VARIED PHYSIOLOGICAL RESPONSES OF THE FACULTATIVE γ-*PROTEOBACTERIUM*, *SHEWANELLA ONEIDENSIS* MR-1, AND THE δ-*PROTEOBACTERIUM DESULFOVIBRIO VULGARIS* HILDENBOROUGH TO OXYGEN

by

Anitha Sundararajan

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 2011

©COPYRIGHT

by

Anitha Sundararajan

2011

All Rights Reserved

APPROVAL

of a dissertation submitted by

Anitha Sundararajan

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citation, bibliographic style, and consistency and is ready for submission to The Graduate School.

Dr. Matthew W. Fields

Approved for the Department of Microbiology

Dr. Mark A. Jutila

Approved for The Graduate School

Dr. Carl A. Fox

STATEMENT OF PERMISSION TO USE

In presenting this dissertation in partial fulfillment of the requirements for a doctoral degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. I further agree that copying of this dissertation is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this dissertation should be referred to ProQuest Information and Learning, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute my dissertation in and from microform along with the non-exclusive right to reproduce and distribute my abstract in any format in whole or in part."

Anitha Sundararajan

April 2011

DEDICATION

To Amma and Appa, for their endless love and support.

ACKNOWLEDGEMENTS

Not many have the privilege of attending graduate school without enduring difficulties. My experience was worth every second and this was possible because of all the people that made it happen for me in the past seven years of my life.

First and foremost, I would like to thank Dr. Matthew Fields, my mentor. Thank you for your continuous support though these years. I thank you for being so passionate about Science and your selfless nature to share it with your students. Thank you for giving me all of those endless opportunities to attend scientific conferences, meetings which have helped me better my scientific perspective. Hats off to your patience, dedication and ability to groom naïve students to budding scientists. In short, if I had to re-live the past six or so years in your lab as a graduate student, I would most gladly do it. I would especially like to thank my committee members, Dr. Geesey, Dr. Camper and Dr. Franklin for all their valuable inputs. My friends have played a significant role in making sure I was not alone during my journey. My friends from India, Ohio and Bozeman- I thank all of you with all my heart. I would like to thank all my labmates for all their help, friendship and advice and the countless laughs. I would also like to thank my friends at the CBE. Folks at the Department of Microbiology (MSU and MU) have been wonderful to work with. I would like to thank Mom and Dad for letting me follow my dreams. I thank Anu, my lovely sister who has been so giving and generous; Ram, my brother and Trishu, my incredibly cute niece. Last but not the very least, I would like to thank Thiru. You have been truly amazing and I cannot emphasize enough how every little or big things you have done for me has impacted in shaping me to be a better person.

TABLE OF CONTENTS

1. INTRODUCTION 1

Evolution of molecular oxygen and emergence of life 1
Shewanella sp. And MR-1 3
Hypothetical and conserved hypothetical proteins in MR-1 4
Signal transduction in bacteria 6
Signaling domains 7
PAS 7
GGDEF and EAL 8
Sulfate-reducing bacteria 9
Discovery and History 9
Where do SRBs live? 11
Oxidative stress response in SRBs 11
Protective mechanisms towards oxygen exposure 13
Thesis Motive 14
References 15

2. A *SHEWANELLA ONEIDENSIS* MR-1 SENSORY BOX PROTEIN IS INVOLVED IN AEROBIC AND ANOXIC GROWTH 23

Contribution of authors and co-authors 23
Manuscript information page 24
Abstract 25
Introduction 26
Materials and Methods 28
Sequence comparisons 28
Mutant construction 29
ΔSO3389 complementation 30
Growth 31
Growth for transcriptomic analysis 32
Transcriptomics 32
Dissolved oxygen and oxidation-reduction potential measurements 33
SDS-PAGE and cytochrome analysis 33
Fumarate reductase 34
Quantitative PCR 34
Total heme quantitation 35
Results and Discussion 36
SO3389 in *Shewanella* 36
Phylogenetic relationships 36
Growth Phenotypes 38

TABLE OF CONTENTS – CONTINUED

Complementation....40
c-type cytochrome content....43
Gene expression....45
Fumarate reductase activity....48
Structural similarity to PAS O_2/redox sensors....49
References....55

