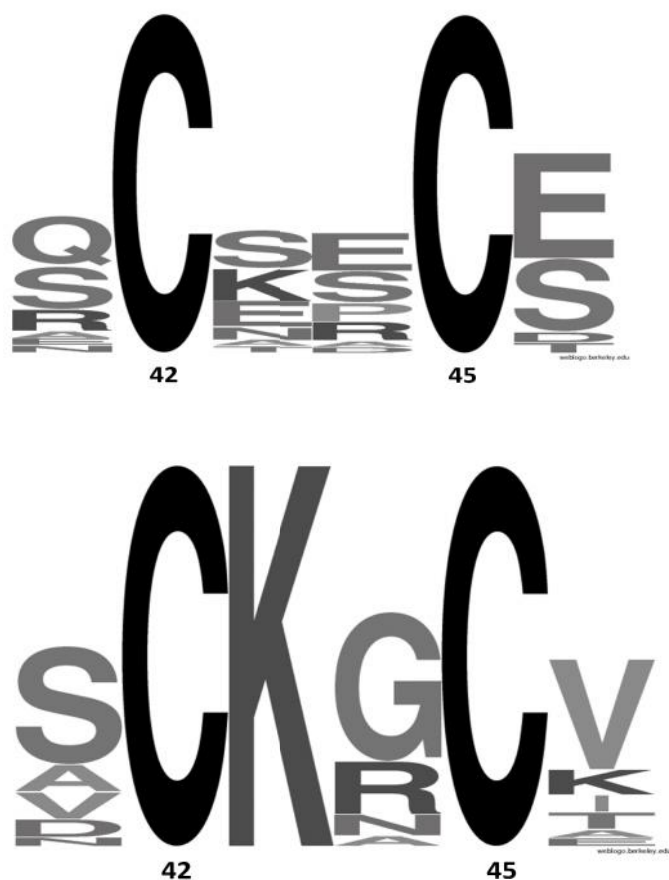
Fig4. 1. LOGO diagrams comparing the amino acids in the N-terminal CXXC motifs of MtrB
homologs identified in the genomes of 22 metal-reducing *Shewanella* strains (top panel) and 20
CXXC-containing MtrB homologs in γ-*proteobacteria* outside the genus *Shewanella* (bottom
panel) (corresponding to amino acid positions 42-45 of *S. oneidensis* MtrB). Strain designations
are listed in Table S1.

Literature searches were conducted to determine the dissimilatory metal reduction capability of the host strains harboring each of the 52 MtrB homologs (Table S3). Correlations between the similarity of the 52 MtrB homologs and the ability of the corresponding host strains to catalyze dissimilatory metal reduction were not observed. The 52 MtrB homologs found outside the *Shewanella* genus were subsequently ranked according to e-value, ranging from the MtrB homolog of the metal-reducing γ-*proteobacterium Ferrimonas balearica* (e-value of 7.00e-145) to the MtrB homolog of the metal-reducing δ-*proteobacterium Geobacter metallireducens* (e-value of 0.28). ClustalW analyses of the 52 MtrB homologs (Table S3) indicated that N-terminal length varied from 4-132 amino acids, while the number of C-terminal β-sheets varied from 22-32 sheets. MtrB homologs of the γ-*proteobacteria Ferrimonas*, *Aeromonas*, and *Vibrio* were represented in 20 of the top 21 MtrB homologs, and each of the 20 *Ferrimonas*, *Aeromonas*, and *Vibrio* homologs contained an N-terminal CXXC motif (Fig. 1, Table S3). The threshold e-value for MtrB homologs containing an N-terminal CXXC motif was 4.00e-43 displayed by the MtrB homolog of *Vibrio vulnificus* YJ016. *Ferrimonas* and *Aeromonas* species are facultatively anaerobic γ-*proteobacteria* capable of dissimilatory metal reduction [151-153], while *Vibrio* species have not been previously examined for dissimilatory metal reduction activity. Of the top 21 MtrB homologs, only the MtrB homolog of the γ-*proteobacterium Nitrosococcus halophilus* Tc4 lacked an N-terminal CXXC motif (Table S3). *N. halophilus* Tc4 is a nitrifying chemolithotroph that obligately respires oxygen as terminal electron acceptor [154]. These results indicate that N-terminal CXXC motifs are found in MtrB homologs of γ-*proteobacteria* capable of
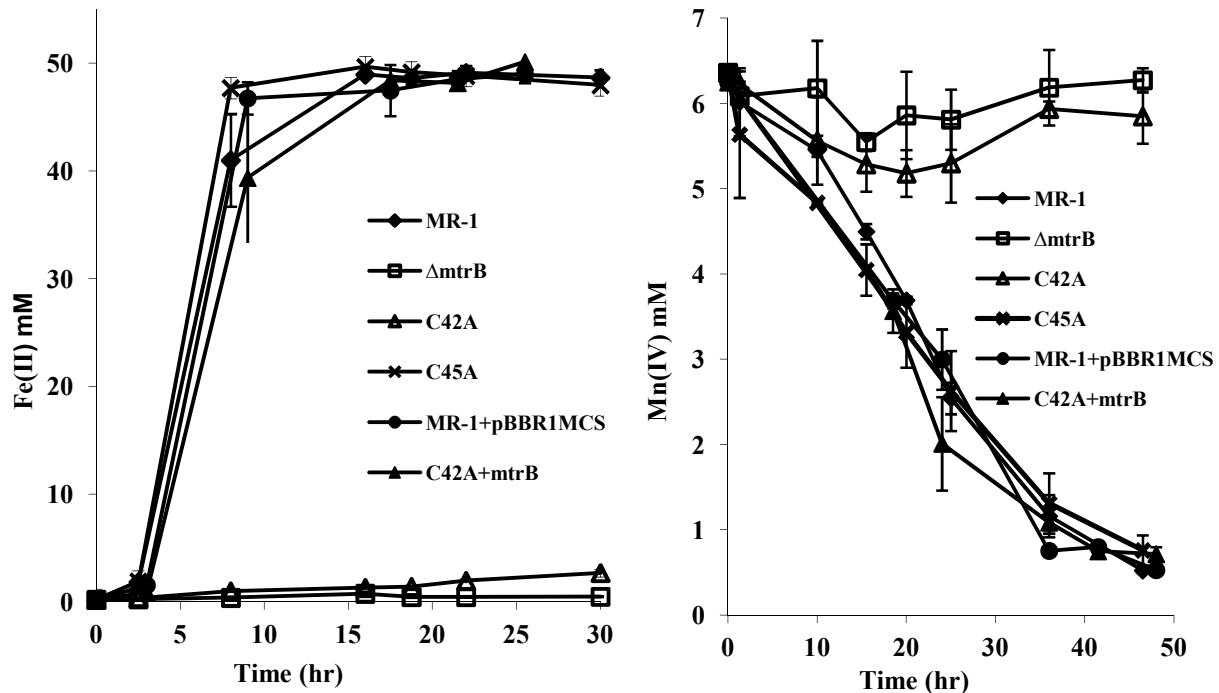
dissimilatory metal reduction, while N-terminal CXXC motifs are missing from the MtrB homolog of an obligately aerobic, non-metal-reducing γ-*proteobacterium*.

The remaining 29 MtrB homologs were found in one *Acidobacterium*, one NC10 group strain, and 27 α-, β-, γ-, and δ-*proteobacteria* (Table S3). None of the remaining 29 MtrB homologs contained an N-terminal CXXC motif. α- and β-*proteobacteria* were represented in 18 of the 29 MtrB homologs lacking an N-terminal CXXC motif, including the MtrB homologs of the Fe(II)-oxidizing β-*proteobacteria Dechloromonas aromatica, Gallionella capsiferriformans,* and *Sideroxydans lithotrophicus* [155-157]. CXXC motifs were also missing from the N-terminus of PioB, the MtrB homolog of the Fe(II)-oxidizing α-*proteobacterium Rhodopseudomonas palustris* [53], and from the MtrB homolog of the γ-*proteobacterium Halorhodospira halophila*, a sulfur-oxidizing anoxygenic phototroph [158]. Three of the 29 MtrB homologs lacking an N-terminal CXXC motif were found in metal-reducing bacteria, including the β-*proteobacterium Rhodoferax ferrireducens* [159] and the δ-*proteobacteria Geobacter* sp. M21, *G. metallireducens* and *G. uraniireducens* [160]. These results indicate that MtrB homologs of metal-reducing γ-*proteobacteria* contain an N-terminal CXXC motif that is missing from MtrB homologs of non-metal-reducing γ-*proteobacteria* and from all bacteria outside the γ-*proteobacteria*, including those catalyzing dissimilatory metal reduction or oxidation reactions.

The first conserved cysteine in the N-terminal CXXC motif of MtrB is required for dissimilatory metal reduction by *S. oneidensis*

To determine if the N-terminal CXXC motif of MtrB was required for dissimilatory metal reduction, the N-terminal CXXC motif of *S. oneidensis* MtrB was selected for site-directed mutational analysis, and the resulting CXXC mutants were tested for dissimilatory metal reduction activity. *S. oneidensis* mutant strain C42A was unable to reduce Fe(III) or Mn(IV) as terminal electron acceptor (i.e., displayed metal reduction-deficient phenotypes identical to Δ*mtrB*; Fig. 2), yet retained wild-type respiratory activity on all non-metal electron acceptors, including O_2, NO_3^-, NO_2^-, $S_2O_3^{2-}$, fumarate, DMSO, and TMAO (Fig. S3). *S. oneidensis* mutant strain C45A, on the other hand, displayed wild-type reduction activity of all electron acceptors, including Fe(III) and Mn(IV) (Figs. 2 and S3). The involvement of C42 in metal reduction activity was confirmed via restoration of wild-type metal reduction activity to C42A transconjugates provided with wild-type *mtrB* on pBBR1MCS (Fig. 2). These findings indicate that the first, but not the second, cysteine in the N-terminal CXXC motif of MtrB is required for dissimilatory metal reduction by *S. oneidensis*. These findings also indicate that overlapping MtrB function is not provided by the MtrB paralogs MtrE, DmsF, and SO4359, or that these paralogs are expressed under metal-reducing conditions different than those employed in the present study [118, 144].

The involvement of C42 in metal reduction by *S. oneidensis* and the absence of the corresponding N-terminal CXXC motif in MtrB homologs of metal-reducing *Rhodoferax* and *Geobacter* species indicate that the molecular mechanism of metal reduction by γ-, β- and δ-*proteobacteria* differs in at least one fundamental aspect. The biochemical function of C42 in metal reduction by *S. oneidensis* is currently unknown. Based on the participation of CXXC motifs in metal binding, redox sensing, and disulfide

Figure 4.2 Dissimilatory metal reduction activity of strains *S. oneidensis* wild-type, wild-type containing pBBR1MCS, Δ*mtrB*, C45A, C42A, and C42A complemented by wild-type *mtrB* with either Fe(III) (left panel) or Mn(IV) (right panel) as terminal electron acceptor. Values are the means of two parallel but independent anaerobic incubations; error bars represent standard deviations. Some error bars cannot be seen due to small standard deviations.

bond formation [145-147], potential roles for C42 include the binding of metals or co-factors required for electron transport by the MtrCAB complex, sensing redox conditions via sulfur redox chemistry, or enhancing MtrB interaction with other cysteine-containing metabolites and proteins via heterologous disulfide bond formation. Current work is focused on examining these possibilities during metal reduction by *S. oneidensis*.

Prediction of dissimilatory metal reduction activity by γ-*proteobacteria* with unknown metal reduction capability.

As described above, 20 of the top 21 MtrB homologs were identified in the genera *Ferrimonas*, *Aeromonas*, and *Vibrio* (Table S3). Although *Ferrimonas* and *Aeromonas*

species are known to catalyze dissimilatory metal reduction [152, 153, 161], the

dissimilatory metal reduction capability of *Vibrios* is not well studied. The ability to

predict dissimilatory metal reduction by a γ-*proteobacterium* with unknown metal

reduction capability was tested with *Vibrio parahaemolyticus*, a pathogen whose genome

encodes an MtrB homolog with an N-terminal CXXC motif. A CSEC motif was

identified in the N-terminus of the *V. parahaemolyticus* MtrB homolog VP1218

(87QD1_VIBPA; Table S3). Subsequent anaerobic incubations demonstrated that *V.*

parahaemolyticus reduced Fe(III) and Mn(IV) as terminal electron acceptors (Fig. 3),
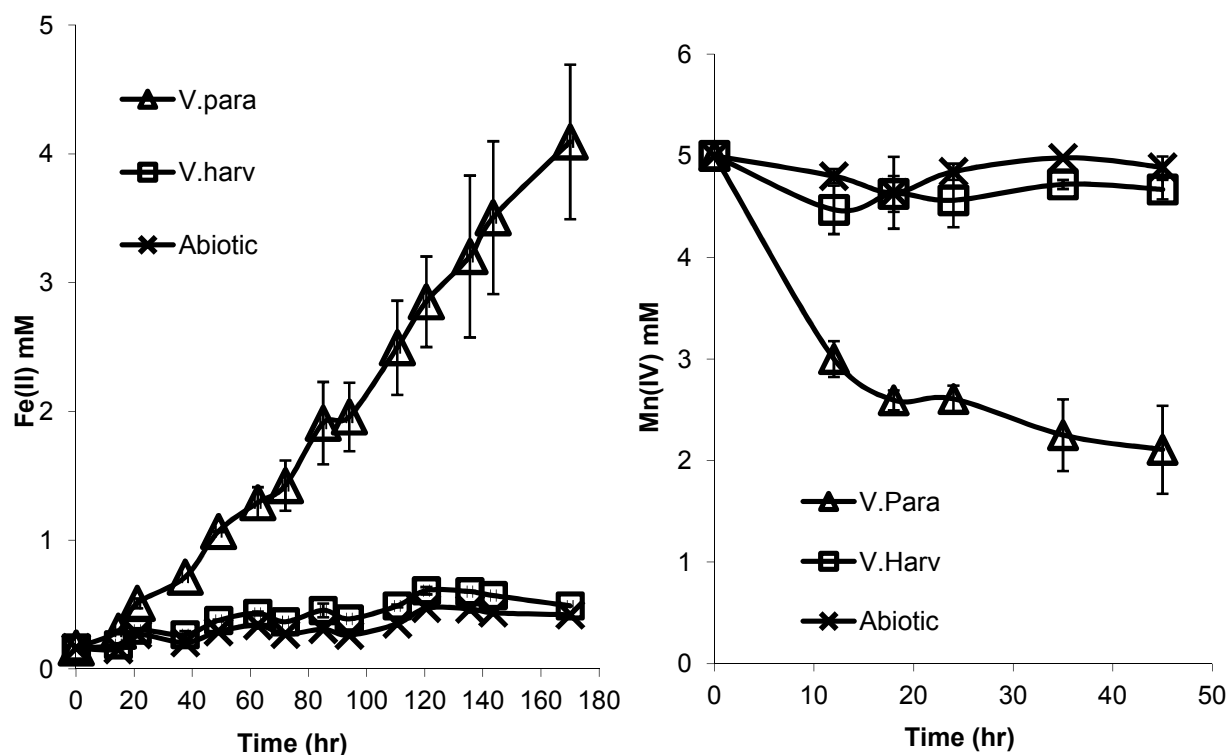
Figure 4.3 Dissimilatory metal reduction activity of *V. parahaemolyticus* and *V. harveyi* wild-type strains with either Fe(III) (left panel) or Mn(IV) (right panel) as terminal electron acceptor. Values are the means of two parallel but independent anaerobic incubations; error bars represent standard deviations. Some error bars cannot be seen due to small standard deviations.

while *V. harveyi*, a *Vibrio* control strain lacking the MtrB homolog, was deficient in Fe(III) and Mn(IV) reduction activity (Fig.4.3).

Results of the present study indicate that MtrB homologs of metal-reducing γ-*proteobacteria* contain an N-terminal CXXC motif that is missing from the MtrB homologs of and NC10 group strains, non-metal-reducing γ-*proteobacteria*, and all α-, β-, and δ-*proteobacteria*, including those catalyzing dissimilatory metal reduction or oxidation reactions. The N-terminal CXXC motif of MtrB is required for dissimilatory metal reduction by the representative metal-reducing γ-*proteobacterium S. oneidensis*, and the ability to predict dissimilatory metal reduction by a γ-*proteobacterium* with unknown metal reduction capability was confirmed with *Vibrio parahaemolyticus*, a pathogen whose genome encodes an MtrB homolog with an N-terminal CXXC motif. MtrB homologs with N-terminal CXXC motifs may thus represent a molecular signature unique to metal-reducing members of the γ-*proteobacteria*, with the potential for further development as a biomarker for tracking the presence and activity of metal-reducing γ-*proteobacteria* in natural and engineered systems.

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Supplementary Table 4.S1. Amino acid sequence identity (ID), similarity (Sim), expect-value (e-value), N-terminal CXXC motif (CXXC motif), number of amino acid residues in the N-terminus (N-term length), and number of β-sheets in the C-terminus (No. β-sheets) of the MtrB homologs identified in the genomes of 22 metal-reducing *Shewanella* strains.

Microorganism	Locus Tag	ID (%)	Sim (%)	e-value	CXXC motif	N-term length	No. β-sheets
S. oneidensis MR-1	SO_1776	100	100	0	CKGC	54	28
Shewanella sp. MR-4	Shewmr4_2512	93	97	0	CKGC	46	28
Shewanellasp. ANA3	Shewana3_2678	93	97	0	CKGC	46	28
Shewanella sp. MR-7	Shewmr7_2580	93	97	0	CKGC	46	28
Shewanella sp. HN41	SOHN41_ 01518	90	96	0	CKGC	50	28
Shewanella sp. W3-18-1	Sputw3181_ 2625	89	96	0	CKGC	46	28
S. baltica OS223	Sbal223_2767	88	96	0	CKGC	54	28
S. baltica OS185	1.1 Shew185_1576	88	96	0	CKGC	54	28
S. baltica OS183	Sbal183DRAFT_1149	88	96	0	CKGC	54	28
S. baltica OS625	Sbal625DRAFT_1717	88	96	0	CKGC	54	28
S. baltica OS195	Sbal195_1610	88	96	0	CKGC	54	28
S. baltica OS155	Sbal_1587	88	96	0	CKGC	54	28
S. putrefaciens CN32	Sputcn32_1476	84	91	0	CKGC	46	30
S. woodyi ATCC 51908	Swoo_3127	72	84	0	CKRC	82	25
S. loihica PV-4	Shew_2527	71	83	0	CKRC	52	26
S. sediminis HAW-EB3	Ssed_1523	68	83	0	CKRC	58	26
S. piezotolerans WP3	Swp_3280	67	83	0	CKRC	54	27
S. benthica KT99	KT99_05757	66	83	0	CKRC	81	25
S. pealeana ATCC 700345	Spea_2700	67	82	0	CKNC	54	26
S. halifaxensis HAW-EB4	Shal_2786	65	81	0	CKNC	55	26

S. frigidimarina NCIMB 400	Sfri_2639	67	81	0	CKAC	80	28
S. amazonensis SB2B	Sama_120	63	79	0	CKGC	53	27

Supplementary Table 4.S2. Amino acid sequence identity (ID), similarity (Sim), expect-value (e-value), N-terminal CXXC motif (CXXC motif), number of amino acid residues in the N-terminus (N-term length), and number of β-sheets in the C-terminus (No. β-sheets) of the three MtrB paralogs identified in the genome of *S. oneidensis* MR-1.

Locus Tag	ID (%)	Sim (%)	E value	CXXC motif	N-term length	No. β sheets
SO_1776 MtrB	100	100	0	CKGC	54	28
SO_1781 MtrE	36	55	4.00E-127	CKQC	61	24
SO_1428 Extracellular DMSO respiration system	35	54	4.00E-114	CKSC	49	28
SO_4359 DMSO reductase system	25	43	1.00E-38	CKQC	50	27

Supplementary Table 4.S3. Phylogenetic affiliation (Class), amino acid sequence identity (ID, %), similarity (Sim, %), expect-value (e-value), N-terminal CXXC motif, (CXXC motif) number of amino acid residues in the N-terminus (N-term length), number of β-sheets in the C-terminus (No. β-sheets), and reported dissimilatory metal reduction or oxidation activity of the host strain (metal redox) for 52 MtrB homologs displaying similarity to *S. oneidensis* MtrB.

Microorganism	Class	Locus Tag	ID	Sim	E-value	CXXC motif	N-term length	No. β-sheets	Metal redox
Ferrimonas balearica DSM 9799	γ	Fbal_1355	41	57	2.00E-148	CKRC	79	26	Fe(III) reduction[1]
Ferrimonas balearica DSM 9799	γ	Fbal_2477	39	56	7.00E-145	CKRC	76	24	
Ferrimonas balearica DSM 9799	γ	Fbal_1363	28	49	1.00E-84	CERC	77	25	
Nitrosococcus halophilus Nc 4	γ	Nhal_1654	29	49	3.00E-83	- - -	5	32	
Aeromonas veronii AMC34	γ	HMPREF1168_03370	29	47	5.00E-70	CASC	51	25	
Aeromonas hydrophila ATCC 7966	γ	AHA_2766	27	47	3.00E-65	CKSC	51	26	Fe(III) reduction[2]
Aeromonas hydrophila SSU	γ	HMPREF1171_01918	27	47	7.00E-65	CKSC	51	26	
Aeromonas hydrophila ML09-119	γ	AHML_14660	27	46	2.00E-64	CKSC	51	26	
Aeromonas aquariorum	γ	WP_010633518.1	27	46	4.00E-61	CNSC	51	26	
Aeromonas diversa	γ	G114_06847	28	45	4.00E-60	CNAC	45	27	
Vibrio parahaemolyticus BB22OP	γ	VPBB_1142	27	44	1.00E-51	CSEC	46	22	
Vibrio sp. Ex25	γ	VEA_003778	26	43	2.00E-49	CSEC	46	22	
Vibrio parahaemolyticus 10329	γ	VP10329_20255	27	44	8.00E-49	CSEC	46	22	
Vibrio parahaemolyticus RIMD 2210633	γ	NP_797597.1	27	43	9.00E-49	CSEC	46	22	Fe(III) reduction[3]
Vibrio sp. EJY3	γ	VEJY3_05885	26	46	2.00E-48	CTDC	46	26	
Vibrio alginolyticus E0666	γ	C408_1584	26	43	2.00E-48	CSEC	46	22	
Vibrio parahaemolyticus AQ4037	γ	VIPARAQ4037_2777	27	43	3.00E-48	CSEC	46	22	
Vibrio vulnificus	γ	VVMO6_	26	43	9.00E-	CEPC	46	26	

MO6-24/O		03631			46				
Vibrio vulnificus CMCP6	γ	VV2_0135	26	42	1.00E-45	CEPC	46	26	
Vibrio vulnificus YJ016	γ	VVA_0644	26	42	5.00E-43	CEPC	51	26	
Halorhodospira halophila SL1	γ	Hhal_2380	24	43	1.00E-31	- - -	39	29	
Dechloromonas aromatica RCB	β	Daro_1403	23	41	8.00E-31	- - -	41	25	Fe(II) oxidation[4]
Rhodovulum sp. PH10	α	A33M2094	26	44	9.00E-26	- - -	73	29	
Azoarcus sp. KH32C	β	AZKH_4036	24	41	2.00E-25	- - -	136	23	
Azoarcus sp. KH32C	β	AZKH_4372	24	42	6.00E-25	- - -	38	27	
Gallionella capsiferriformans ES-2	β	Galf_2003	22	39	2.00E-22	- - -	106	28	Fe(II) oxidation[5]
Rhodanobacter fulvus Jip2	γ	UU9_10127	24	38	1.00E-21	- - -	97	31	
Candidatus Methylomirabilis oxyfera	NC10	DAMO_0819	22	39	5.00E-20	- - -	49	29	
Nitrosococcus halophilus Nc 4	γ	Nhal_1191	22	39	1.00E-18	- - -	4	34	
Magnetospirillum magneticum AMB-1	α	Amb3018	23	41	1.00E-18	- - -	48	26	
Sideroxydans lithotrophicus ES-1	β	Slit_2496	22	40	2.00E-17	- - -	43	28	Fe(II) oxidation[6]
Azoarcus sp. KH32C	β	AZKH_1287	23	40	2.00E-17	- - -	44	28	
Thauera linaloolentis	β	C666_11635	23	41	4.00E-17	- - -	81	26	
Rhodanobacter thiooxydans	γ	UUA_11468	23	38	2.00E-16	- - -	99	29	
Geobacter uraniireducens Rf4	δ	Gura_3627	23	38	3.00E-15	- - -	33	31	Fe(III) reduction[7]
Pseudoxanthomonas spadix BD-a59	γ	DSC_09260	23	37	5.00E-15	- - -	30	30	
Geobacter sp. M21	δ	GM21_0398	22	42	1.00E-13	- - -	29	29	
Rhodoferax ferrireducens T118	β	Rfer_4081	21	39	1.00E-11	- - -	71	24	Fe(III) reduction[8]
Rhodopseudomonas palustris BisB18	α	RPC_2959	22	41	5.00E-11	- - -	72	28	