3. DELETION OF A MULTI-DOMAIN PAS PROTEIN CAUSES PLEIOTROPIC EFFECTS IN *SHEWANELLA ONEIDENSIS* MR-1....61

Abstract....61
Introduction....62
Materials and Methods....64
Growth....64
Mutant construction....65
ΔSO3389 complementation....65
Iron reduction assay....66
Biofilm analysis....67
Motility assay....67
MPN for determining suppressor....67
Protein estimation....68
Activity gels....68
Scanning electron microscopy....69
Epiflourscence microscopy....69
Results and Discussion....70
References....85

4. SUBSTRATE-BASED DIFFERENTIAL RESPONSE OF *DESULFOVIBRIO VULGARIS* HILDENBOROUGH PLANKTONIC CELLS TO DISSOLVED OXYGEN....87

Abstract....87
Introduction....88
Materials and Methods....90
Strains and growth....90
Growth conditions....90
GlgA DVU2244 deletion....91
PerR DVU3095 deletion....93
Medium Preparation....93
Inoculation procedure....94

TABLE OF CONTENTS – CONTINUED

Viability experiments....95
Protein and carbohydrate measurements....95
Extraction of internal/external carbohydrates by centrifugation method....96
Sulfide assay....96
ATP measurements....96
Results....97
D. vulgaris growth with lactate and pyruvate....97
Effect of DO on *D. vulgaris* planktonic, lactate-grown cells....98
Effect of DO on *D. vulgaris* planktonic, pyruvate-grown cells....101
Total vs. internal carbohydrate measurements....104
ATP measurements of lactate and pyruvate-grown cells....106
Δ*glg*A in the presence of DO....107
GlgA Growth....107
Effect of DO on Δ*glg*A planktonic, lactate-grown cells....107
Effect of DO on Δ*glg*A planktonic, pyruvate-grown cells....108
Effect of DO on Δ*per*R planktonic, lactate and pyruvate-grown cells....110
Discussion....111
References....118

5. RESPONSE OF *DESULFOVIBRIO VULGARIS* HILDENBOROUGH BIOFILM CELLS TO DISSOLVED OXYGEN....122

Abstract....122
Introduction....123
Materials and Methods....125
Growth conditions....125
Biofilm growth under nutrient limiting conditions....125
Biofilm growth in an annular reactor with varying rpm....126
Protein and carbohydrate measurements....127
Optimization of dissolved oxygen levels in 10ml anaerobic tubes....128
Biofilm cultivation for re-inoculation....128
SEM for biofilm cells exposed to DO....129
Results and Discussion....130
Growth of biofilms in annular reactors....130
CDC reactor biofilm cultivation....132
SEM to test morphology of biofilm cells in the presence of DO....141
References....144

TABLE OF CONTENTS – CONTINUED

6. EPILOGUE 147

References 162

APPENDIX A: Supplemental Figures 166

LIST OF TABLES

Table Page

1. Strains and vectors 31

2. Comparison of $\log_2$ expression levels for selected genes when wild-type or mutant cells were compared between aerobic and anoxic conditions 46

3. Peptide sequence identity between PAS2 domain of SO3389 and PAS domains of FAD-based redox sensors in *Methylococcus capsulatus* (Mmo), *Azotobacter vinelandii* (Az), *Escherichia coli* (Ec) 50

4. 580/600nm ratios which signify biofilm quantitation in WT and mutant during four consecutive aerobic transfers after which culture was transferred to anoxia....80

5. Motility assay of WT and mutant at 4 aerobic transfers before culture was transferred to anoxia....80

6. Primers used for mutagenesis of Δ*glgA* 92

7. Viability of *D. vulgaris* planktonic cells grown with lactate at 1.86mg/l DO determined by most probable number (MPN)....100

8. Growth rates of *D. vulgaris* planktonic cells grown in the presence of different concentrations of DO with lactate and pyruvate as substrate 102

9. ATP concentrations of lactate and pyruvate *D. vulgaris* planktonic cells at 0.3 and 0.8mg/l DO at early log (0.3 OD) and late log (0.8) phase....106

10. List of genes up-expressed in *D. vulgaris* planktonic cells grown in the presence of lactate within the first 20 min of exposure to air 114

11. Growth rates of *D. vulgaris* cells grown with 60mM lactate or pyruvate as carbon/energy sources and 50mM sulfate as the electron acceptor. Source of inoculum was vortexed scraped biofilm cells grown with the respective electron donors 141

LIST OF FIGURES

Figure Page

1. Phanerozoic (current eon on geological time scale where maximum animal life exists, 543 million years ago – present) history of atmospheric oxygen concentrations 2