Rhodopseudomonas palustris BisA53	α	RPE_0832	22	39	3.00E-10	- - -	48	32	
Burkholderiales bacterium JOSHI_001	β	BurJ1Draft _0794	23	37	1.00E-09	- - -	32	28	
Thauera linaloolentis	β	C666_ 11670	21	38	2.00E-09	- - -	104	24	
Rhodopseudomonas palustris DX-1	α	Rpdx1_ 0795	21	41	1.00E-08	- - -	46	28	
Thioflavicoccus mobilis 8321	γ	Thimo_ 2811	22	36	2.00E-08	- - -	132	26	
Rhodopseudomonas palustris TIE-1	α	Rpal_0816 (pioB)	21	38	1.00E-05	- - -	46	28	Fe(II) oxidation[9]
Rhodopseudomonas palustris CGA009	α	RPA_0745	21	38	1.00E-05	- - -	46	28	
Thiocystis violascens DSM198	γ	Thivi_3491	22	40	4.00E-05	- - -	6	24	
Rubrivivax benzoatilyticus	β	RBXJAZT_ 07418	21	38	5.00E-05	- - -	92	22	
Thiorhodococcus drewsii	γ	ThidrDRAF T_0680	20	36	3.00E-04	- - -	68	32	
Nitrosococcus oceani	γ	NOC27_ 3054	24	39	6.00E-04	- - -	25	20	
Solibacter usitatus Ellin6076	Acido	Acid_6725	28	56	0.006	- - -	38	26	
Geobacter metallireducens GS15	δ	Gmet_174 5	24	47	0.28	- - -	119	26	Fe(III) reduction[10]

[1]Nolan *et al.*, 2010, Nakagawa *et al*., 2005; [151-153] [3]This study; [4]Chakraborty *et al*., 2005, [5,6]Emerson & Moyer, 1997, Hedrich *et al*., 2011, [7]Shelobolina *et al*., 2008, [8]Finneran *et al*., 2003, [9]Jiao & Newman 2007, [10]Bond *et al*., 2005

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MtrB
S. sediminis HAW-EB3           MKFKLNLVTLALLANAGVAGTLMTSTAVAAEGYGLQNANTSKVKFEKWVC 50
S._benthica_KT99               MKFKLNLVTLALLANAG-----FVSTAMAAGGYGIQDANTDKVKFDKWAC 45
S._woodyi_ATCC_51908           MKFKLNLVTLALLANAG-----IASTAMAAGGYGLQNANTDKVKFDKWDC 45
S._pealeana_ATCC_700345        MKFKLNLVTLALLTNAG---MLS-GAAIAADGYGIQNANTEKVKFEKWVC 46
S._halifaxensis_HAW-EB4        MKFKLNLVTLALLTNAG---MFMSGAAIAADGYGIQNANTEKVKFDKWVC 47
S. piezotolerans WP3           MKFKLNLVTLALLASAG---AVAPSIAMA-DGYGIQNANTDKVKFEKWDC 46
S. loihica PV-4                MKFKLNVVTLALIANAG----IVIPGMALADGYGIQNANTEKVKFDNWAC 46
Shewanella_sp._MR-4            MKFKLNLITLALLANTS--------LAVAADGYGLANANTDKVKLSAWSC 42
Shewanella_sp._MR-7            MKFKLNLITLALLANTG--------LAVAADGYGLANANTDKVKFAAWSC 42
Shewanella_sp._ANA-3           MKFKLNLITLALLANTG--------FAVAADGYGLANANTDKVKLSAWSC 42
S_oneidensis_MR-1              MKFKLNLITLALLANTG--------LAVAADGYGLANANTEKVKLSAWSC 42
Shewanella_sp._HN-41           MKFKLNLITLALLANTG--------FAVAADGYGINNANTDKVKFSAWSC 42
Shewanella_sp._W3-18-1         MKFKLNLITLALLANTG--------FAVAADGYGIANANTEKVKLSAWSC 42
S._putrefaciens_CN-32          MKFKLNLITLALLANTG--------FAVAADGYGIANANTEKVKLSAWSC 42
S._baltica_OS223               MKFKLNLITLALLANTG--------FAIAADGYGLANANTEKVKMSAWSC 42
S._baltica_OS155               MKFKLNLITLALLANTG--------FAIAADGYGLANANTEKVKMSAWSC 42
S._baltica_OS625               MKFKLNLITLALLANTG--------FAIAADGYGLANANTEKVKMSAWSC 42
S._baltica_OS195               MKFKLNLITLALLANTG--------FAIAADGYGLANANTEKVKMSAWSC 42
S._baltica_OS185               MKFKLNLITLALLANTG--------FAIAADGYGLANANTEKVKMSAWSC 42
S._baltica_OS183               MKFKLNLITLALLANTG--------FAIAADGYGLANANTEKVKMSAWSC 42
S._frigidimarina_NCIMB_400     MNTKLNLITLALLTSTS--------FSLMADGYGLANAKTDNIKYDAWNC 42
S._amazonensis_SB2B            MRFQLNMITLALLAASA---------PALAGGYSLNNANTDKVKFDAWAC 41
                               *. :**:****:: :.              **.: :*:*.::*   * *


S. sediminis HAW-EB3           KRCEVETGFAGTVGVGVGYNDSDDIRSANAFAAEDEFAGKVDADLTYAGK 100
S._benthica_KT99               KRCVVETGVNGTVGVGMGYNDSDDIRSANAFAAEDEFAYKVDADLKYISE 95
S._woodyi_ATCC_51908           KRCVVETGVNGTVGVGVGYNDSNDIRSANAFAAENEFAGKVDADLTYVGK 95
S._pealeana_ATCC_700345        KNCKVEKGVSGTVGIGAGYSDSDDIRSANAFATEDGFAGKVDADVKYTGE 96
S._halifaxensis_HAW-EB4        KNCKVEKGVAGTVGIGAGYSDSDDIRSANAFATEDGFAGKVDADVKYTGE 97
S._piezotolerans_WP3           KRCTVETGYSGSIGVGVGYNDSDDIHSANAFAAEDEFAGKVDADLKYSGK 96
S. loihica PV-4                KRCKIETGVTGTIGAGVGYNDSDDIRSANAFAAENEFVGKVDADVSYISE 96
Shewanella_sp._MR-4            KGCVVETGVSGTVGVGVGYNGEEDIRSANAFGSKNEVAGKFDADLAYRGE 92
Shewanella_sp._MR-7            KGCVVETGVSGTVGVGVGYNGEEDIRSANAFGSKNEVAGKFDADLAYRGE 92
Shewanella_sp._ANA-3           KGCVVETGVSGTVGVGVGYNGEEDIRSANAFGSKNEVAGKFDADLAYRGE 92
S_oneidensis_MR-1              KGCVVETGTSGTVGVGVGYNSEEDIRSANAFGTSNEVAGKFDADLNFKGE 92
Shewanella_sp._HN-41           KGCVVETGTSGTVGVGVGYNSEEDIRSANAFGSSNEVAGKLDADLSFKGE 92
Shewanella sp. W3-18-1         KGCIVETGVSGTVGVGVGYNSEDDIRSANAFGTSNEVAGKFDADLAYSGE 92
S._putrefaciens_CN-32          KGCIVETGVSGTVGVGVGYNSEDDIRSANAFGTSNEVAGKFDADLAYSGE 92
S._baltica_OS223               KGCVVETGTSGTVGVGVGYNSEEDIRSANAFGTSNEVAGKLDADVTFRGE 92
S._baltica_OS155               KGCVVETGTSGTVGVGVGYNSEEDIRSANAFGTSNEVAGKLDADVTFRGE 92
S._baltica_OS625               KGCVVETGTSGTVGVGVGYNSEEDIRSANAFGTSNEVAGKLDADVTFRGE 92
S._baltica_OS195               KGCVVETGTSGTVGVGVGYNSEEDIRSANAFGTSNEVAGKLDADVTFRGE 92
S. baltica_OS185               KGCVVETGTSGTVGVGVGYNSEEDIRSANAFGTSNEVAGKLDADVTFRGE 92
S._baltica_OS183               KGCVVETGTSGTVGVGVGYNSEEDIRSANAFGTSNEVAGKLDADVTFRGE 92
S._frigidimarina_NCIMB_400     KACAVETGTTGNVGVGIGYNSEDDINSANAFNSSNQTAGKIDADIKYRGN 92
S._amazonensis_SB2B            KGCTLETGTQGNIGIGVGYQDTDDVRSANSFGSDEEVPYKVDADVRHVTE 91
                               * * :*.*  *.:* * **.. :*:.***:* :.:    *.***: .  :
```

Fig.4. S1. Multiple sequence alignments generated by ClustalW analysis of the N-termini of MtrB homologs identified in the genomes of 22 metal-reducing *Shewanella* strains. N-terminal CXXC motifs are found at amino acid positions corresponding to C42-C45 of *S. oneidensis* MtrB.

```
SO_1776_MtrB       -----MKFKLNLITLALLANTG----LAVAADGYGLANANTEKVKLSAWSCKGCVVETGT
51
SO_1781_MtrE       MQIVNISTPKVCFSLTLLAWTMSGVLNTAHAEGYEIQKANRSGVKNEAWSCKQCQPQTGR
60
SO_1428_DmsF       -----MSFKLNIITLGLLAATS-----GVSAADFSVHKANLQGLKLDAYQCKSCIGEKRY
50
SO_4359            -----MKLSKTTIALAMAGFCF-----QAYALDTTFVNKEPQLVDIKNWTCKQCS-EKTM
49

                        :.       ::* : .              . * .   . : : . :. . : ** * .:.


SO_1776_MtrB       SGTVGVGVGYNSEEDIRSANAFGTSN-EVAGKFDADLNFKGEKGYRASVDAYQLGMDGGR
110
SO_1781_MtrE       QGNVSATLAHNDGDDSRFGNRTGIDKDGLVGAIGADMKYKAESGYQTSLMADKLGFDTGS
120
SO_1428_DmsF       QGELQLSAGWAENDDIHAGNAFGDASDGMRAAMDADVRYRN-AGYEANVQAYQLGLENSY
109
SO_4359            SGNMQVGVAHTDSDNKRTLNSLGTTA-GLDALVDANVKMRT-GSQQLHAKAYMTDPNIAY
107