2. Input signals and output of c-di-GMP metabolism 9

3. Genome region view and domain architecture of SO*3389* 37

4. Phylogram based upon PAS domains of SO*3389* with a neighbor-joining method within the Microbes On Line workbench 38

5. (**A**) Growth curves of wild-type MR-1 and △SO3389 cells in aerobic, defined, minimal medium with lactate (150 rpms). **(B)** Growth rates of wild-type and mutant planktonic cultures at different shake speeds (rpms) 39

6. **(A**) Dissolved oxygen measurements for WT and △SO3389 and oxidative-reductive potential (mV) measurements for WT and △SO3389 versus time when shaking aerobic cultures were incubated statically. **(B)**The decrease in DO (mg/l) was measured for cultures growing in lactate medium for wild-type and mutant at 0 rpm and 150 rpm 41

7. Growth curves of wild-type MR-1 and △SO3389 cells in anaerobic, defined, minimal medium with lactate and fumarate 42

8. Growth curves of wild-type MR-1, ΔSO3389, wild-type pBBR1MCS-2, ΔSO3389 pBBR1MCS-2 and Δ3389:: pBBR1MCS-2-SO3389 in defined minimal medium with lactate as the electron donor and fumarate as the electron acceptor 43

9. SDS-PAGE gels of spheroplast and periplasmic-shock fractions of wild-type and △SO3389 cells stained with Coomassie blue (**A**) or cytochrome-c heme stain (**B**) 44

LIST OF FIGURES-CONTINUED

Figure Page

10. Alignment of *M. capsulatus* MmoS PAS1, *M. capsulatus* MmoS PAS2, *A. vinelandii* NifL PAS, *E. coli* Dos PAS, *B. japonicum* FixL PAS, SO3389 PAS1, and SO3389 PAS2 51

11. Structure of the *M. capsulatus* MmoS **(A)** (PDB: 3EWK) and SO3389 **(B)** PAS domains and the structural superposition **(C)** 52

12. Anaerobic growth of WT and ΔSO3389 upon transfer from aerobic culture. **(A)** Growth on 120mM lactate as electron donor and 120mM fumarate as electron acceptor. **(B)** Growth on 20mM lactate and 40mM fumarate 71

13. Growth of MR-1 WT and ΔSO3389 mutant in medium containing Fe(III) as electron acceptor. **(A)** Growth of MR-1 and ΔSO3389 at 24h. **(B)** Growth of MR-1 and ΔSO3389 at 48h. **(C)** Fe(III) reduction quantified by Ferrozine assay in MR-1 WT and ΔSO3389 over a 60h time period 72

14. Crystal violet stained biofilms formed at 150rpm at approximately 35-40h by **(A)** MR-1 WT and **(B)** ΔSO3389 73

15. Comparison of biofilm formation between wild-type and mutant cultures at varying shake speeds (rpm). 73

16. Epifluorescent microscopy of MR-1WT **(A)** and △SO3389 **(C)** biofilms grown in defined, minimal medium with lactate for 40 h. Scanning electron micrographs of wild-type **(B)** and △SO3389 **(D)** biofilms grown in defined, minimal medium 75

17. A colony of **(A)** wild-type or **(B)** △SO3389 cells on 0.3% motility agar inoculated from exponential phase aerobic cultures 76

LIST OF FIGURES-CONTINUED

Figure Page

18. Anoxic growth of WT and ΔSO3389 (suppressor) in the presence of fumarate as electron acceptor 77

19. Anoxic growth of WT (with fumarate) and mutant suppressor strains (S) in the presence of fumarate and DMSO as electron acceptors 78

20. Fumarate reductase activity gel of wild-type and △SO3389 cells during transition (**A**) and anoxic conditions (**B**) in defined medium 79

21. Crystal violet stained biofilms formed by **(A)** WT and **(B)** ΔSO3389. Shake flasks were examined for biofilm formation at all four transfers....80

22. Fumarate reductase activity for wild-type and five separate suppressor strains, S1, S2, S3, S4 and S5 and WT during anoxic growth 81

23. Growth of *Desulfovibrio vulgaris* Hildenborough washed and unwashed cells in the presence of 60mM **(A)** lactate and **(B)** pyruvate as carbon and energy sources and 50mM sulfate as the electron acceptor....98