                   .* :    .   . :: :  *  *       : . ..*::. :    . .     *    . : .
```

Fig. 4.S2. Multiple sequence alignments generated by ClustalW analysis of the N-termini of three CXXC-containing MtrB paralogs identified in the *S. oneidensis* genome. The three MtrB paralogs included another member of the Mtr gene cluster (MtrE; SO1781), and two proteins involved in extracellular DMSO reduction (DmsF; SO1428), and SO4359.

A. O$_2$

B. DMSO

C. TMAO

D. Fumarate

MR-1
Δ*mtrB*
C42A
C45A
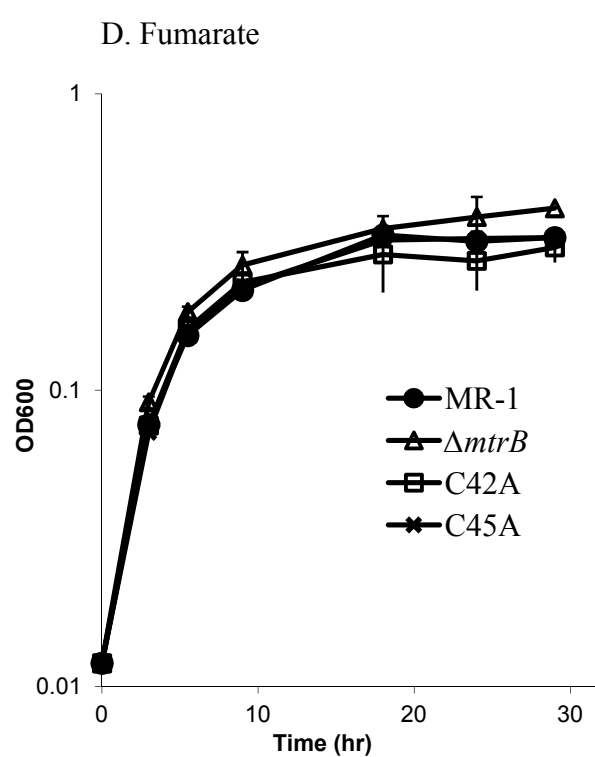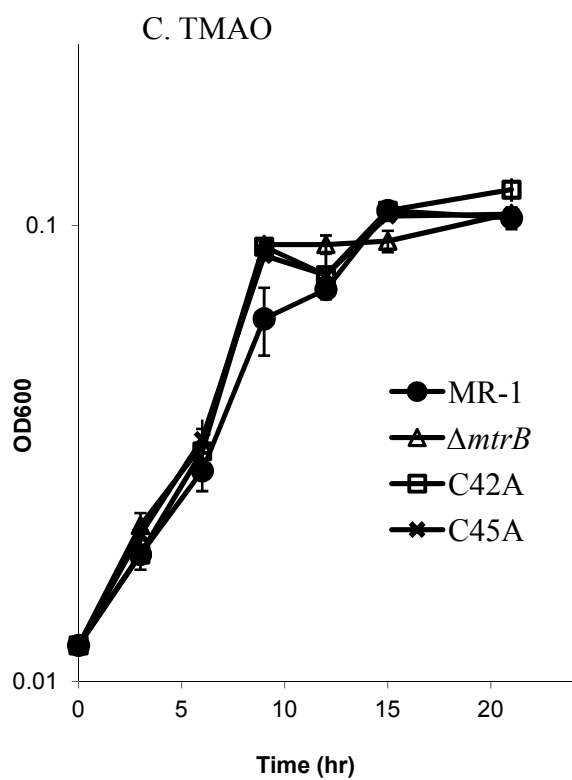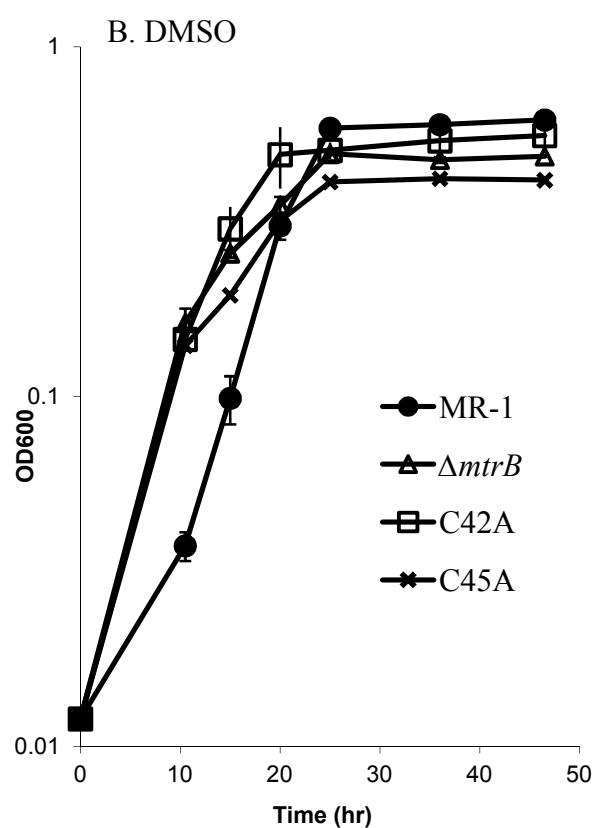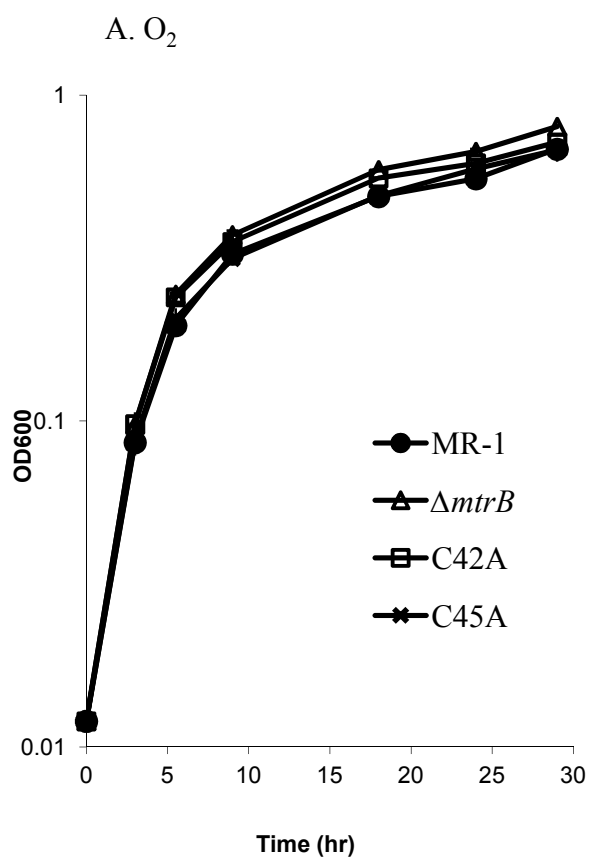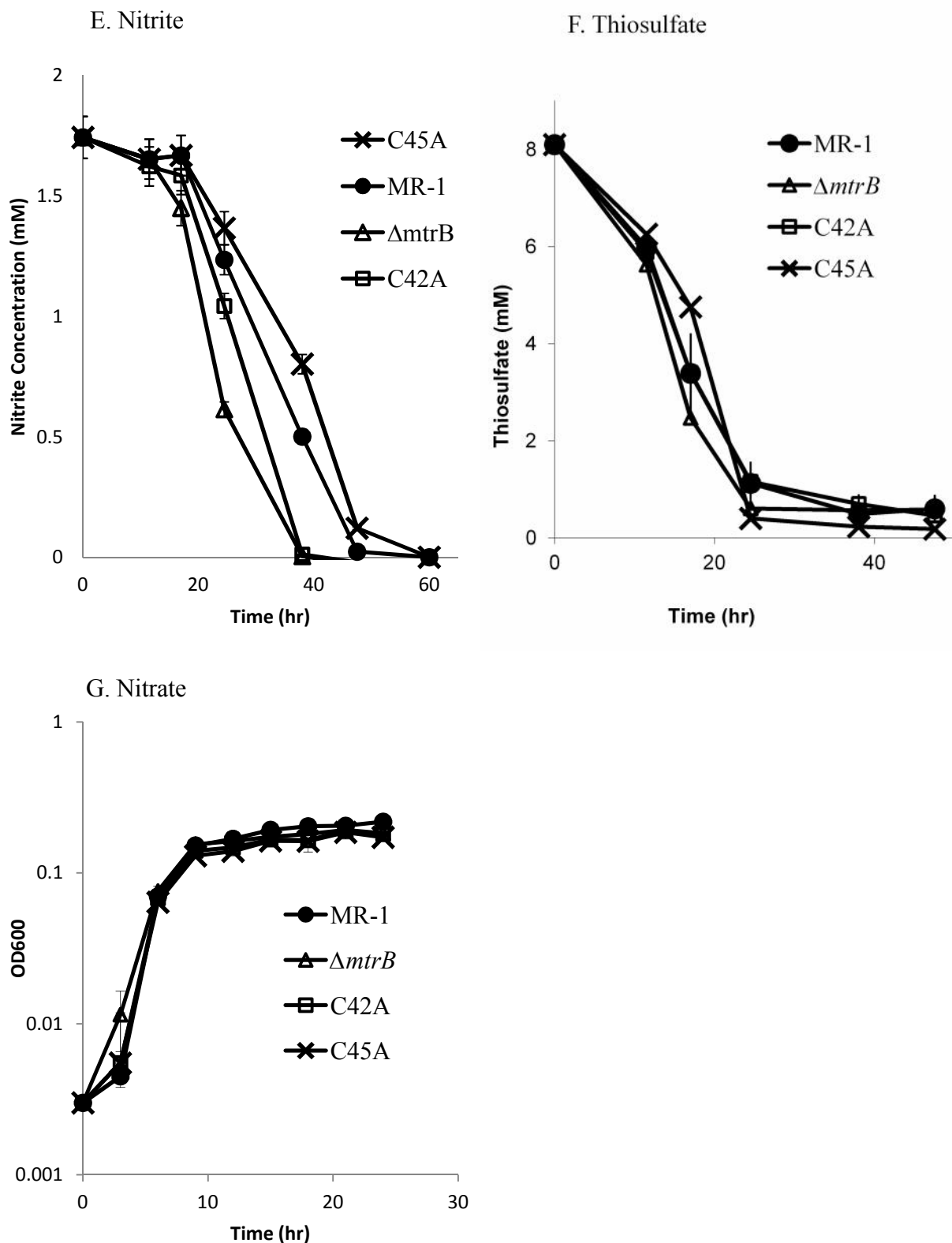
Figure 4. S3. Growth of *S. oneidensis* MR-1 wild-type (●), Δ*mtrB* (Δ), C42A (□), and C45A (×) mutant strains with either O$_2$ (A), DMSO (B), TMAO (C), fumarate (D), nitrite (E), thiosulfate (F), or nitrate (G) as electron acceptor. Values are the means of two parallel but independent anaerobic incubations; error bars represent standard deviations. Some error bars cannot be seen due to small standard deviations.

CHAPTER 5

Design of a flavin mononucleotide (FMN)-based

fluorescent protein reporter for in vivo detection of

intracellular FMN concentrations in *Shewanella oneidensis*

Summary

Applications of the Green Fluorescent Protein (GFP) to examine molecular events in single microbial cells are limited by the oxygen-dependent autocatalytic maturation of the GFP chromophore. GFP applications are thus restricted to aerobic microorganisms and are not suitable for in situ studies of molecular events in anaerobic microorganisms. A novel group of flavin mononucleotide (FMN)-based fluorescent reporter proteins (FbFPs), have been developed as replacements for GFP. FbFPs do not require oxygen for chromophore maturation, and can thus be applied under both aerobic and anaerobic conditions to monitor molecular events in single microbial cells. FbFPs require FMN as cofactor, which also suggests that FbFP fluorescence may be used as an in vivo reporter of internal FMN concentrations. The FbFP reporter system constructed in Chapter 5 provided a novel technology for monitoring internal FMN concentrations in single *S. oneidensis* cells during anaerobic growth on an array of terminal electron acceptors, including O_2, fumarate, and Fe(III).

Introduction

      Phycobiliprotein, a photosynthetic antenna pigment isolated from cyanobacteria, was the first fluorescent protein tested for application as an intracellular fluorescent reporter [1]. The applications of phycobiliprotein, however, were limited due to the requirement of tetrapyrrole as cofactor [2]. Green fluorescent protein (GFP), on the other hand, does not require a cofactor for fluorescence and can be readily expressed in a variety of microorganisms [2-4]. GFP was first recombinantly expressed in the prokaryotic and eukaryotic model organisms *Eschericia coli* and *Caenorhabditis elegans,* respectively [5]. GFP facilitates the study and in situ visualization of complex molecular events in single cells and organisms using flow cytometry or fluorescent microscopy [4, 6, 7]. Reflecting the significance of the discovery, the Nobel Prize in Chemistry in 2008 was recently awarded for "the discovery and development of the green fluorescent protein, GFP" [3, 6]. GFP was first isolated from the jellyfish *Aequorea aequorea* [8]. The fluorescent properties of GFP have since been enhanced by improvements in fluorescent efficiency, thermostability, photostability, and alteration of emission wavelength [2, 3, 6, 7]. GFP variants now emit a wide range of fluorescent colors that nearly span the entire continuum of the visible spectrum [6, 7]. GFP is now widely used to examine molecular events in situ, including gene expression, recombinant protein localization, promoter screening, and monitoring changes in intra- or extracellular conditions [4].

      GFP applications, however, are limited by the oxygen-dependent autocatalytic maturation of the GFP chromophore [9]. GFP applications are thus restricted to aerobic systems [10, 11] and are not suitable for in situ studies of molecular events in anaerobic microorganims. A novel group of fluorescent proteins, termed flavin mononucleotide (FMN)-based fluorescent proteins (FbFPs), have been developed as replacements for GFP [10]. FbFPs do not require oxygen for chromophore maturation, and can thus be applied under both aerobic and anaerobic conditions [10]. FbFPs originate from LOV (light, oxygen, voltage) domain-containing bacterial

photoreceptor proteins that exhibit weak intrinsic autofluorescence when irradiated with blue light (see Figure 1.5 in Chapter 1) [12-14].

Two of the photoreceptor FbFPs, YtvA from *Bacillus subtilis* and SB2 from *Pseudomonas putida,* have been engineered to produce fluorescent quantum yields comparable to GFP variants [10]. Site directed mutagenesis of the photoactive cysteine in the LOV domain in both the truncated YtvA and wild type SB2 proteins, followed by codon optimization, resulted in a 25-fold increase in fluorescent strength [4]. Recently, FbFPs have been used to monitor the presence of single anaerobic bacterial cells with promising results [11, 15-17]. FbFPs require FMN as cofactor, which also suggests that FbFP fluorescence may be used as an in vivo reporter of internal FMN concentrations. FbFP fluorescence thus provides a novel technology for monitoring internal FMN concentrations in vivo in individual *S. oneidensis* cells during flavin-based electron shuttling to external Fe(III) oxides. The main objectives of the present study were to i) design a FMN-based FbFP reporter system for in vivo monitoring of molecular events in single *S. oneidensis* cells grown under anaerobic conditions, and ii) to employ the newly developed FbFP reporter system to monitor internal FMN concentrations in single *S. oneidensis* cells during anaerobic growth on an array of terminal electron acceptors, including O_2, fumarate, and Fe(III).

Materials and methods

Bacterial strains and cultivation conditions

Bacterial strains and plasmids used in the present study are listed in Table 1. For genetic manipulations, all *E. coli* and *S. oneidensis* strains were cultured at 30°C in Luria Bertani medium (10 g L^{-1} NaCl, 5 g L^{-1} yeast extract, 10 g L^{-1} tryptone). For aerobic and anaerobic growth experiments, all *S. oneidensis* strains were cultured in a defined salts medium (M1) supplemented with 20 mM lactate as carbon/energy source [18]. Electron acceptors were synthesized as previously described [19-23] and added at the following final concentrations: Fe(III) citrate, 50 mM; fumarate, 15 mM; anhydrous Fe(III)-oxide (HFO), 40 mM. For BS2 expression on pBAD202, arabinose was amended at 2 mM, kanamycin was supplemented at 50 μg mL^{-1}.

Table 5. 1. Strains and plasmids used in the present study

Strains	Features	Source
Shewanella oneidensis MR-1	Wild-type strain	ATCC
S.oneidensis+pBAD_BS2	*S. oneidensis* containing pBAD_BS2	This study
S.oneidensid+pBAD	*S. oneidensis* containing pBAD202	This study

Plasmids	Features	Source
pBAD202	Arabinose inducible expression vector	Invitrogen
pBAD_BS2	pBAD202 containing FbFP BS2 gene	This Study
Evoglow BS2 plasmid	Plasmid containing FbFP BS2 gene	Evocatal, Germany

Construction of the FbFP expression vector pBAD_BS2

The BS2 gene encoding FbFP, was PCR-amplified from Evoglow BS2 plasmid (Evocatal, Germany) using the following primers: BS2_pBad_For, 5'-CACCATGGCGTCGTTCCAGTCG TTCGG-3' and BS2_Rev_Stop, 5'-TTACTCGAGCAGCTTTTCATATTCCTTCTGC-3'. The BS2 gene was cloned into expression vector pBAD202 (Invitrogen) according to manufacturer's instructions. The resulting construct (pBAD_BS2) was electroporated (0.55kV, 200Ω) into *S. oneidensis* electrocompetent cells that were previously washed with 1 M Sorbitol.

Analytical procedures

Cell growth was monitored by direct cell optical density or by measuring Fe(III) reduction. Fe(III) reduction was monitored by measuring HCl-extractable Fe(II) production with ferrozine [24]. Control experiments consisted of incubations with *S.oneidensis* MR-1 wild type strain with and without the empty pBAD202 plasmid.

In vivo **fluorescence measurement, fluorescence imaging of living cells, and spectrophotometric analysis**

Confocal laser scanning microscopy (LSM 510, Carl Zeiss, Germany) was used for *in vivo* fluorescence imaging of *S. oneidensis* cells expressing BS2-FbFP. Cell cultures (8 µL) were placed on a microscope slide and illuminated with laser light at a wavelength of 458 nm, and the emission was detected in the range of 475-525 nm. For anaerobic cell cultures, slides were

prepared anaerobically in a Coy anaerobic chamber during processing. Documentation and single cell fluorescent intensity measurements were carried out using the Zen 2011 software (Carl Zeiss Microscopy). Bulk fluorescent signals were measured photometrically on a plate reader by irradiating 200 µl of samples with light of 449 nm and detecting fluorescence emissions at 495 nm.

Determination of intracellular flavin concentration and protein concentration

Aliquots of cell cultures (10 mL) were harvested by centrifugation, the cell pellet was washed twice with Tris-HCl buffer and resuspended in 0.5 ml Tris-HCl buffer. Harvested cells were lysed by sonication (2 cycles of 30 s on, 15 s off, 70% power) followed by centrifugation to separate the cells debris. Flavins concentration in the cell lysate were measured in Biotek Synergy H4 plate reader with an excitation wavelength of 440 nm and an emission wavelength of 525 nm as previously described [25]. Protein concentrations were determined by the Bradford assay [26].

Results and discussion

The gene encoding BS2-FbFP was cloned into the arabinose inducible plasmid pBAD202 to generate pBAD_BS2. pBAD_BS2 was subsequently mobilized into wild type *S. oneidensis* electrocompetent cells via electroporation to produce the recombinant strain *S. oneidensis* +pBAD_BS2. FbFP expression was examined by incubating S. *oneidensis* + pBAD_BS2 in M1 minimum growth medium with lactate as electron donor and O_2, fumarate, Fe(III) citrate, or anhydrous Fe(III) oxide (HFO) as the terminal electron acceptor.

Effect of FbFP expression on cell growth

Effects of FbFP expression on cell growth were examined by monitoring cell growth during FBFP expression induced by different concentrations of arabinose. Addition of 1-to-4 mM arabinose did not affect aerobic growth (Figure 5.1a). However, concentration of arabinose >4 mM resulted in significantly slower anaerobic growth rates with fumarate and Fe(III) citrate as the terminal electron acceptor (Figure 5.1b, 5.1c). In a parallel experiment, the bulk

a. O2

b. Fumarate
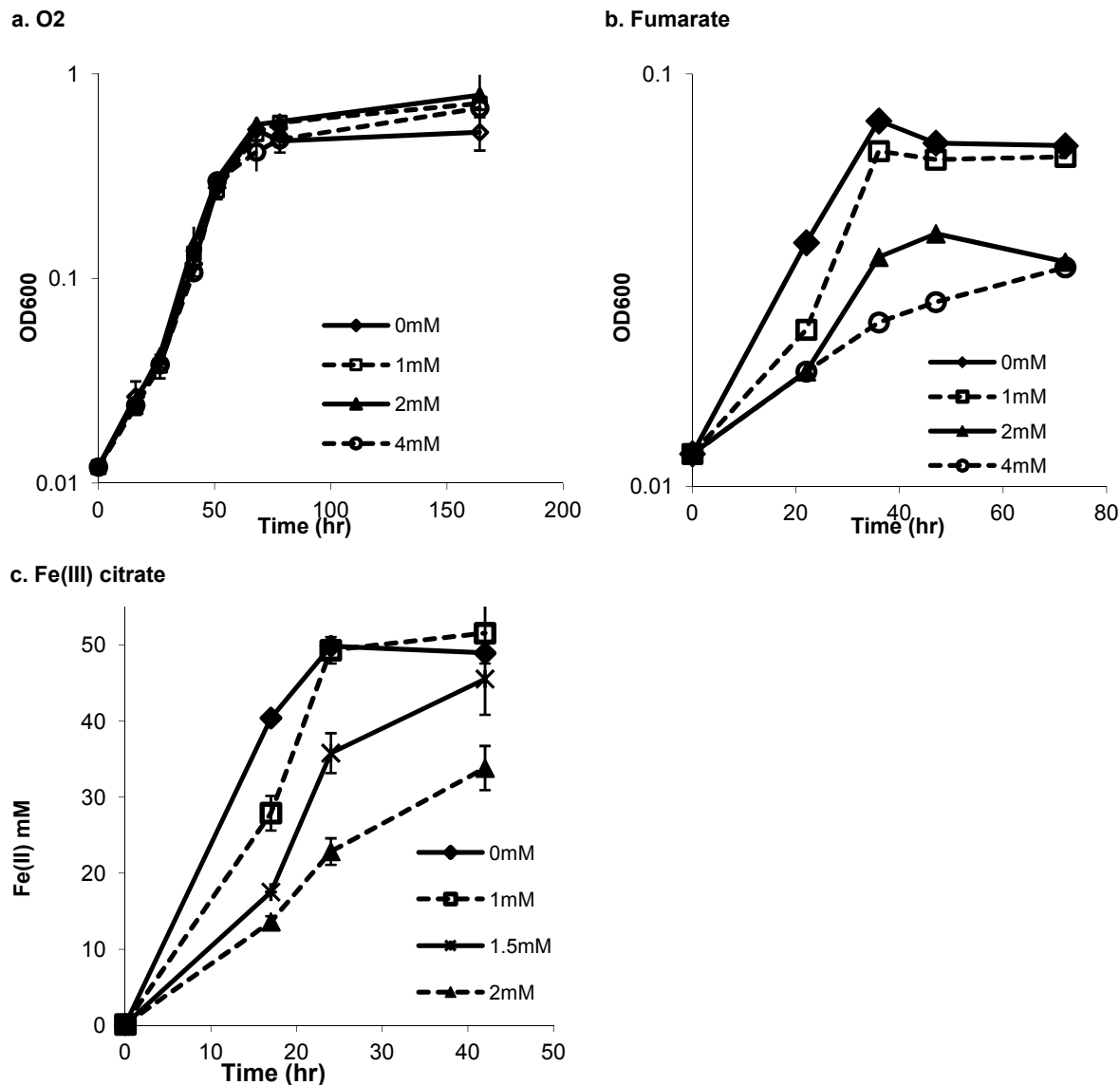
c. Fe(III) citrate

Figure 5.1. Growth of recombinant *S. oneidensis* strain +pBAD_BS2 with (a) O$_2$, (b) fumarate, and (c) Fe(III) citrate as electron acceptor and amended with 0-4 mM arabinose. Cell growth with O$_2$ and fumarate were monitored by OD600 measurements, while growth with Fe(III) was monitored by Fe(II) production.

fluorescence intensity was monitored during aerobic growth. Although the aerobic growth rates were unaffected by addition of different arabinose concentrations, higher arabinose oncentrations displayed stronger fluorescent intensities (Figure 5.2), thus demonstrating that FbFP expression is induced by arabinose.
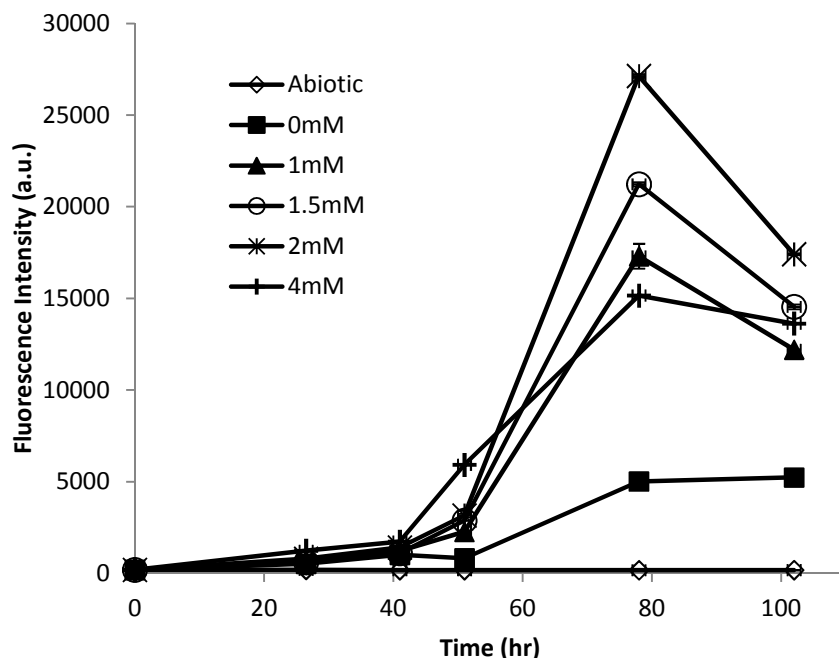
Figure 5.2. Bulk fluorescence emitted by cultures of recombinant strain *S. oneidensis*+pBAD_BS2 amended with 0-4 mM of arabinose.

Bulk fluorescent profiles

Bulk fluorescence emitted by FbFP-expressing *S.oneidensis*+pBAD_BS2 cell cultures with O_2 and fumarate electron acceptor increased during the different growth phases (Figure 5.3). Maximum fluorescence intensities were detected when the cell cultures reached late exponential growth phase. Higher fluorescent intensity was detected with cells grown on fumarate, although the cell density is much lower than O_2-grown cells. The bulk fluorescence intensity emitted by cells grown on different electron acceptors are not equivalent. The bulk fluorescence intensity of cells grown with Fe(III) citrate as electron acceptor were not detectable due to interference of the fluorescent signals by Fe(III) citrate.
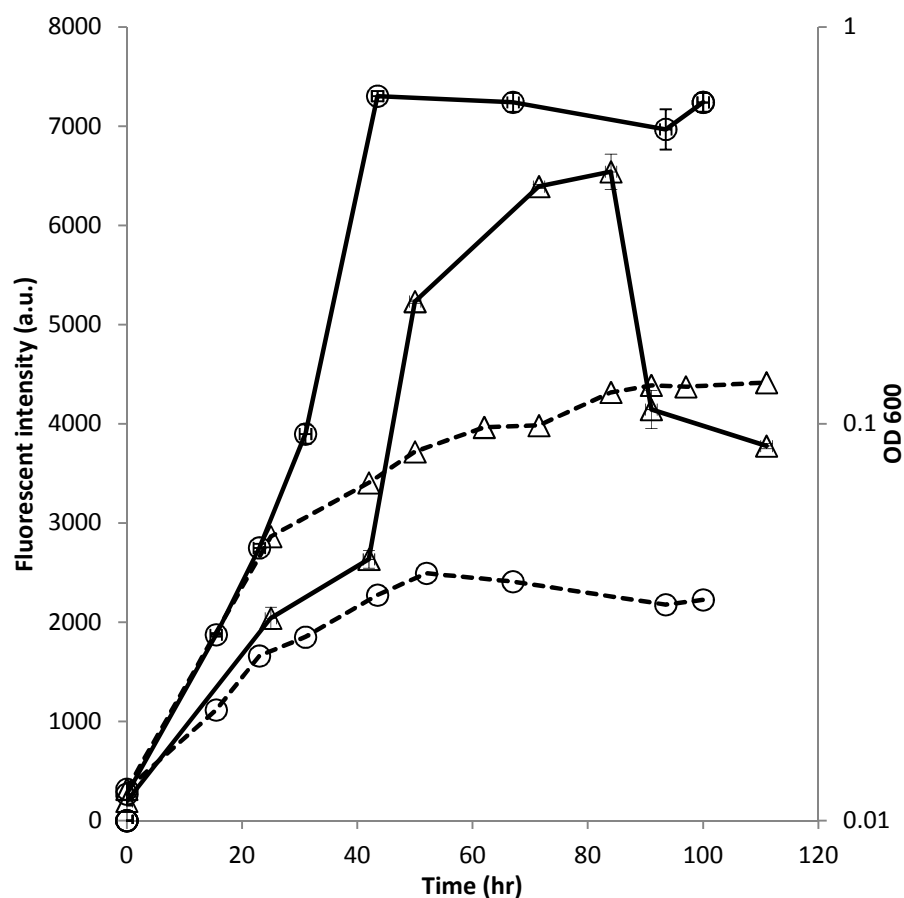
Figure 5.3. Bulk fluorescent profiles for *S. oneidensis*+pBAD_BS2 grown with O$_2$ (triangle) and fumarate (circle) as electron acceptor. Fluorescent intensities are shown in solid lines, while cell growth is shown in dashed lines.

Single cell fluorescence

S. oneidensis growth rates are electron acceptor-dependent, thus bulk fluorescence measurements only provide rough estimates of the fluorescence emitted from individual cells. To gain more insight into the differences in FbFP expression during *S. oneidensis* growth on various terminal electron acceptors, single cell fluorescence for cells grown on different electron acceptors was measured via laser confocal microscopy. Visualization inspection of single cell fluorescence indicated that wild-type *S.oneidensis* cells do not produce detectable fluorescence

under all conditions tested. *S. oneidensis* +pBAD_BS2 produced intense fluorescent signals

during growth on O_2, fumarate, Fe(III) citrate, and Fe(III) oxide, yet the fluorescent signal

intensities of single cells varied according to growth stage and electron acceptor. *S. oneidensis*

cells grown on O_2, fumarate, and Fe(III) oxide displayed the brightest fluorescent signals at late

exponential growth phase (Figure 5.4 a, b, and d), while cells grown on Fe(III) citrate displayed

the brightest fluorescent signal at mid-exponential growth phase (Figure 5.4 c).
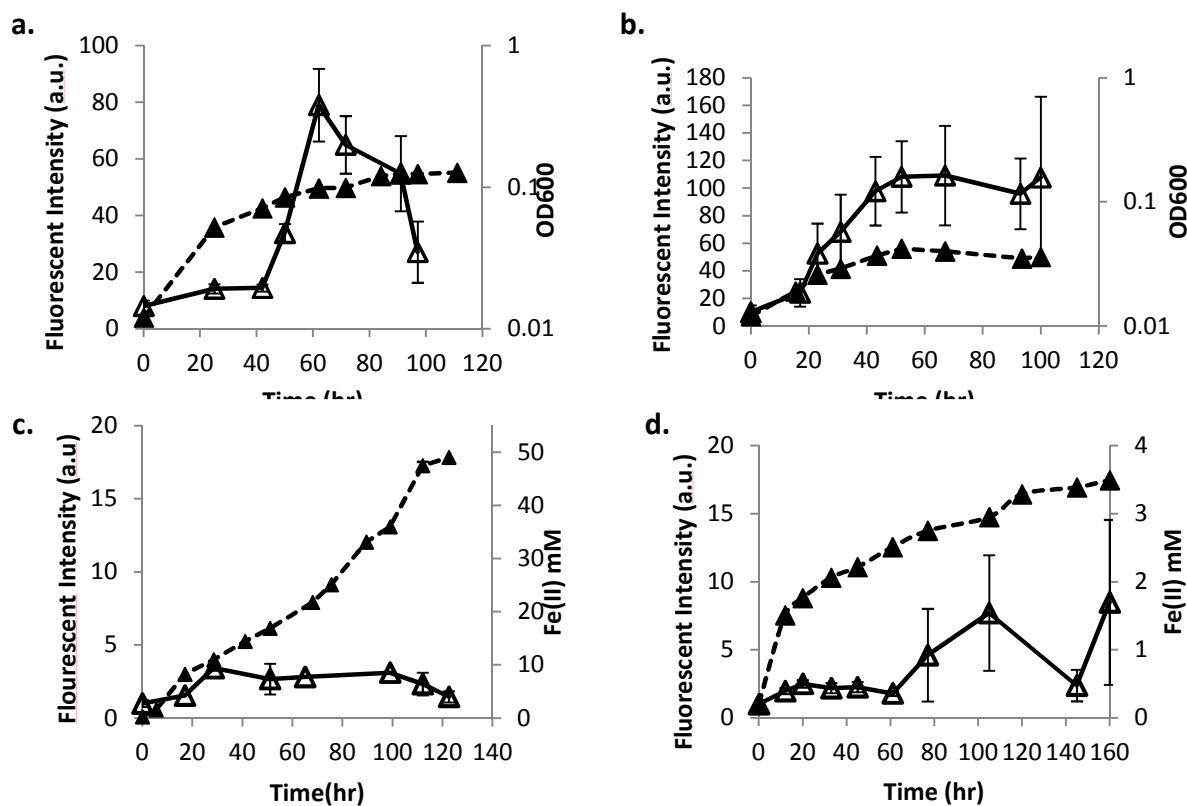
Figure 5.4. Single cell fluorescent intensities emitted from *S. oneidensis*+pBAD_BS2 cells grown with (a) O_2, (b) fumarate, (c) Fe(III) citrate, and (d) Fe(III) oxides. Single cell fluorescent intensities are shown in solid lines, cell growth is shown in dashed line. Error bars represent standard deviations of single cell intensities detected from 15-30 randomly chosen individual cells.

Maximum fluorescence intensity emitted by single cells grown with fumarate as electron

acceptor is slightly higher than cells grown with O_2 as electron acceptor. For cells grown with

Fe(III) citrate and Fe(III) oxide as electron acceptor, the maximum single cell fluorescence

intensities were at least 10-fold lower than the maximum single cell fluorescence intensities emitted by cells grown with O_2 and fumarate as electron acceptor. These results indicate that the FbFP fluorescent signals emitted by *S. oneidensis*+pBAD_BS2 cells is electron acceptor-dependent.
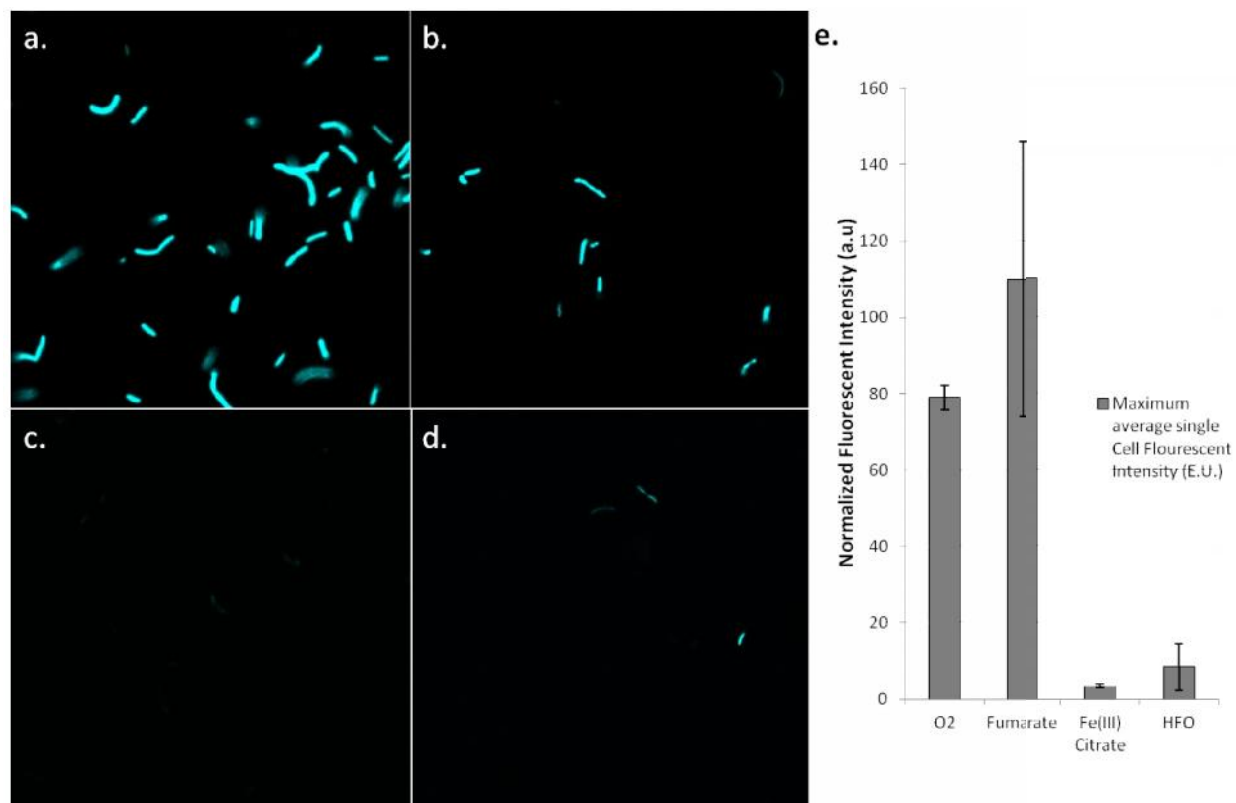
Figure 5.5. Laser confocal microscopy images showing *S. oneidensis*+pBAD_BS2 cells respiring on different electron acceptors. (a) O_2; (b) fumarate; (c) Fe(III) citrate; (d) Fe(III) oxide. FbFP was expressed on pBAD202 expression vector induced by 2 mM arabinose. (e) normalized average fluorescent intensity emitted by 15-30 single cells grown with various electron accetors.

Intracellular flavin

Flavins play an important role during anaerobic Fe(III) oxide respiration by *S. oneidensis* [27]. Although the exact role of flavins is a subject of an ongoing controversy, *S.oneidensis* is

postulated to secrete flavins to function as electron shuttles to external Fe(III) oxides [28]. Alternately, flavins may function as cofactors for the Fe(III) oxide-reducing outer membrane *c*-type cytochrome MtrC. To determine if differences in fluorescence intensities emitted by individual *S. oneidensis* cells grown on different electron acceptors is due to the concentration of intracellular flavin, the intracellular flavin content of both wild type and *S. oneidensis*+pBAD_BS2 cells was determined during growth with O_2, fumarate, and Fe(III) citrate as terminal electron acceptor. Such analyses with Fe(III) oxide-grown cells was not possible due to the inability to separate the *S. oneidensis* cells from the insoluble Fe(III) oxide particles. For cells grown on O_2, fumarate, and Fe(III) citrate, intracellular flavin concentrations correlated with single cell fluorescent signals (Figure 5.6).

Since FbFP employs flavin as cofactor, the increases in single cell fluorescent intensities may be attributed to increased intracellular flavin levels. Wild-type *S. oneidensis* cells (i.e., lacking pBAD_BS2) grown on O_2, fumarate, and Fe(III) citrate contained 2.05 µmol, 1.93 µmol, and 2.88 µmol of flavin per gram protein, respectively (Figure 5.7). Wild-type cells grown on Fe(III) citrate thus contain approximately 40-50% more intracellular flavin than the O_2- and fumarate-grown cells. Surprisingly, the intracellular flavin concentrations in *S. oneidensis*+pBAD_BS2 cells was approximately 10-fold greater than that of the *S. oneidensis* wild-type cells (Figure 5.7). The intracellular concentration of flavins in *S. oneidensis*+pBAD_BS2 cells grown on O_2 and fumarate were nearly identical (22.2 µmol and 22.8 µmol per gram protein, respectively), while *S. oneidensis*+pBAD_BS2 cells grown with

a. O2
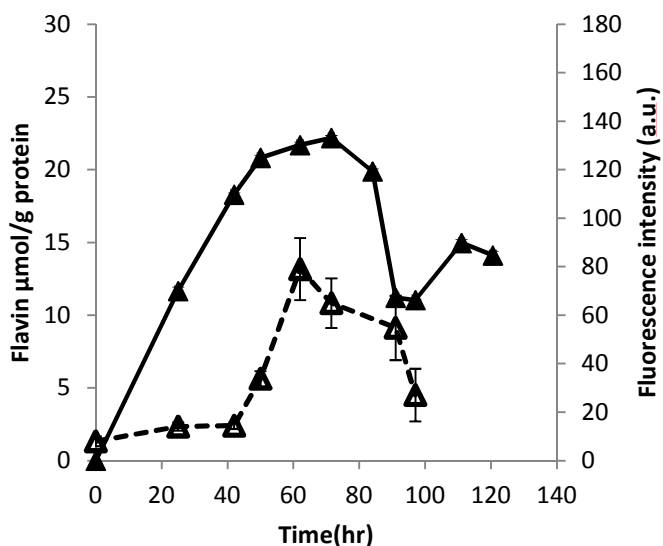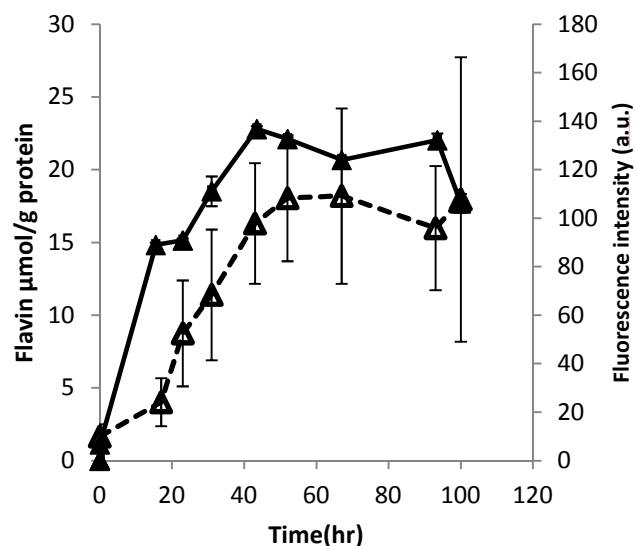
b. Fumarate
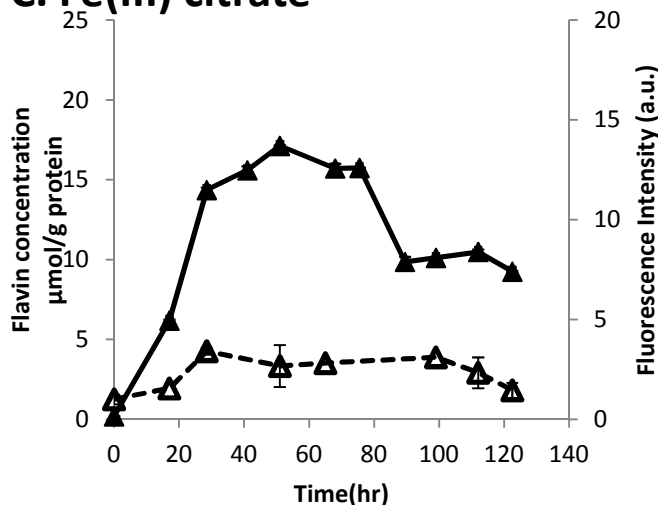
C. Fe(III) citrate

Figure 5.6. Intracelluar flavin and single cell fluorescent signal intensities for *S. oneidensis*+pBAD_BS2 cells grown with (a) O₂, (b) fumarate, and (c) Fe(III) citrate as electron acceptor. Flavin profiles are given as solid lines, while single cell fluorescent signal intensities are given as dashed lines.

Fe(III) citrate displayed 23% lower flavin concentration (17.1 µmol per gram protein) than the

O₂- and fumarate-grown *S. oneidensis*+pBAD_BS2 cells. Reasons for the differences in

intracellular flavin concentrations measured directly in *S. oneidensis* wild-type and *S. oneidensis* +pBAD_BS2 cells are currently under investigation.
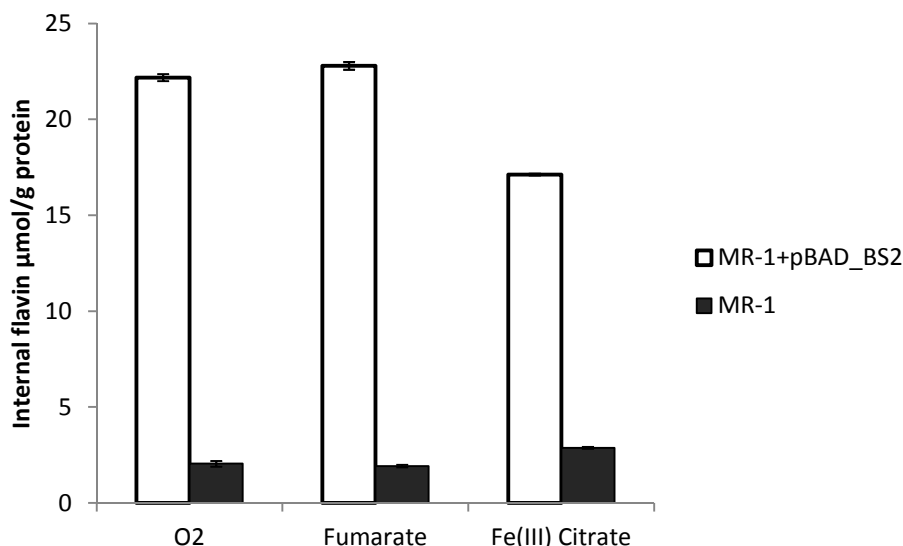
Figure 5.7. Comparison of maximum intracellular flavin concentrations in *S. oneidensis* wild-type and *S. oneidensis*+pBAD_BS2 cells grown on O_2, fumarate, and Fe(III) citrate.

In summary, applications of GFP to examine molecular events in single microbial cells are limited by the oxygen-dependent autocatalytic maturation of the GFP chromophore. GFP applications are thus restricted to aerobic microorganisms and are not suitable for in situ studies of molecular events in anaerobic microorganisms. A novel group of FbFPs, have been developed as replacements for GFP. FbFPs do not require oxygen for chromophore maturation, and can thus be applied under both aerobic and anaerobic conditions to monitor molecular events in single microbial cells. FbFPs require FMN as cofactor, which also suggests that FbFP fluorescence may be used as an in vivo reporter of internal FMN concentrations. The FbFP reporter system constructed in Chapter 5 provided a novel technology for monitoring internal FMN concentrations in single *S. oneidensis* cells during anaerobic growth on an array of terminal electron acceptors, including O_2, fumarate, and Fe(III).

Acknowledgements

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CHAPTER 6

CONCLUSION

S. oneidensis reduced a suite of naturally occurring disulfide compounds commonly found in marine and freshwater environments, including cystine, oxidized glutathione, dithiodiglycolate, dithiodipropionate, cystamine, and dimethyldisulfide to their corresponding thiol forms. Addition of the disulfide compounds to anaerobic *S. oneidensis* cultures greatly accelerated the rate and extent of Fe(III) oxide reduction by *S. oneidensis*. The results of Chapter 2 indicate that thiol-based electron shuttling pathways provide *S. oneidensis* with a more efficient pathway for electron transfer to external Fe(III) oxides during anaerobic Fe(III) oxide respiration. Higher disulfide concentrations may convert the disulfide electron shuttling pathway to an anaerobic respiratory pathway with disulfides as terminal electron acceptor, as opposed to an electron shuttling pathway with Fe(III) oxides as terminal electron acceptor. Thiols can also be absorbed to Fe(III) oxide surface and act as ligands in promoting non-reductive dissolution of Fe(III) oxide. To examine these possibilities, future work will compare thiol accumulation rates as a function of increasing disulfide concentrations in the presence and absence of Fe(III) oxides.

Application of a newly developed disulfide reduction mutant screening technique to random chemical mutants resulted in identification of two respiratory mutants that both mutants contained a point mutation in the gene encoding the outer membrane beta-barrel protein MtrB, which is a central component in the extracellular electron pathway terminating with the reduction of Fe(III), Mn(III), and Mn(IV). The disulfide reduction deficiencies displayed by the disulfide reduction-deficient mutants correlated with their Fe(III) reduction deficiencies. The results of Chapter 3 indicate that disulfide reduction by *S. oneidensis* is catalyzed by the Fe(III)-, Mn(III)-,

and Mn(IV)-reducing Mtr pathway. The results of Chapter 4 indicate that MtrB homologs containing a N-terminal CXXC motif represent a molecular signature unique to metal-reducing members of the *Gammaproteobacteria* with the potential for further development as a biomarker for tracking the presence and activity of metal-reducing γ-*proteobacteria* in natural and engineered systems.

Mtr deletion mutants display impaired disulfide reduction activities. A previously contructed set of mtr deletion mutants *(ΔmtrB, ΔmtrA, ΔmtrC, ΔomcA* and *ΔmtrC-ΔomcA* double mutant) were subsequently tested for disulfide and Fe(III) oxide reduction activities. Interestingly, the Fe(III) oxide reduction deficiencies displayed by the mtr mutants correlated with their disulfide reduction deficiencies. Furthermore, *S. oneidensis* mutants lacking the MtrC and MtrA components of the extracellular electron conduit also displayed disulfide reduction-deficient phenotypes. These results indicate that the disulfide and metal reduction pathways of *S. oneidensis* share the extracellular electron conduit MtrCAB as a common electron transport chain component. The extracellular electron conduit MtrCAB thus appears to be multi-functional, displaying the ability to transfer electrons to extracellular Fe(III) citrate , Fe(III) oxide , flavins , and disulfides . In this manner, *S. oneidensis* may utilize a single terminal reductase complex (MtrCAB) to transfer electrons to multiple external electron acceptors. Alternately, the findings of the present study suggest that Fe(III) reduction is the last step of a multi-step electron shuttling pathway in which *S. oneidensis* secretes disulfides to the cell surface where MtrCAB-catalyzed disulfide reduction reactions produce the corresponding thiols that subsequently reduce oxidized flavin abiotically to their reduced flavin forms, which finally reduce Fe(III) oxide abiotically in the terminal Fe(III) oxide reduction step. Current work is focused on testing the hypothesis that microbial Fe(III) respiration is catalyzed by the multi-

step electron shuttling pathway which begins with enzymatic disulfide reduction at the cell surface and terminates with abiotic Fe(III) oxide reduction outside the cell.

A novel group of flavin mononucleotide (FMN)-based fluorescent proteins (FbFPs), have been developed as replacements for GFP. FbFPs can be applied under both aerobic and anaerobic conditions to monitor molecular events in single microbial cells. The FbFP reporter system constructed in Chapter 5 provided a novel technology for in vivo monitoring of internal FMN concentrations in single *S. oneidensis* cells during anaerobic growth on an array of terminal electron acceptors, including O_2, fumarate, and Fe(III). Limited brightness of FbFPs is still a major setback for its application as GFP replacements. Additional genetic modifications of the FbFP system will be required to further enhance the brightness of the FbFPs expressed under anaerobic conditions, possibly by enhancing FMN cofactor binding efficiency.

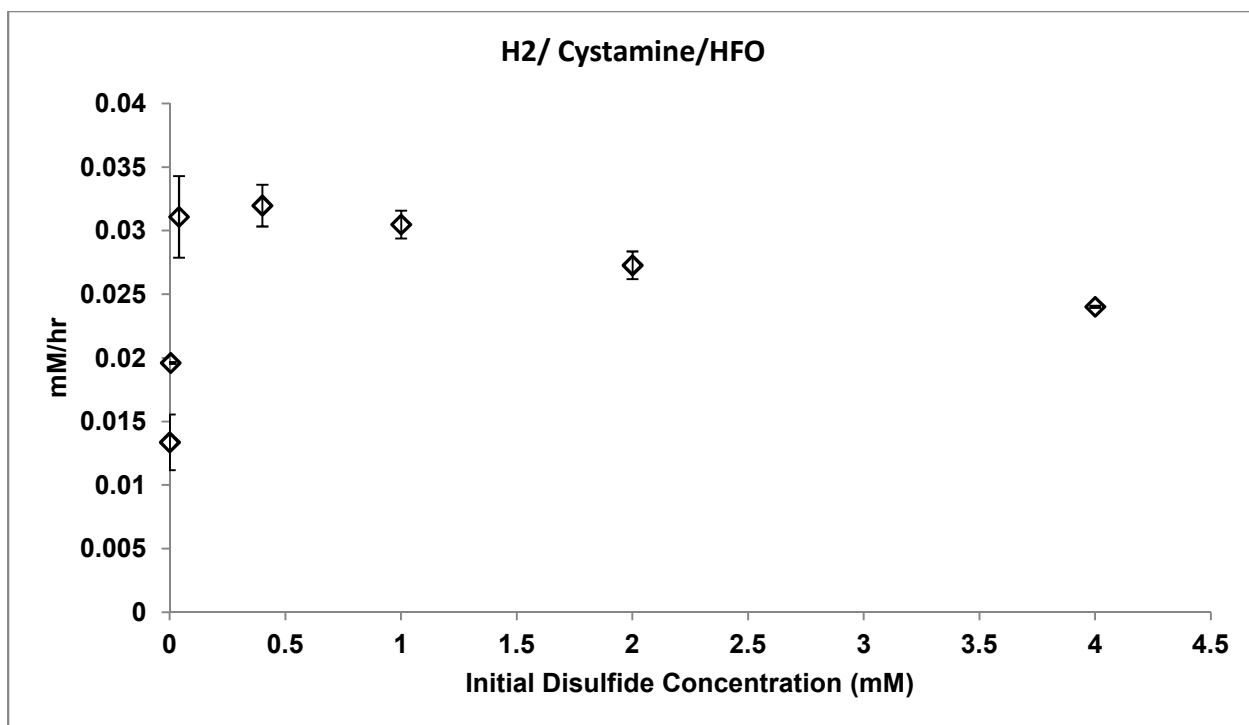**Michaelis-Menten plots used to calculate *V*max-Di and Km-Di for each electron donor/disulfide pair summarized in Table 2.1.**

Formate/CSSC

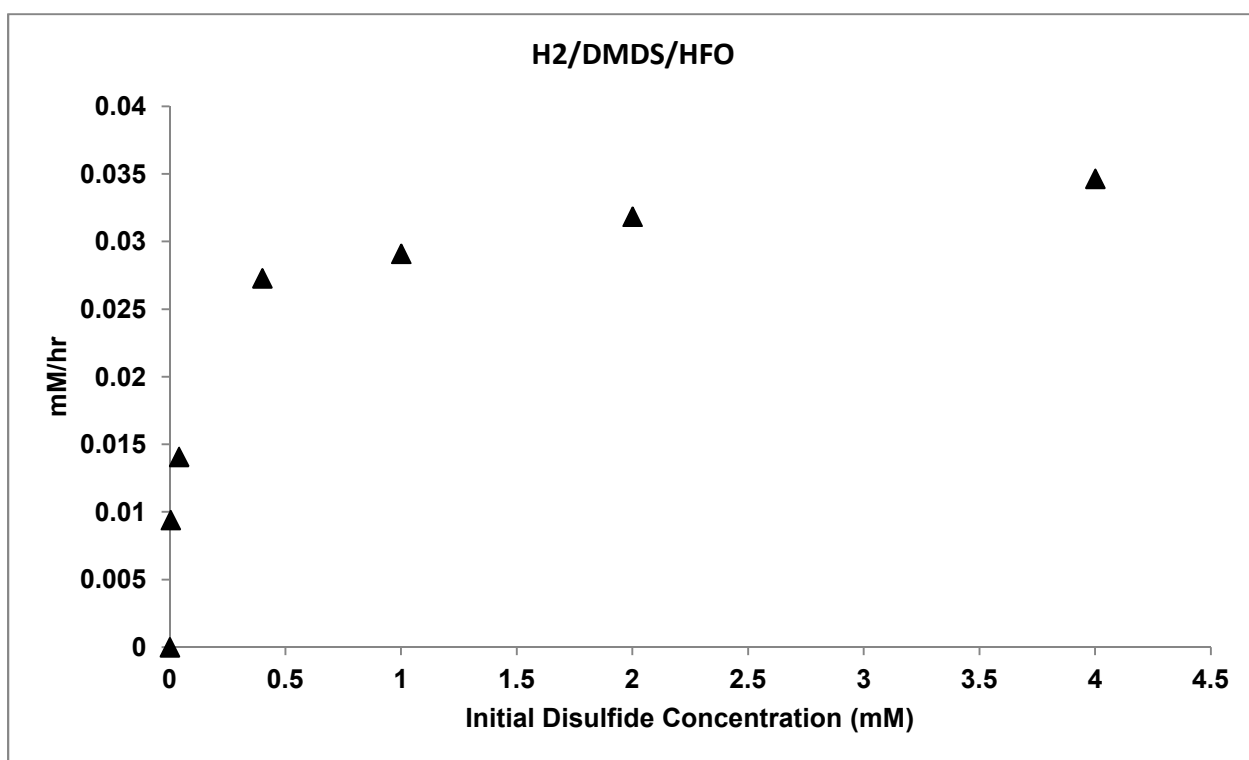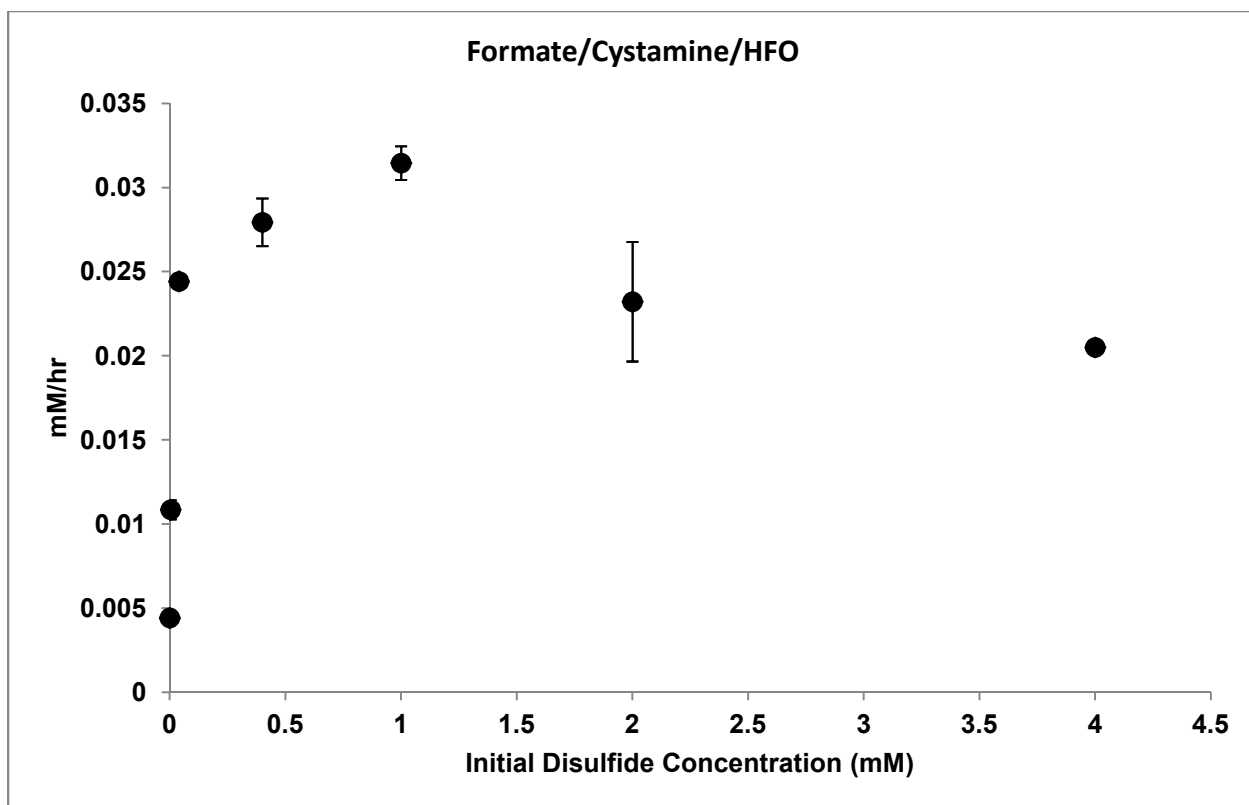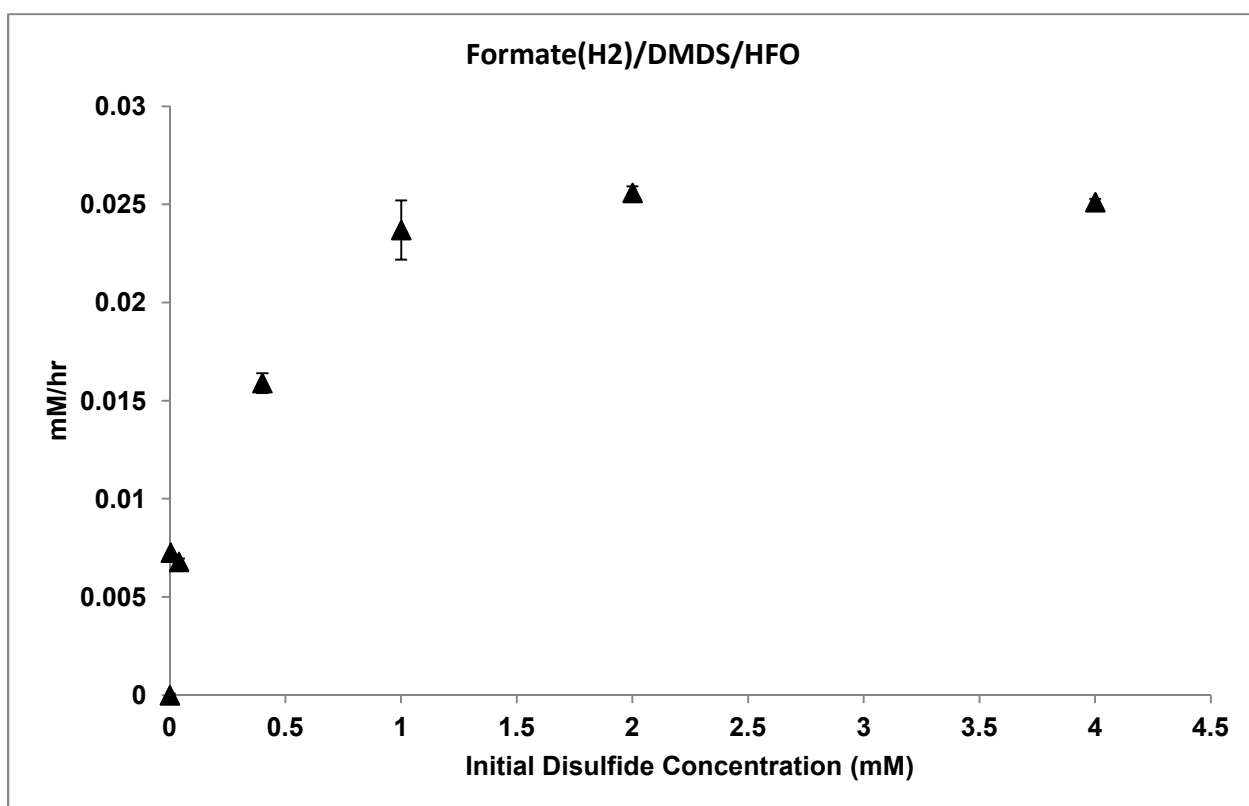**H2/DTDP**

Lactate/DTDP

Formate/DTDP

H2/Cystamine

Lactate/Cystamine

Formate/Cystamine

H2/DMDS

Lactate/GSSG

Formate/GSSG

Michaelis-Menten plots used to calculate *V*max-Fe and Km-Fe foreach electron donor/disulfide pair summarized in Table2.2.
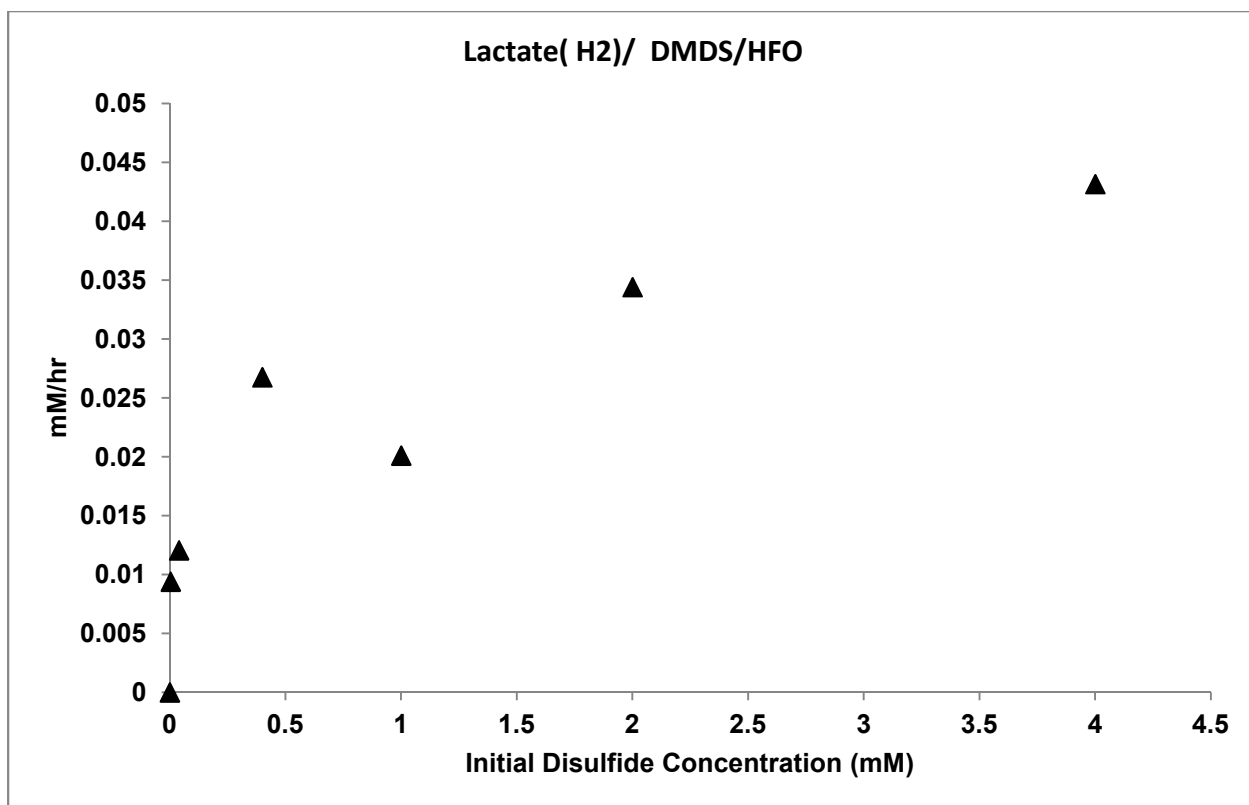
Formate /CSSC /HFO

H2 /DTDP/HFO

Lactate/DTDP/ HFO

Formate/DTDP/HFO

H2/ Cystamine/HFO

Lactate/Cystamine/HFO

Formate/Cystamine/HFO

H2/DMDS/HFO

Lactate(H2)/ DMDS/HFO

mM/hr vs Initial Disulfide Concentration (mM)

Formate(H2)/DMDS/HFO

mM/hr vs Initial Disulfide Concentration (mM)

H2 /DTDG/HFO DTDG

mM/hr

Initial disulfide concentration (mM)

Lactate/ DTDG /HFO

mM/hr

Initial Disulfide Concentration (mM)

Formate/DTDG/HFO

H2/GSSG/HFO
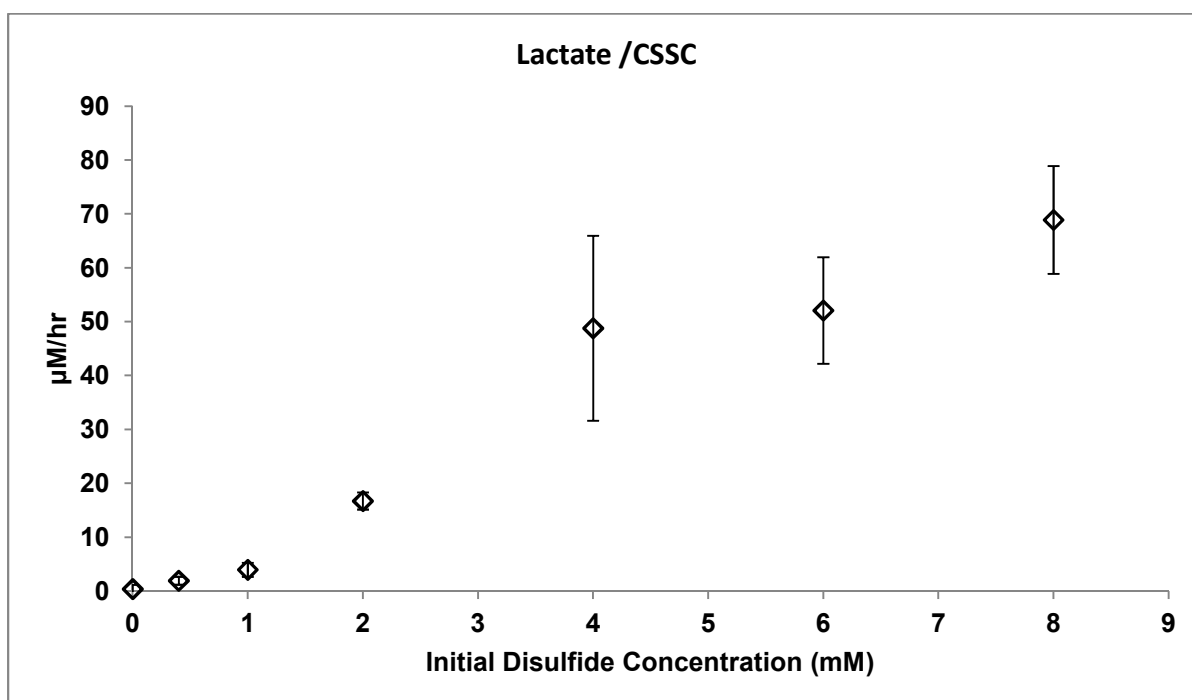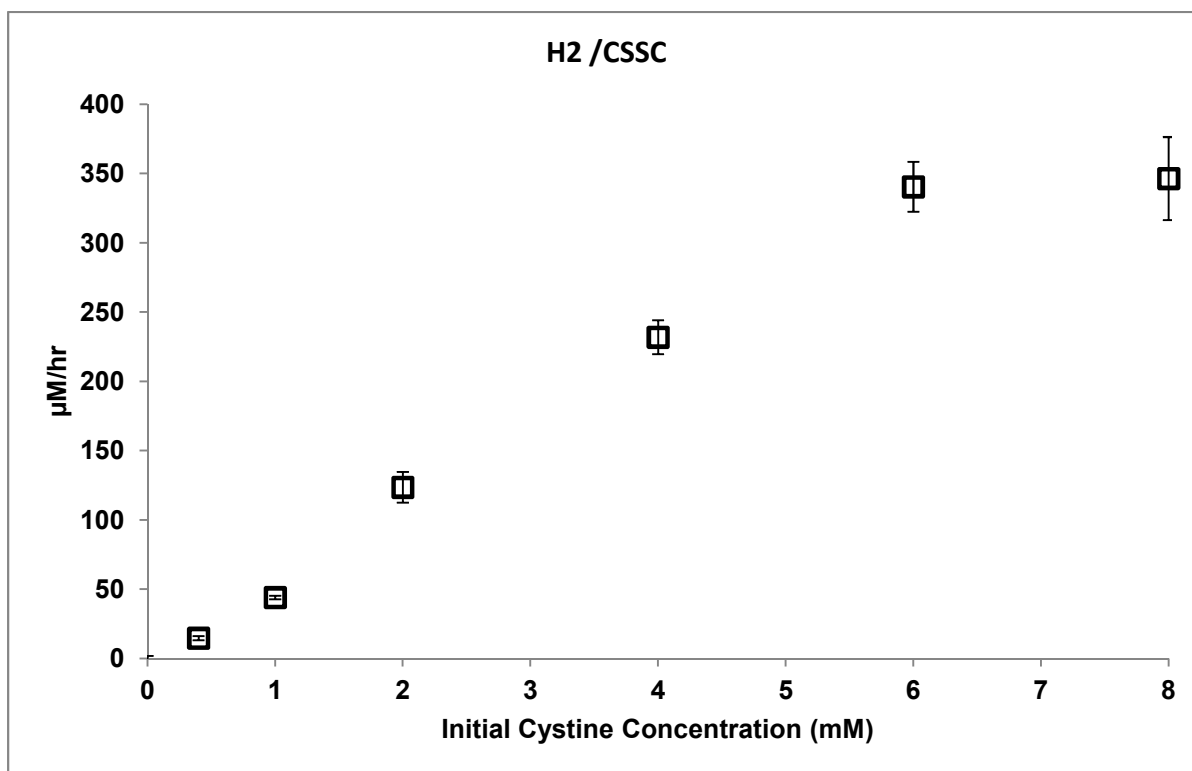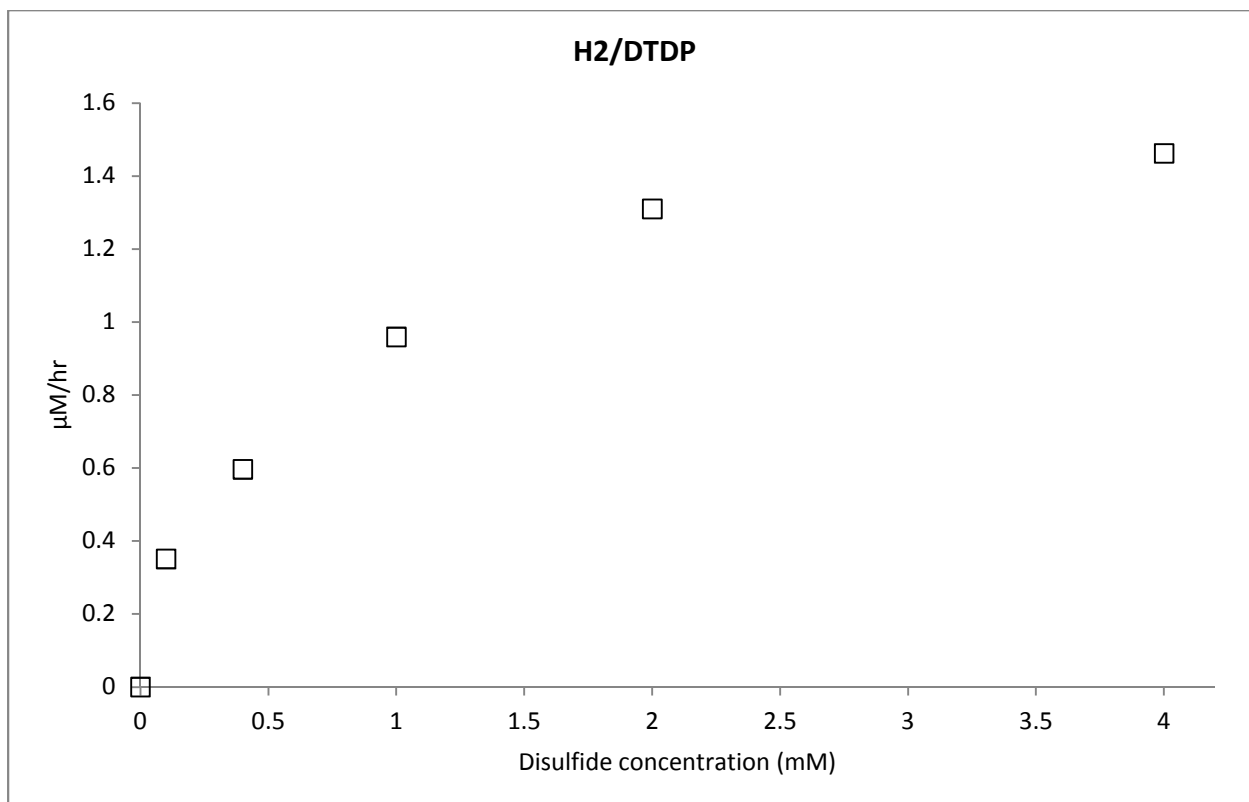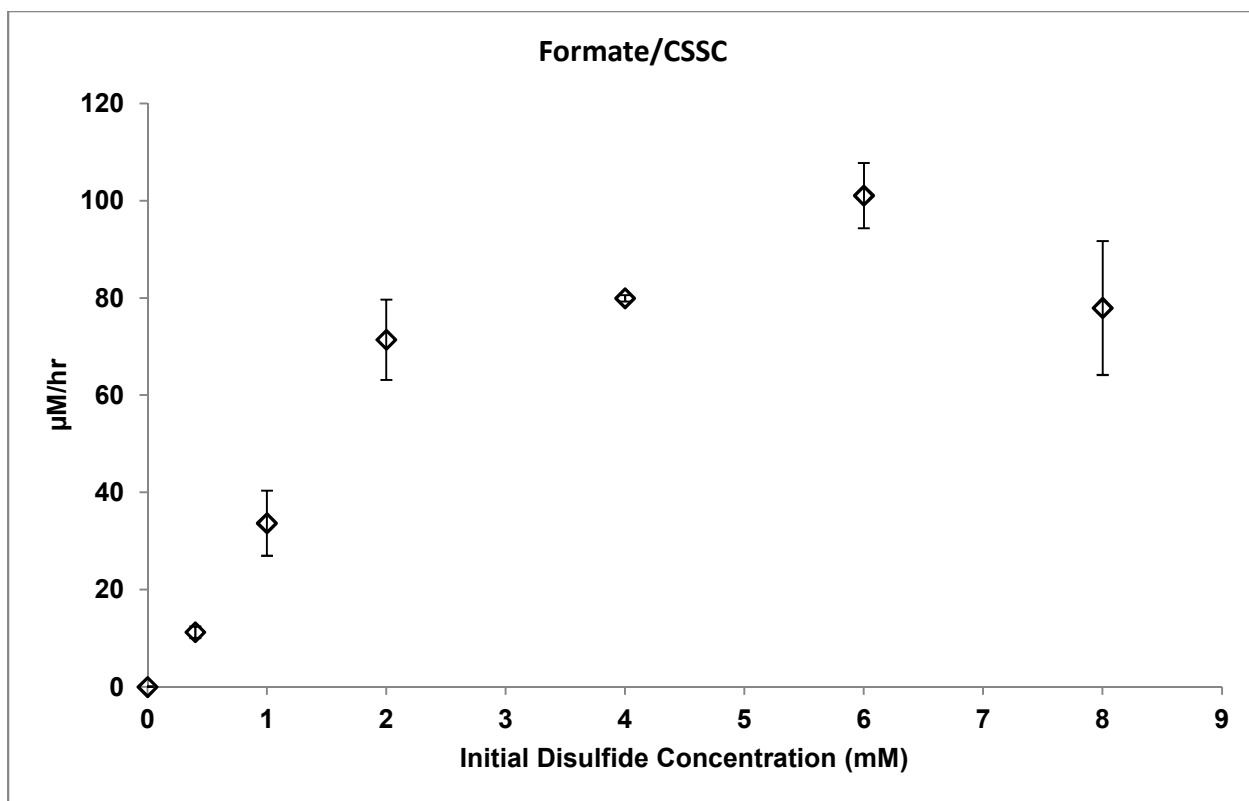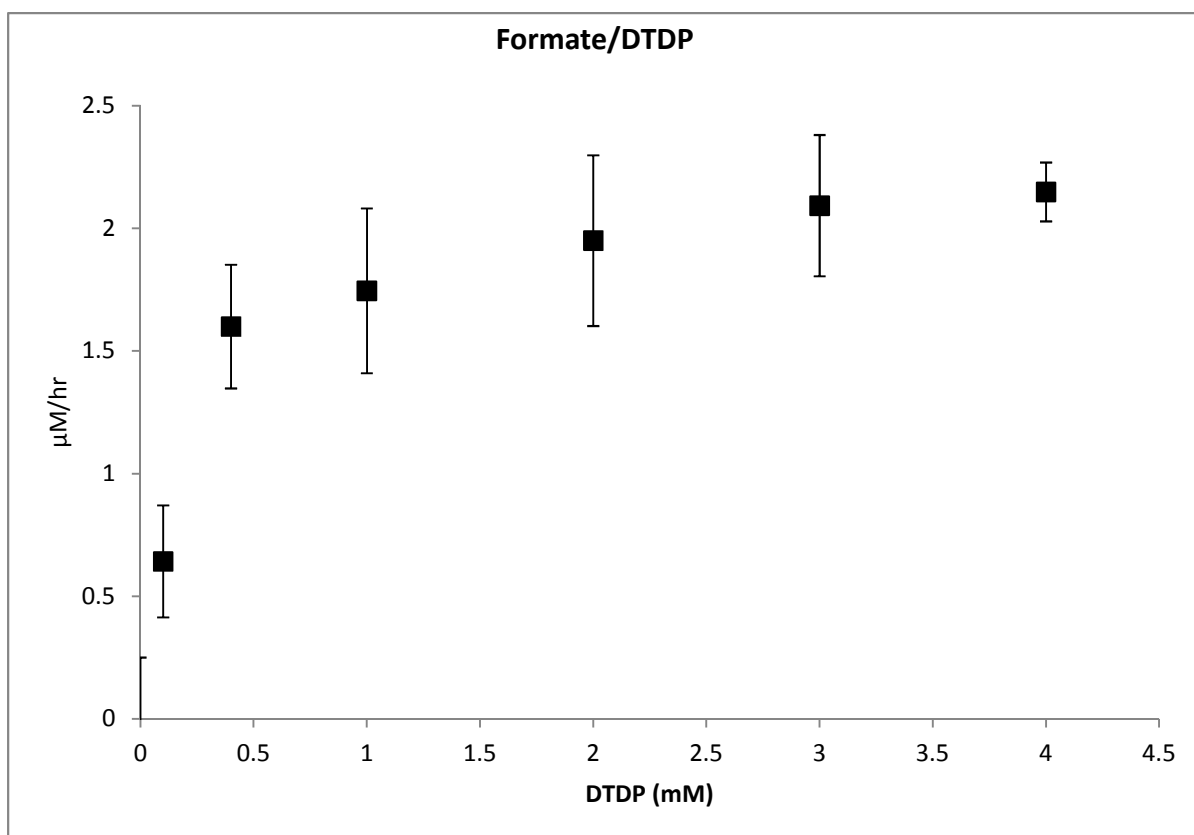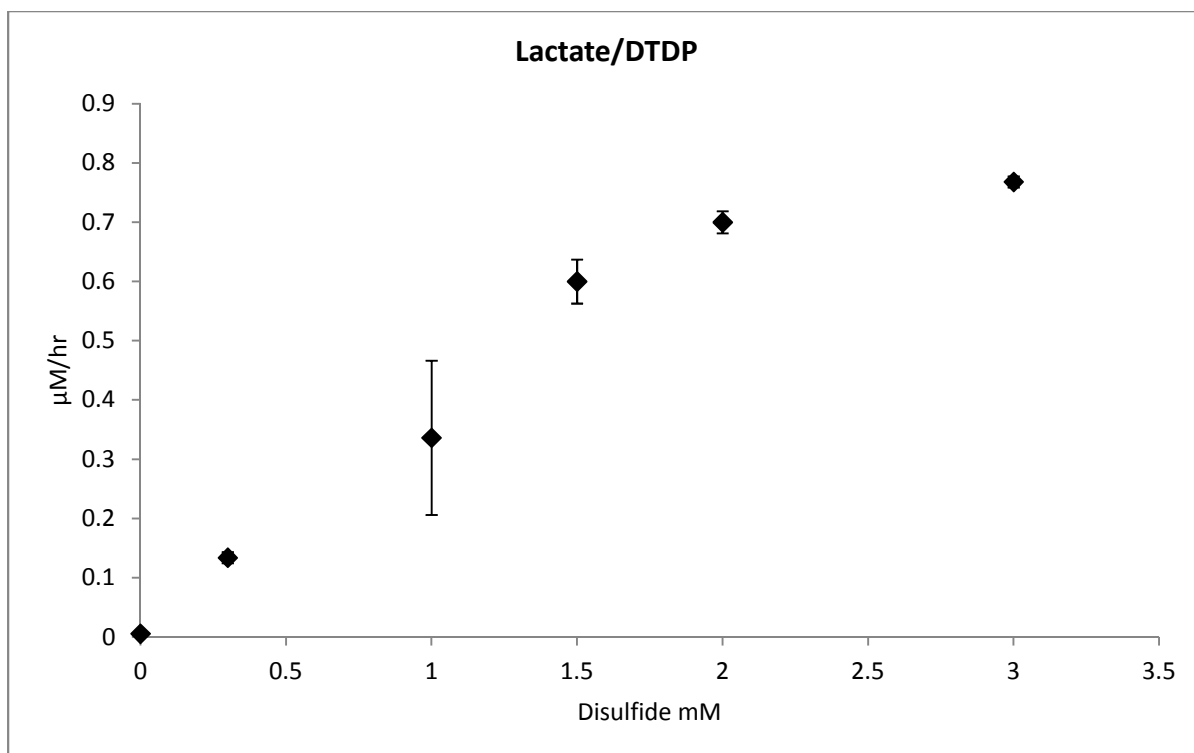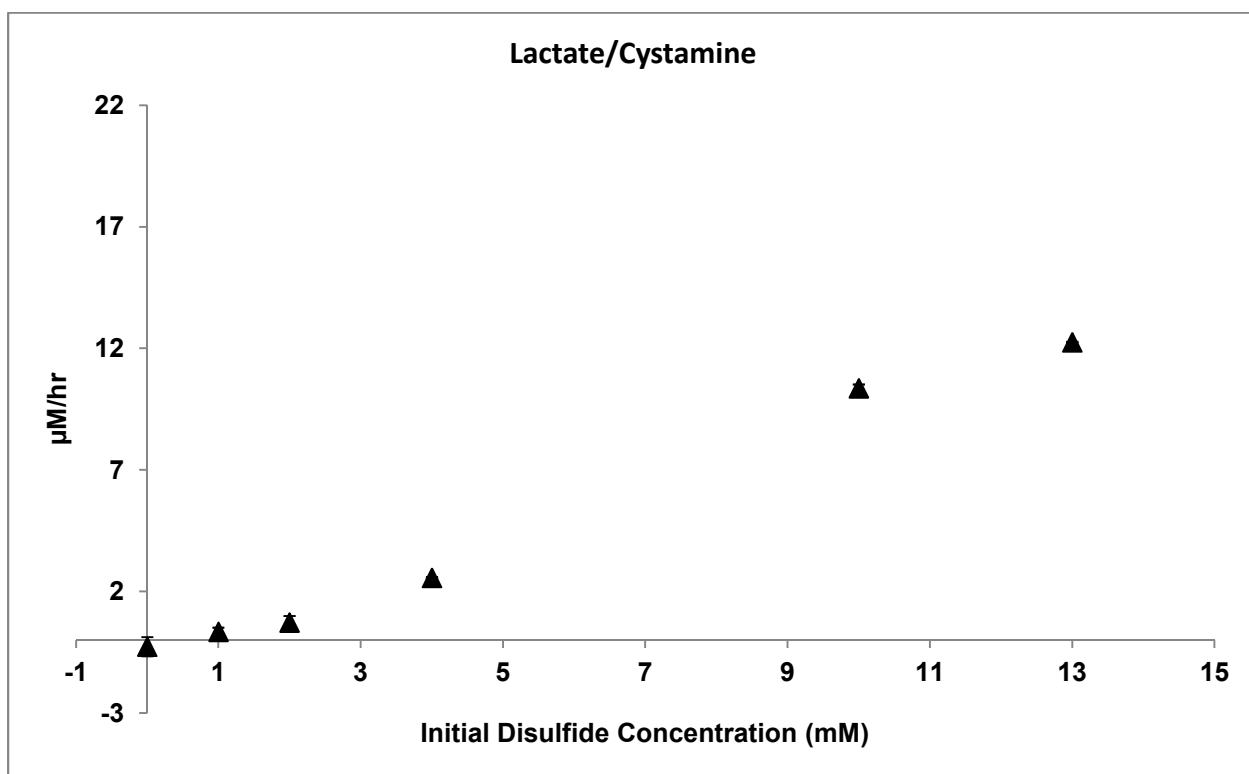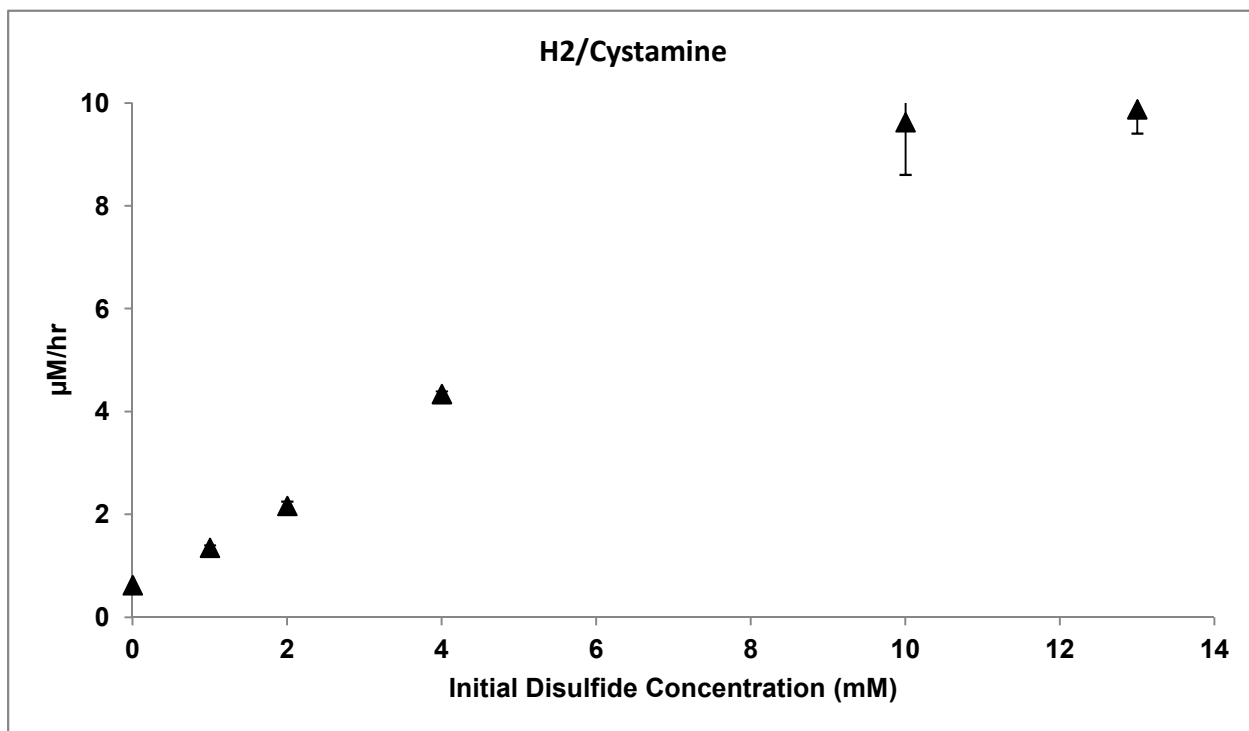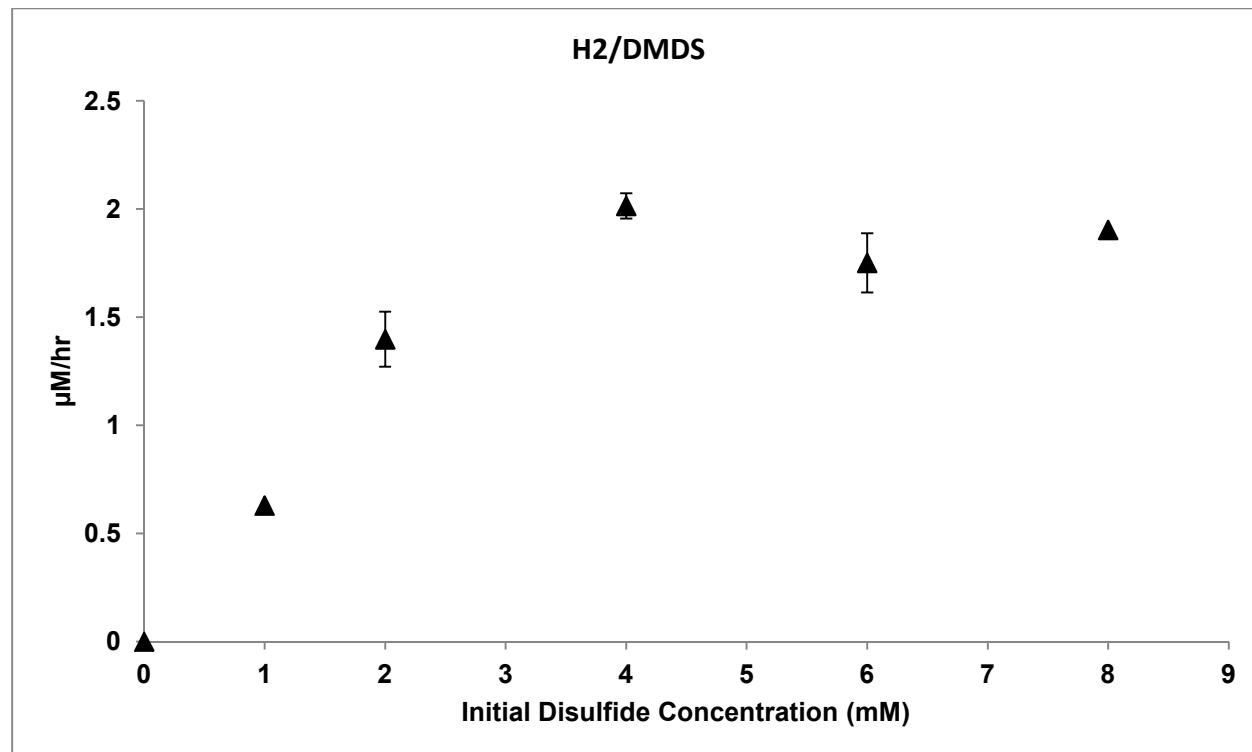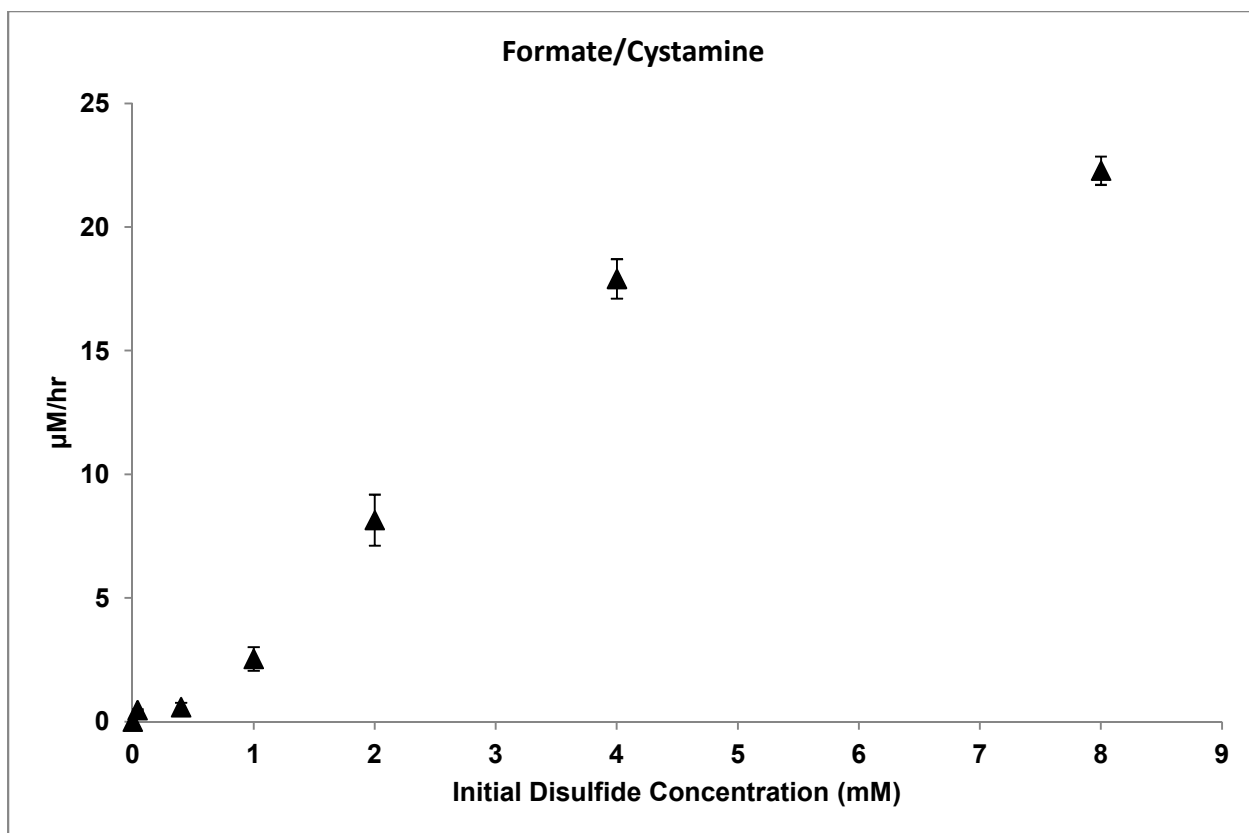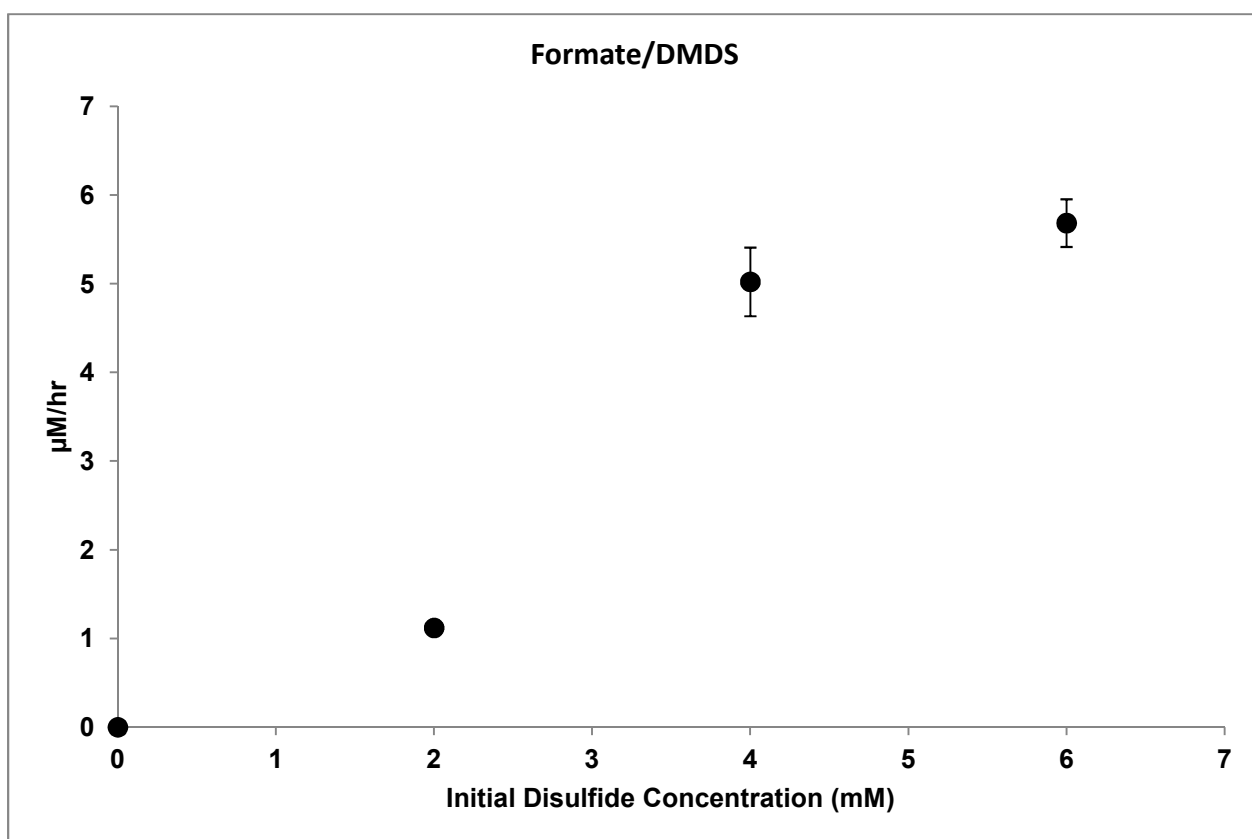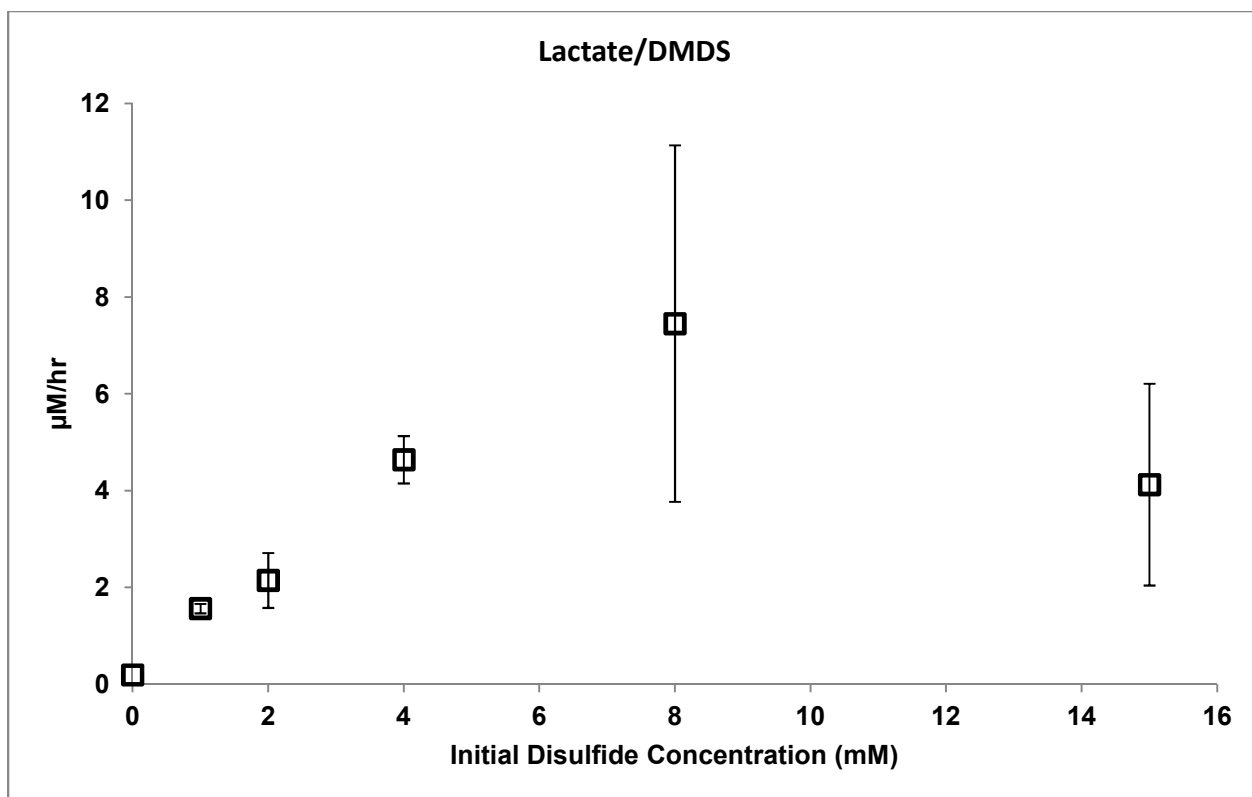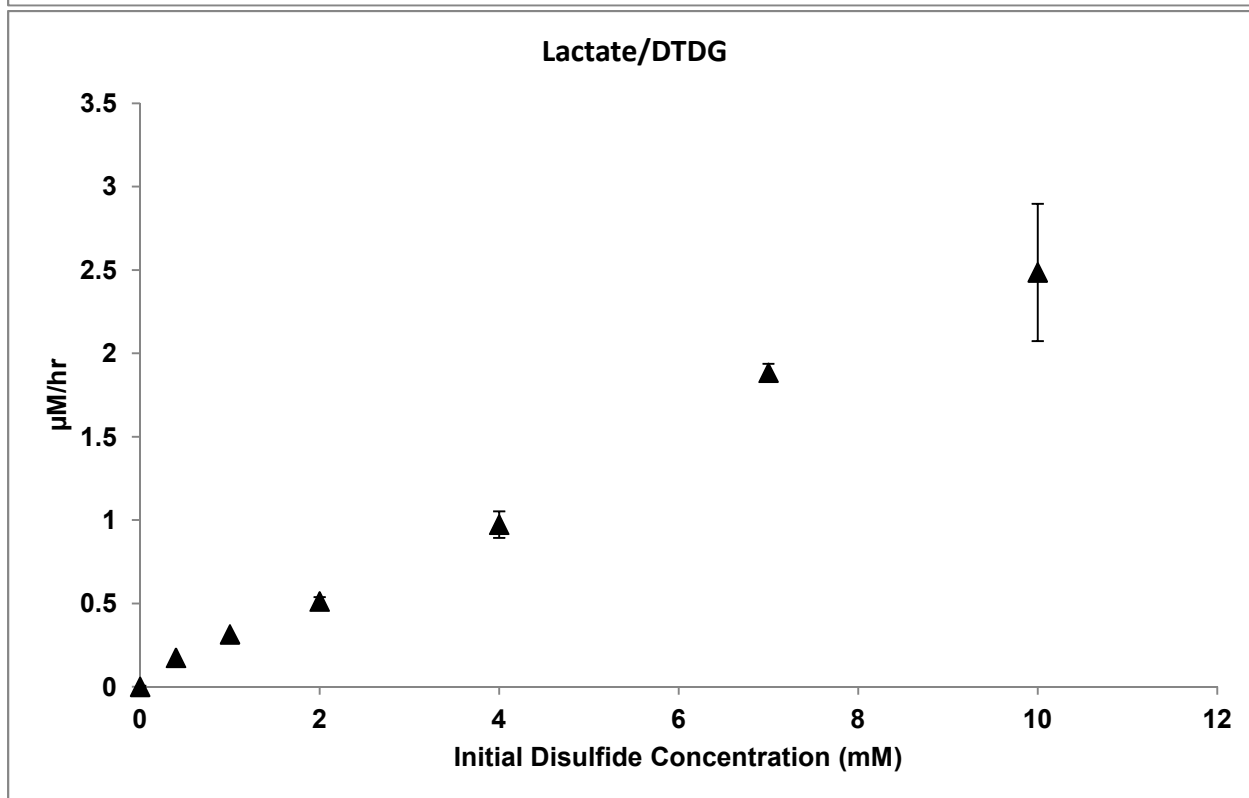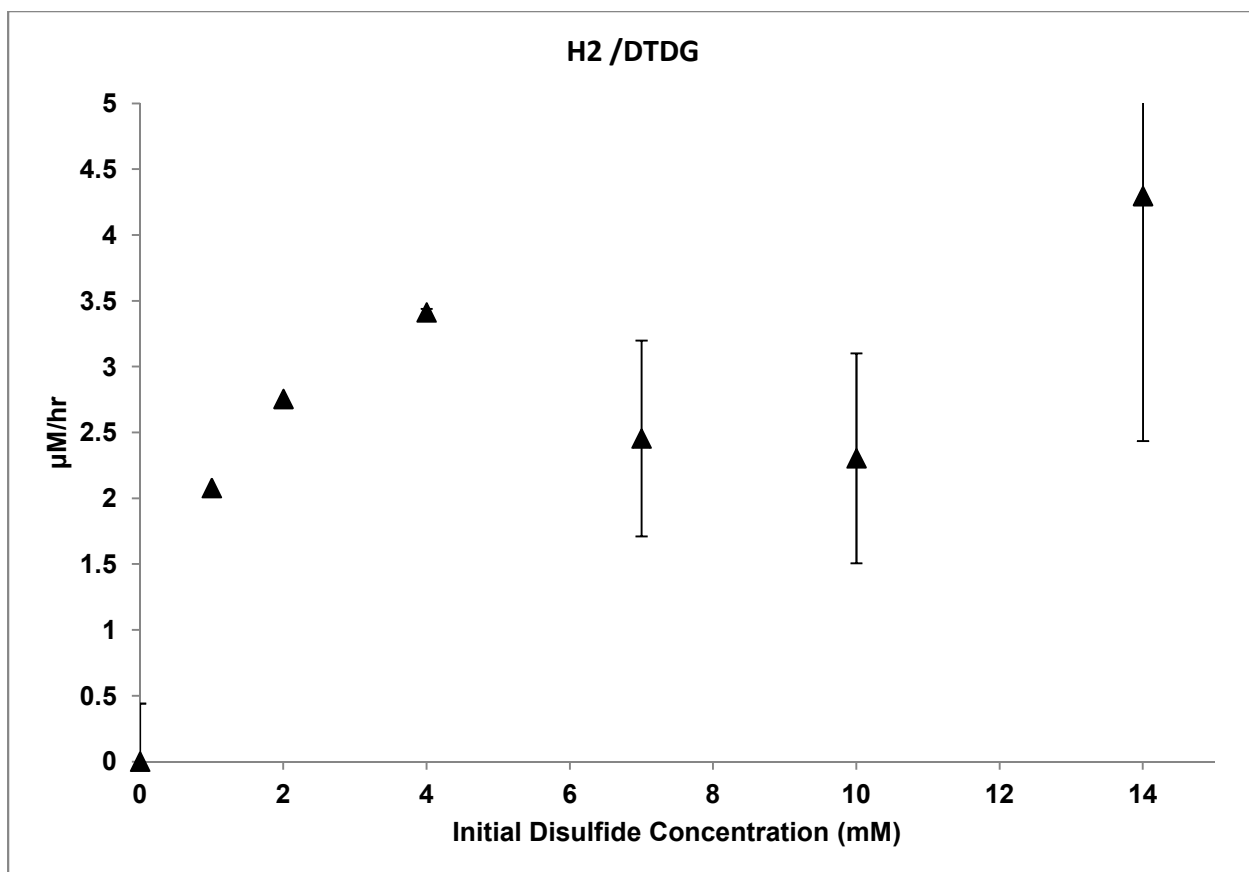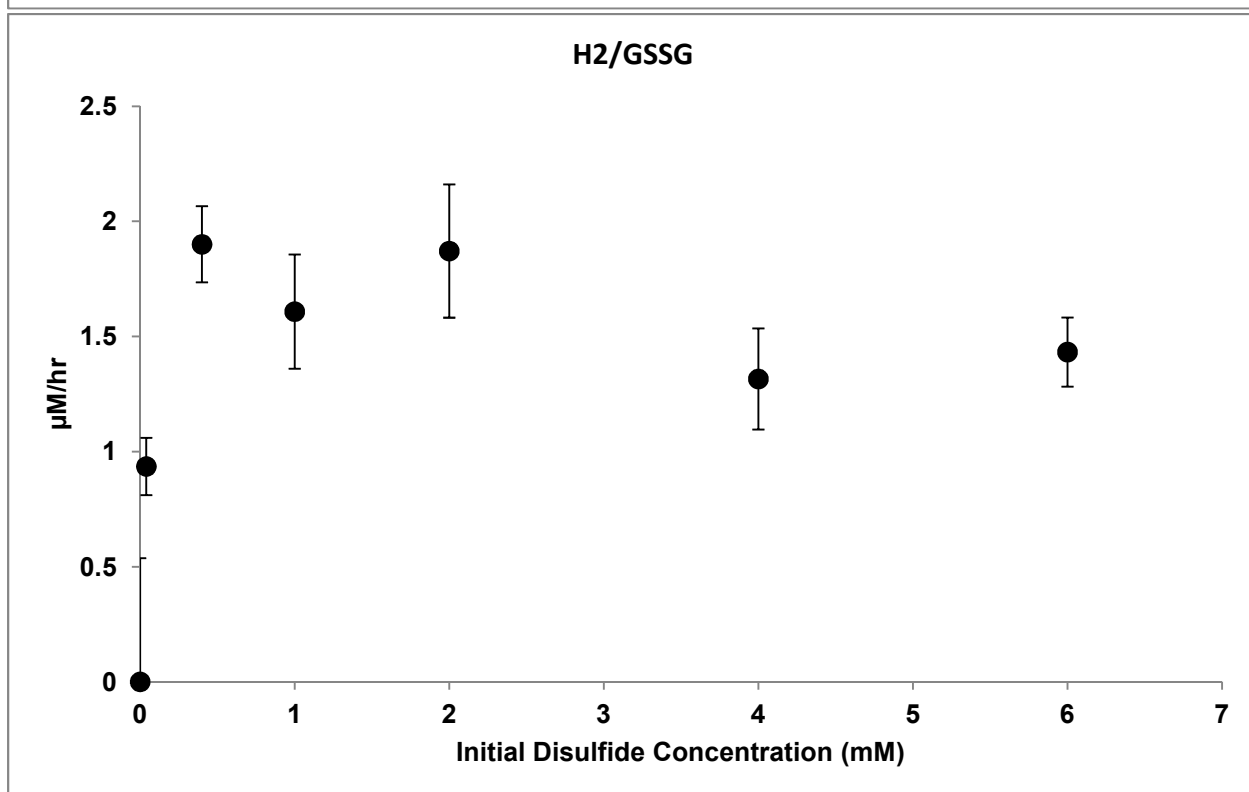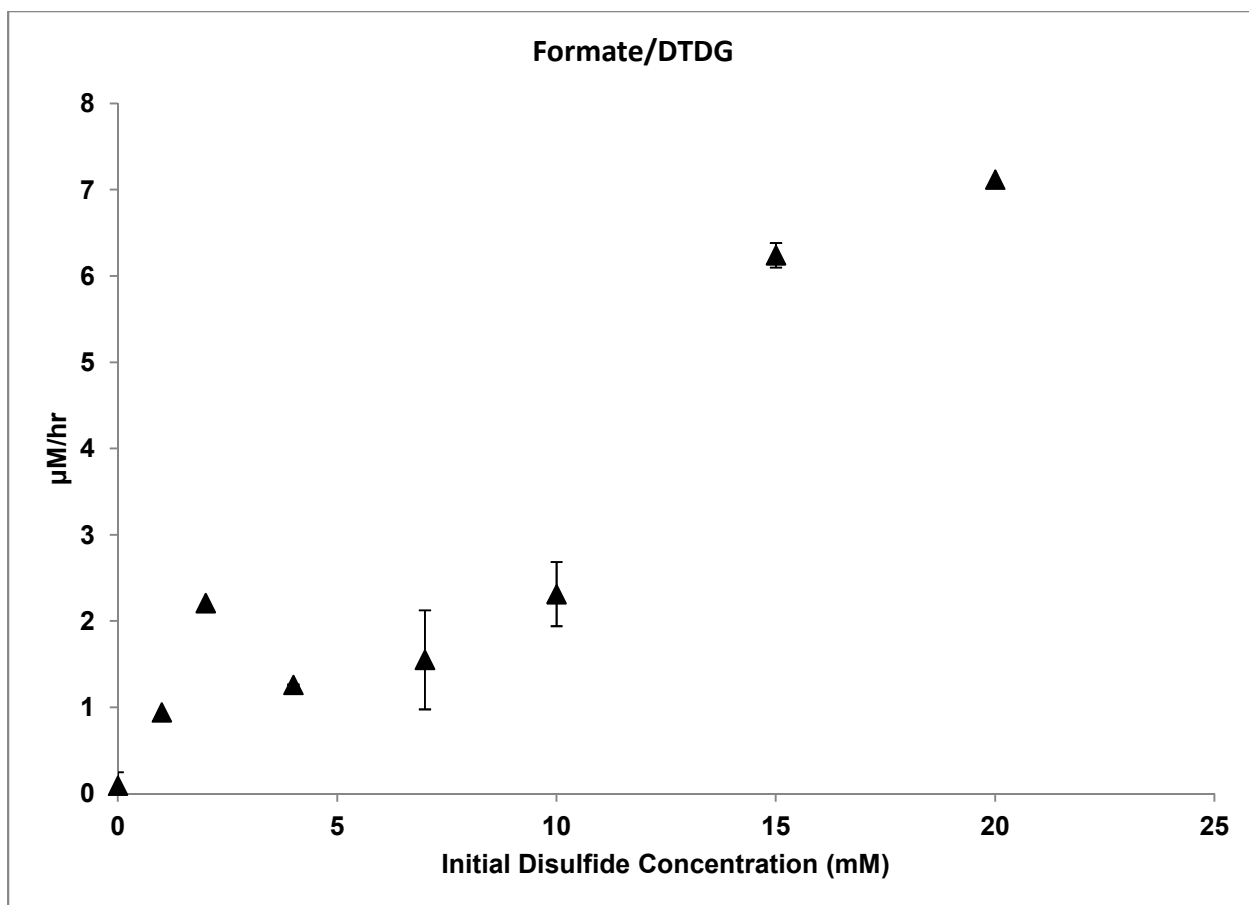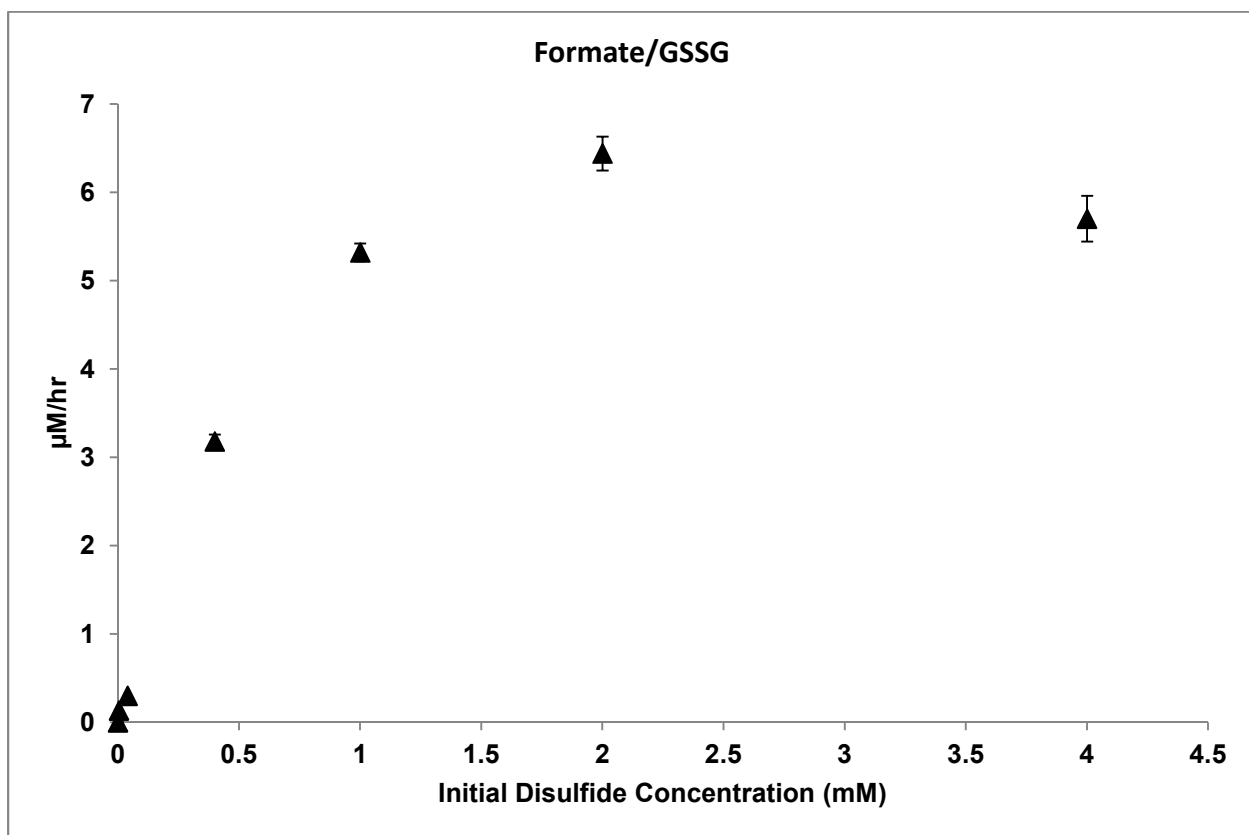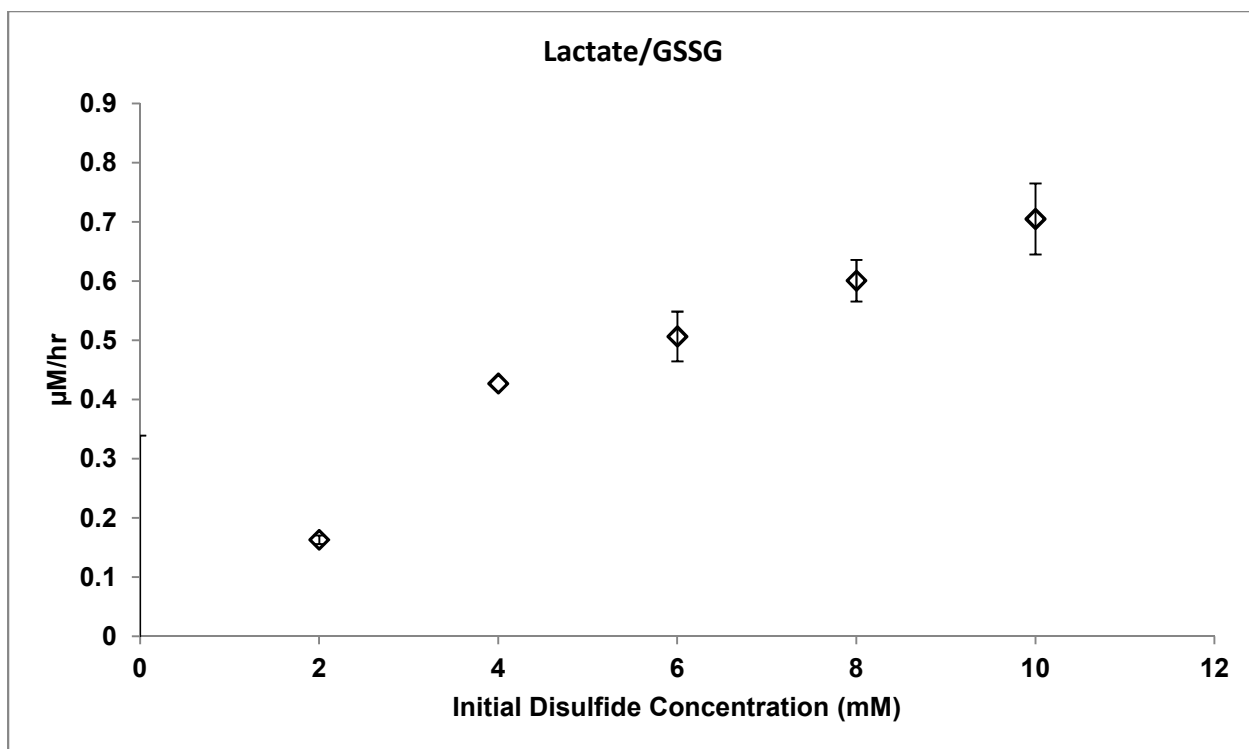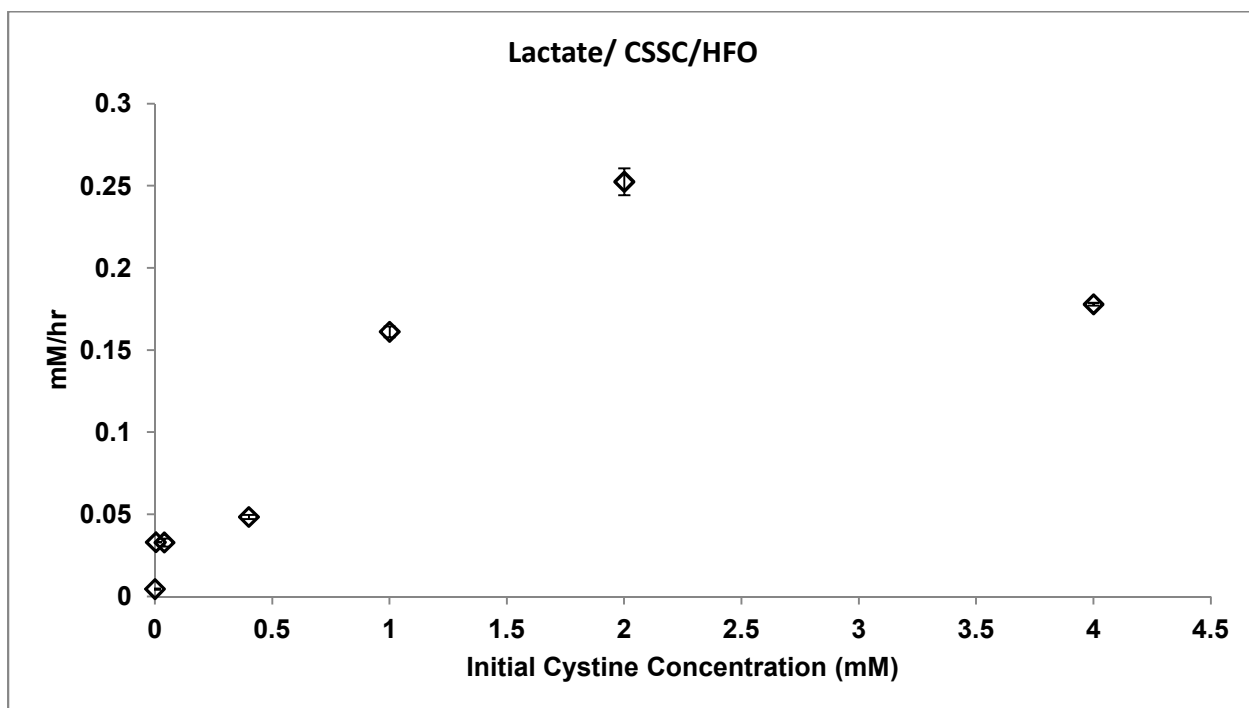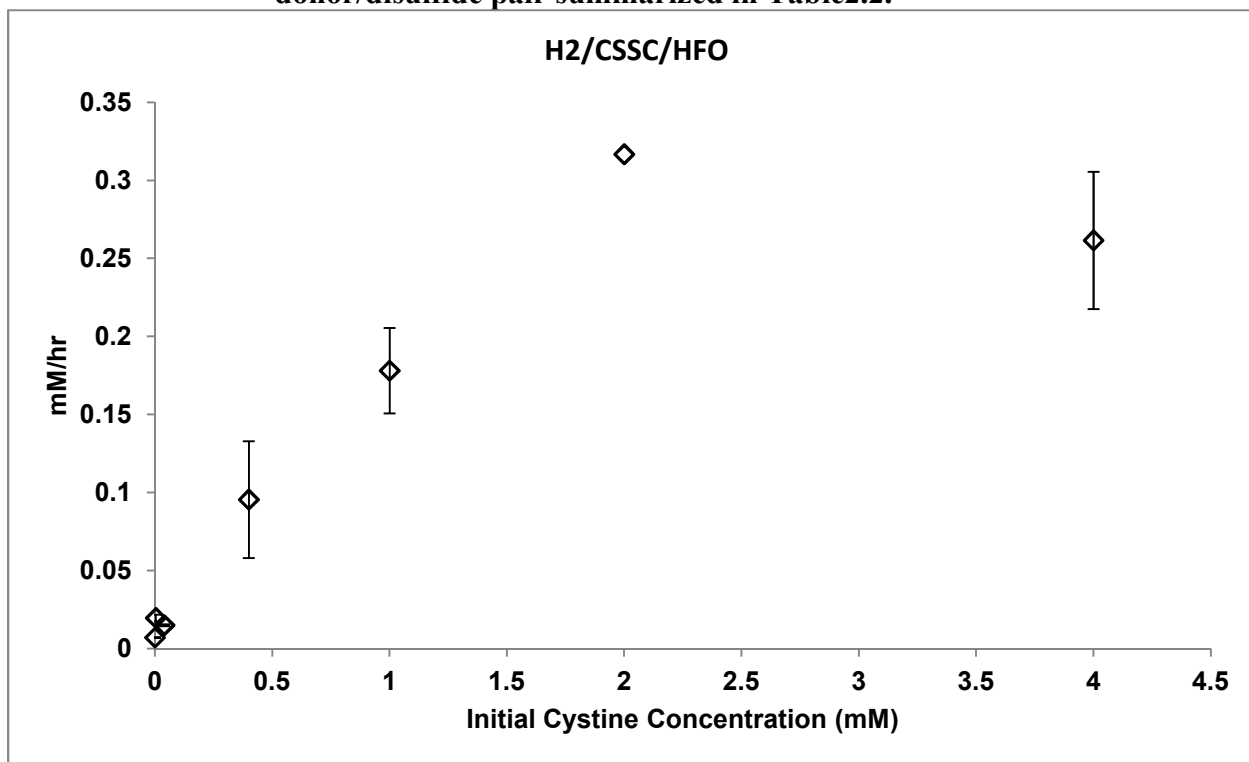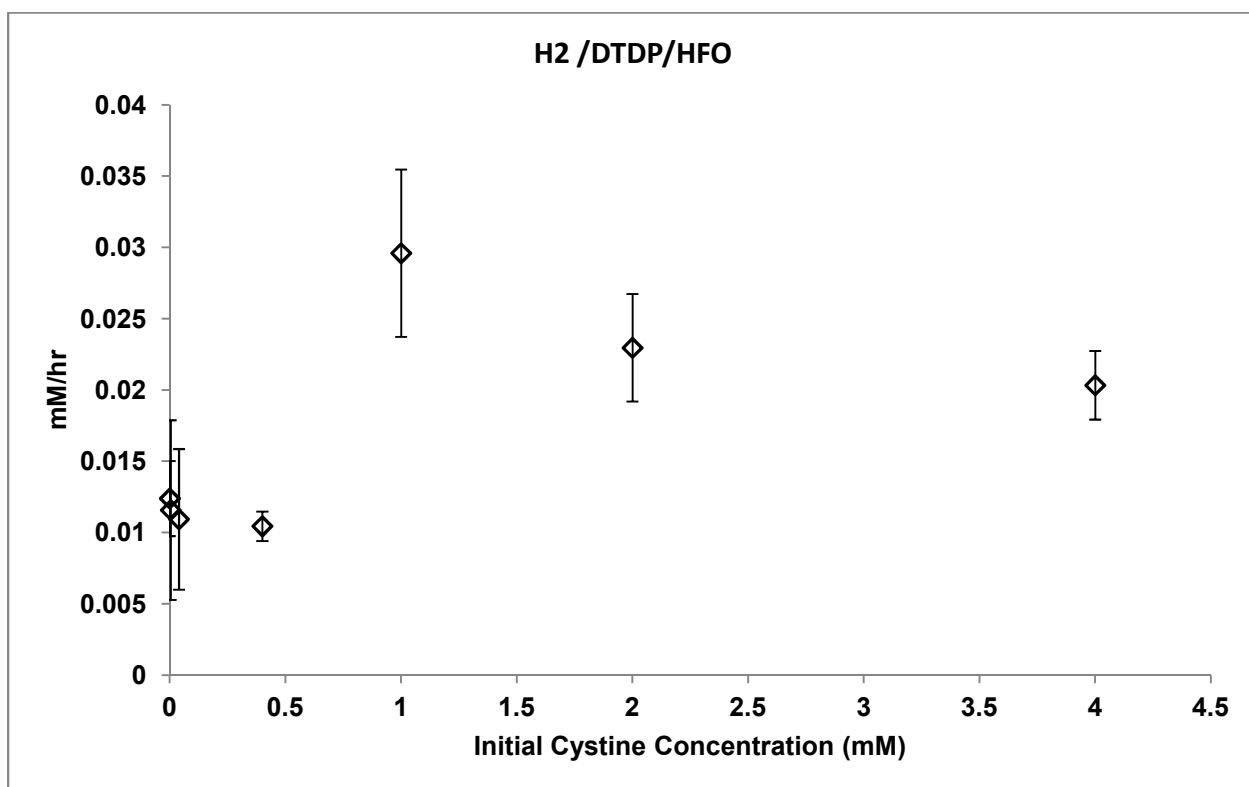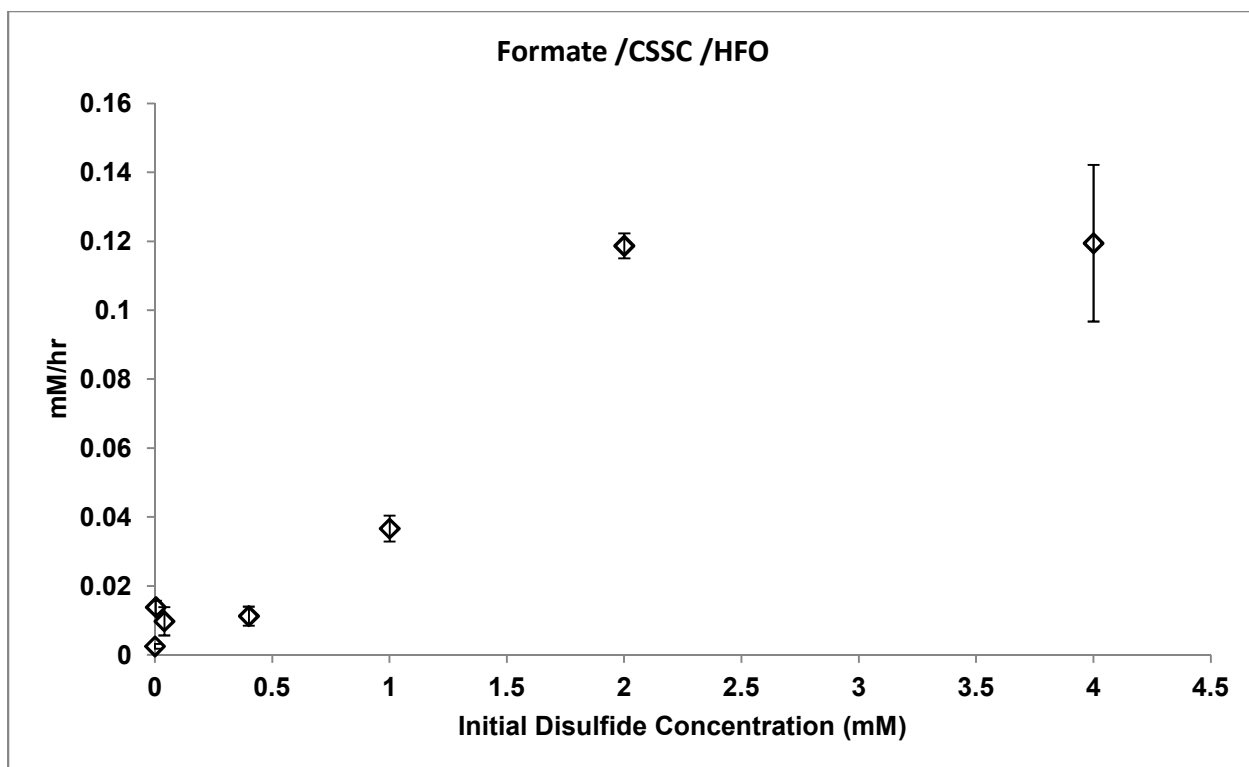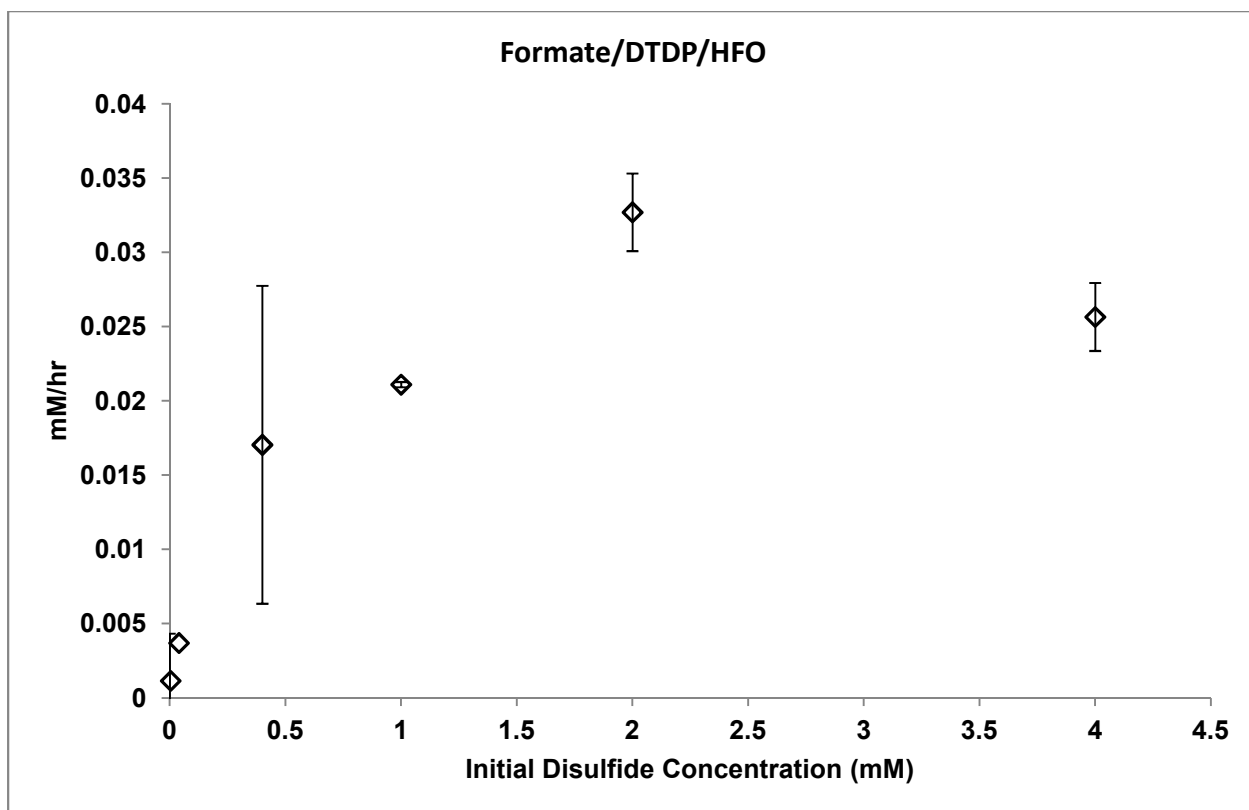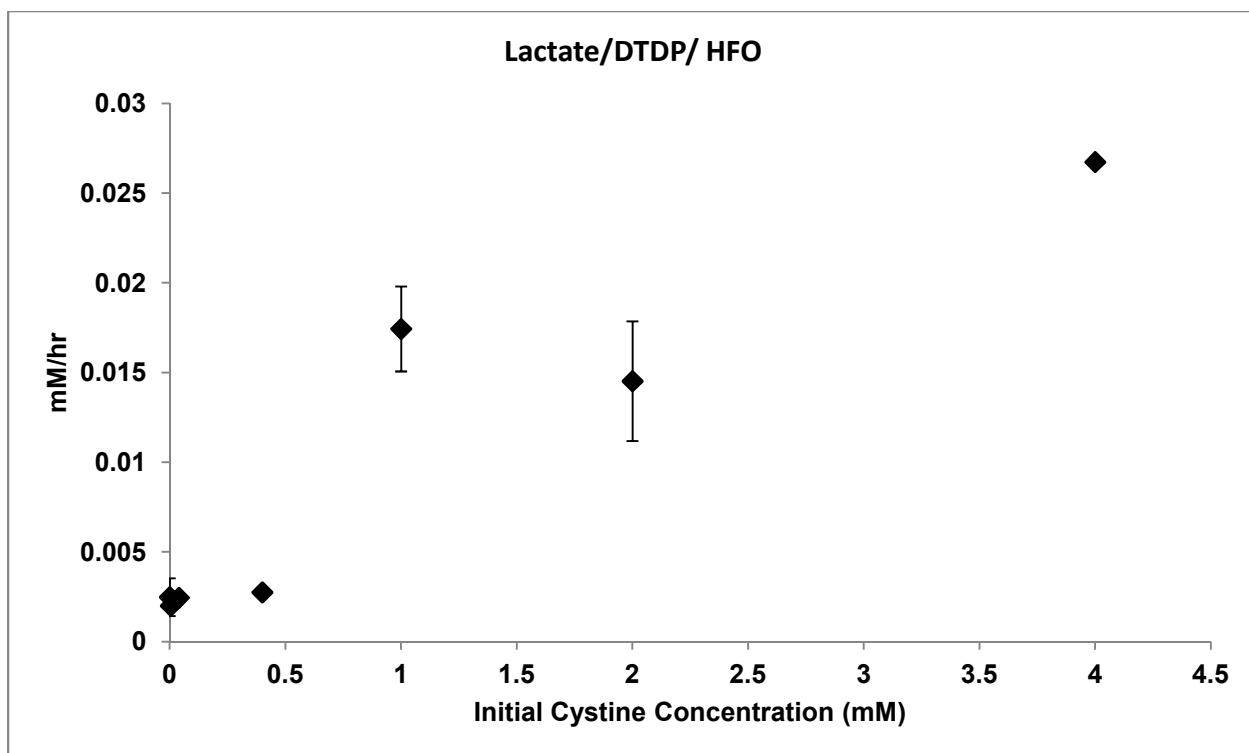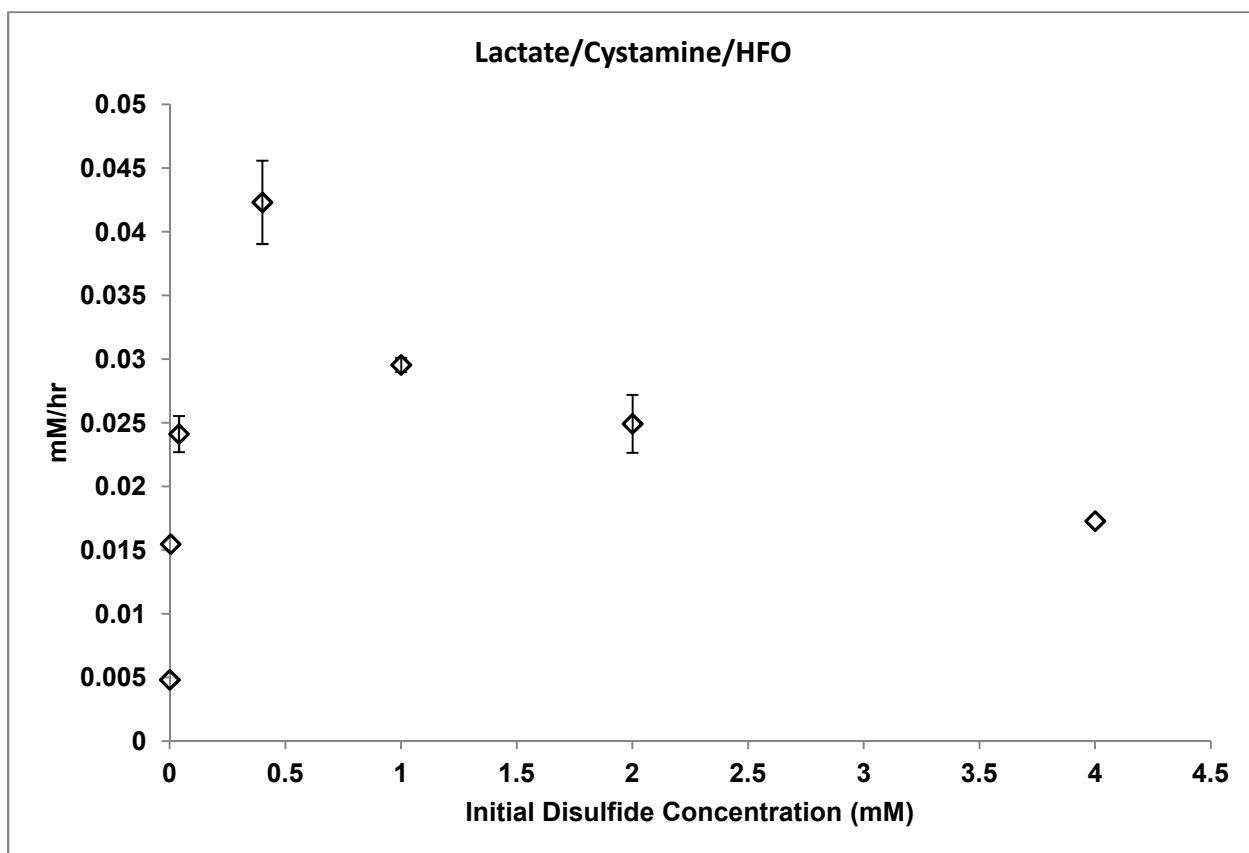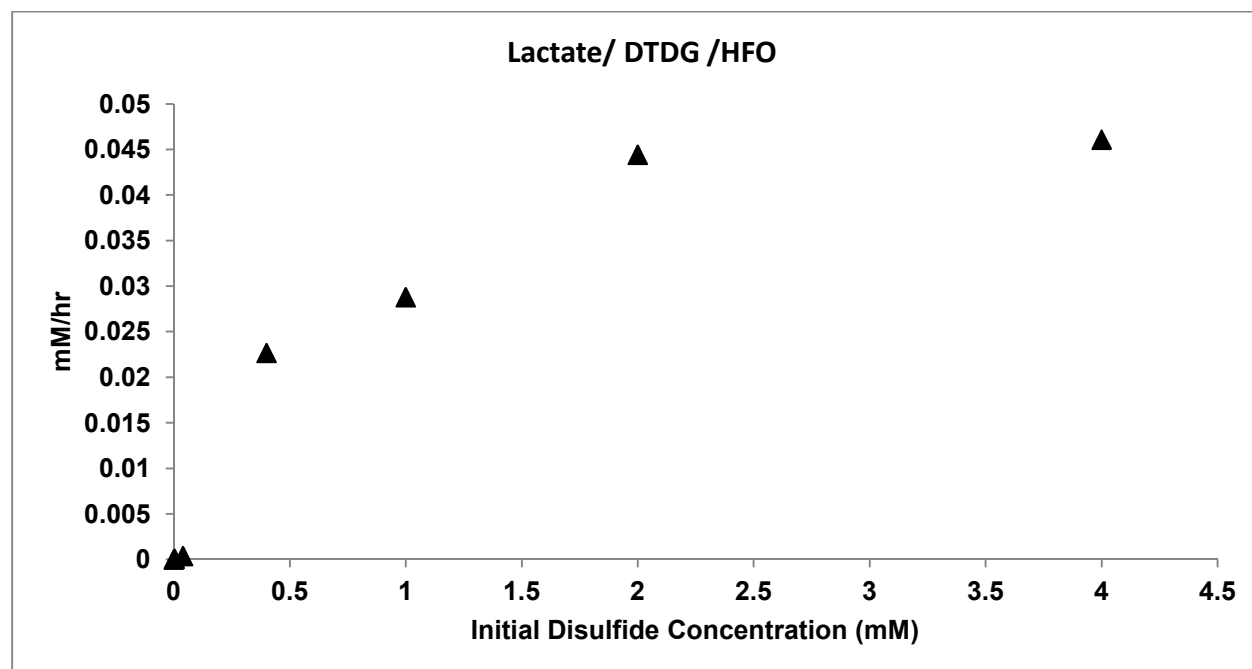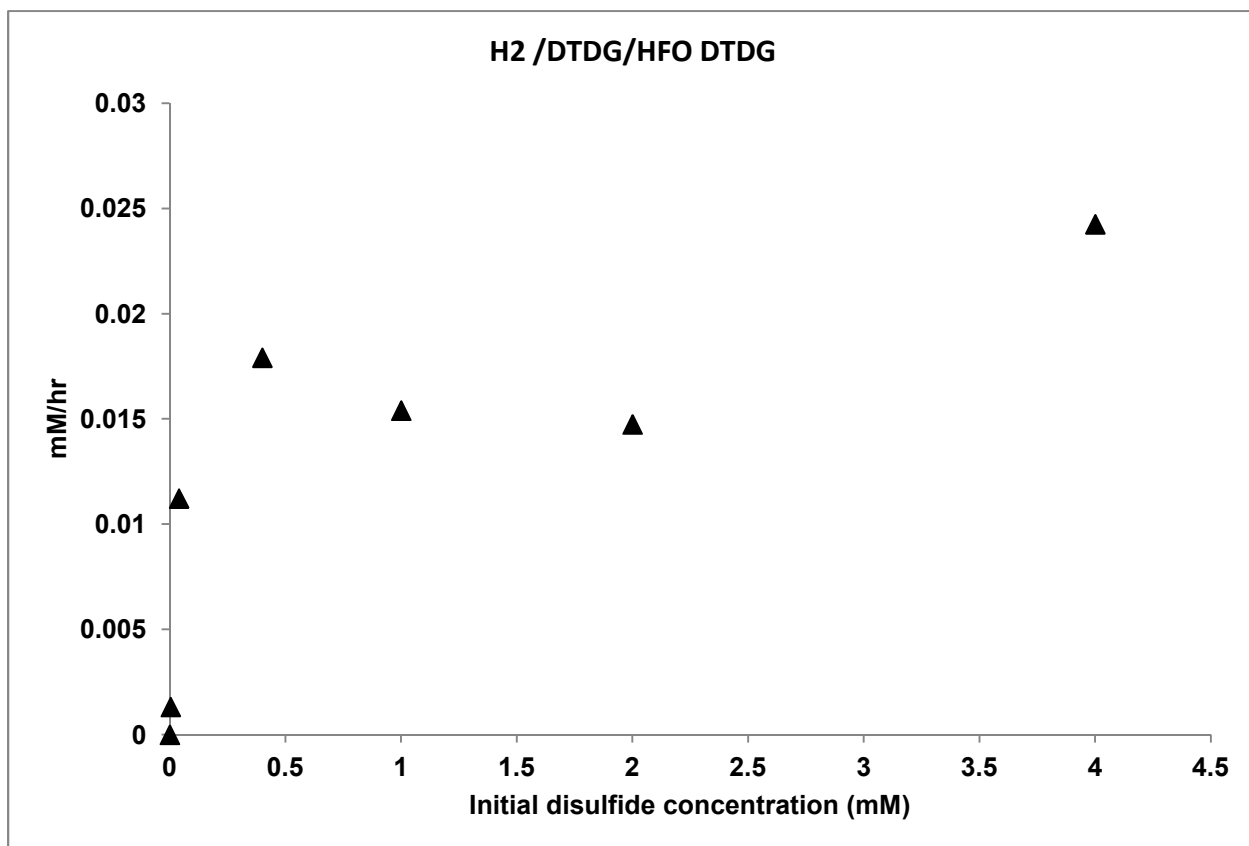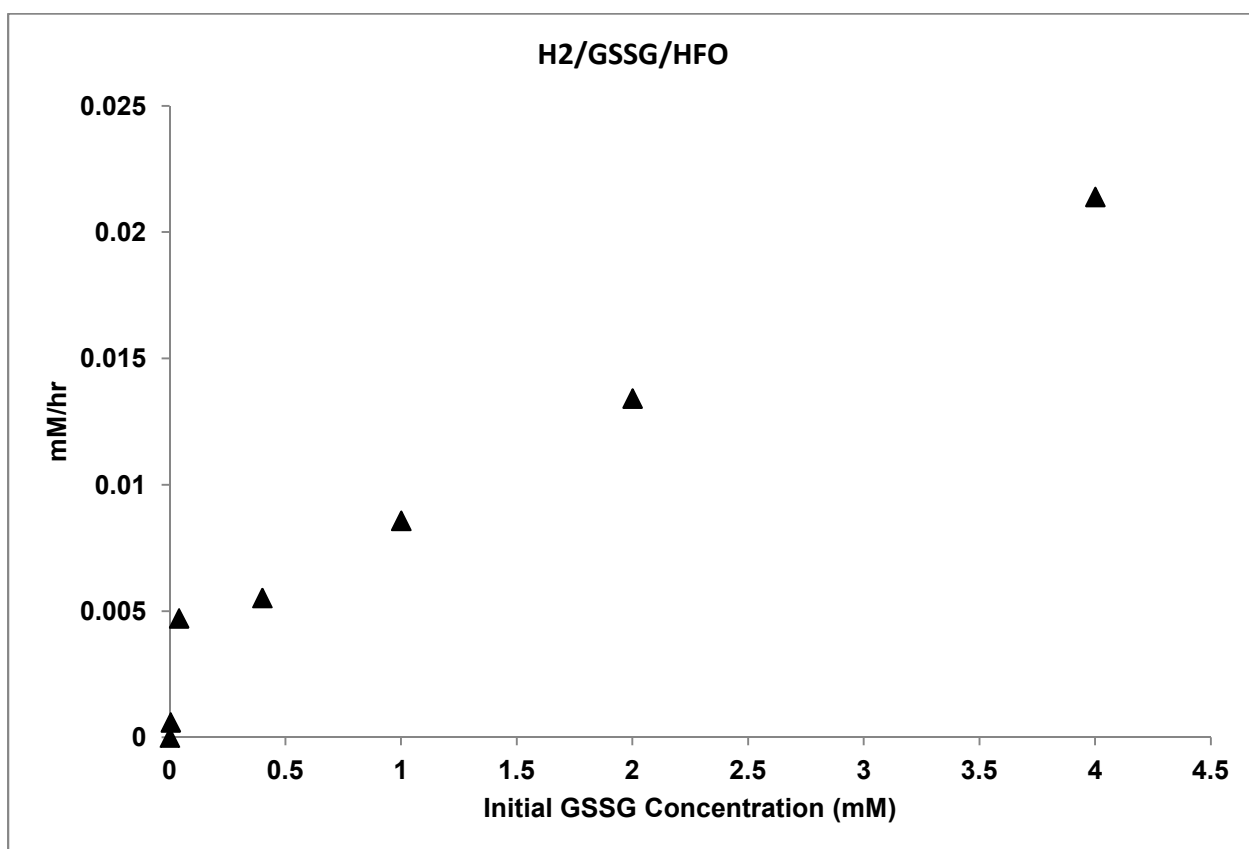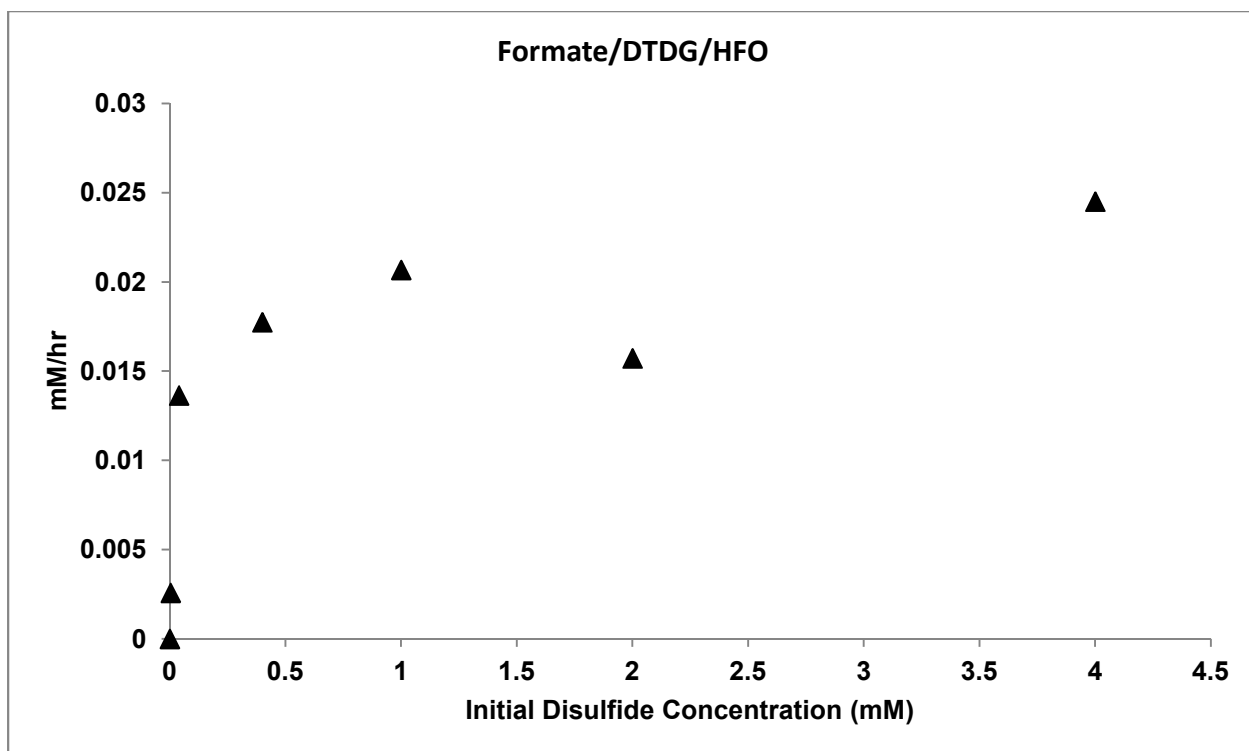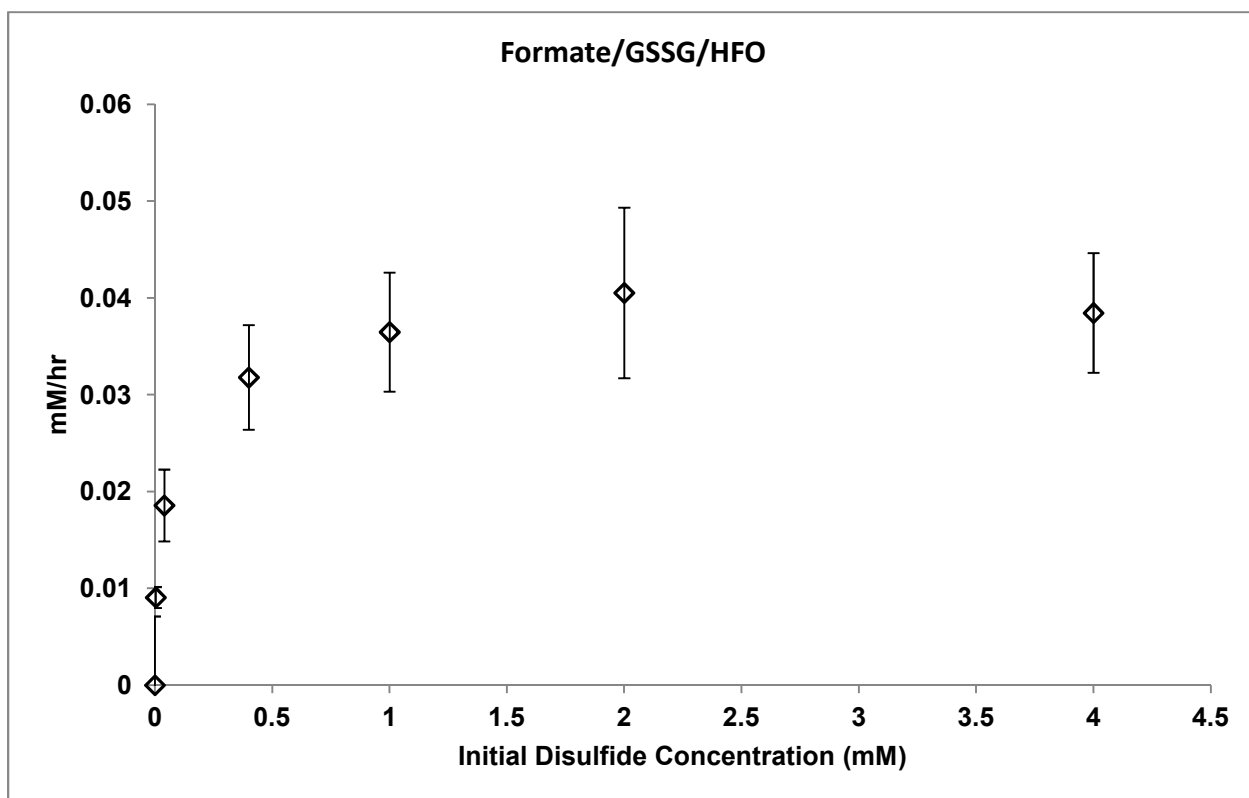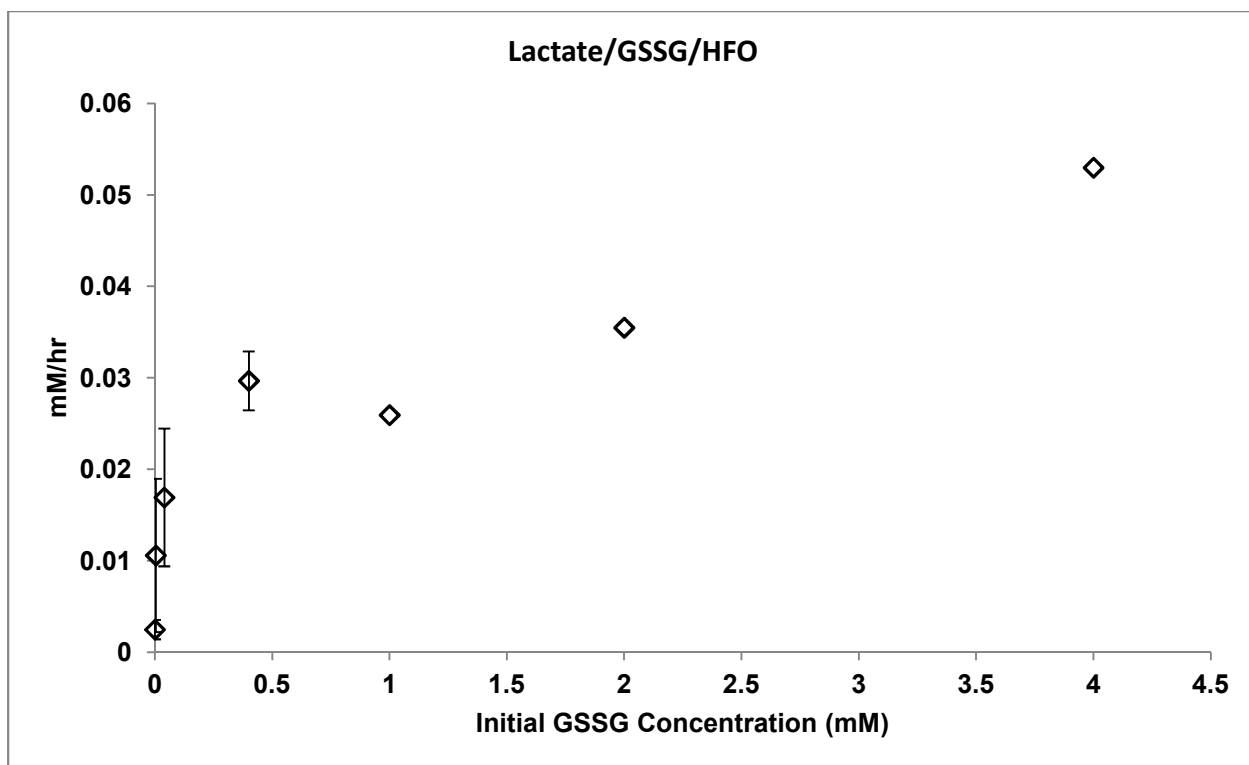
VITA

Seng Kew Wee

Seng Kew Wee was born in Kota Bharu, in the state of Kelantan, Malaysia. He attended vernacular and public schools in his hometown before moving to Japan for his college studies. He received a B.Eng. in Bioengineering from Tokyo Institute of Technology, Yokohama in 2003 and M.Sc. from The University of Tokyo, Tokyo in 2005 before coming to Georgia Tech to pursue a doctorate in 2007. When he is not working on his research, Mr. Wee enjoys playing badminton, he was the president of Georgia Tech Badminton Club, and actively participated in several regional badminton tournaments in Georgia and South Carolina.