24. Growth of *D. vulgaris* planktonic cells in the presence of 0.78mg/l DO with lactate as substrate 99

25. Growth of *D. vulgaris* planktonic cells in the presence of 1.5mg/l DO with lactate as substrate 100

26. Growth of *D. vulgaris* planktonic cells in the presence of 3.14mg/l DO with lactate as substrate 101

27. Growth of *D. vulgaris* planktonic cells in the presence of 0.78mg/l DO with pyruvate as substrate 103

28. Growth of *D. vulgaris* planktonic cells in the presence of **(A)** 1.5, **(B)** 1.86 and **(C)** 3.14 mg/l DO with pyruvate as substrate 104

LIST OF FIGURES-CONTINUED

Figure Page

29. Total vs. internal carbohydrate levels for *D. vulgaris* planktonic cells at 20 and 40h in the presence of lactate and pyruvate 105

30. Growth of Δ*glg*A in the presence of various DO concentrations, i.e, 0.78, 1.5, 1.86 and 3.14mg/l DO with **(A)** lactate and **(B)** pyruvate as substrates 108

31. Total vs. internal carbohydrate levels for Δ*glg*A planktonic cells at 20 and 40h in the presence of **(A)** lactate and **(B)** pyruvate 109

32. **(A)** Growth ofΔ *per*R and WT *D. vulgaris* planktonic cells in the presence of various concentrations of DO with lactate as substrate. **(B)** Growth of Δ*per*R and WT *D. vulgaris* planktonic cells in the presence of various concentrations of DO with lactate as substrate 109

33. Growth of Δ*per*R complement in the presence of different DO concentrations with **(A)** lactate and **(B)** pyruvate as substrates 110

34. **(A)** Protein and carbohydrate concentrations of scraped biofilm cells from coupons recovered from Annular Reactors run at 15, 20 and 40 rpm **(B)** Carbohydrate to protein ratio of biofilm cells at 48, 72, 96 and 120h. Biofilms were grown in annular reactors with 60mM lactate and 50mM sulfate as carbon and energy source and electron acceptor respectively. Reactor was run at 150, 200 and 400rpm 131

35. Graphs representing protein and carbodydrate concentrations of *D. vulgaris* biofilm cells grown in CDC reactors under nutrient limiting conditions. **(A)** Protein and carbohydrate measurements of *D. vulgaris* biofilm grown on 7.5mM lactate and 6.25mM sulfate. **(B)** Protein and carbohydrate measurements of *D. vulgaris* biofilm grown on 7.5mM pyruvate and 6.25mM sulfate **(C)** Carbohydrate to protein ratio of *D. vulgaris* biofilm cells grown in CDC reactor with lactate and pyruvate as substrates 134

LIST OF FIGURES-CONTINUED

Figure Page

36. Growth of *D. vulgaris* cells on 60mM lactate and 50mM sulfate. **(A)** *D. vulgaris* biofilm cells transferred to planktonic mode grown without added oxygen (0.3mg/l), unvortexed and vortexed. **(B)** *D. vulgaris* biofilm cells (unvortexed) in planktonic mode at 0.3mg/l and 0.78mg/l DO. **(C)** Vortexed *D. vulgaris* biofilm cells in planktonic mode at 0.3mg/l and 0.78mg/l DO.....137

37. Growth of *D. vulgaris* cells on 60mM lactate and 50mM sulfate. **(A)** *D. vulgaris* biofilm cells (unvortexed) in planktonic mode at 0.3mg/l and 1.5mg/l DO. **(B)** Vortexed *D. vulgaris* biofilm cells in planktonic mode at 0.3mg/l and 1.5mg/l DO.....138

38. Growth of *D. vulgaris* cells on 60mM pyruvate and 50mM sulfate. **(A)** Vortexed *D. vulgaris* biofilm cells grown in the presence of 0.3mg/l (control) and 0.78mg/l DO. **(B)** Vortexed *D. vulgaris* biofilm cells grown in the presence of 0.3mg/l (control) and 1.5mg/l DO. **(C)** Vortexed *D. vulgaris* biofilm cells grown in the presence of 0.3mg/l (control) and 1.86mg/l DO.....139

39. Electron micrographs of *Desulfovibrio vulgaris* Hildenborough biofilm cells grown on glass coupons in a CDC reactor with 60mM lactate as the electron donor and 50mM sulfate as the electron acceptor. Mag=10,000X **(A)** EM of *D. vulgaris* cells, unexposed to dissolved oxygen at 0h. **(B)** EM of *D. vulgaris* cells, unexposed to DO at 24h **(C)** EM of *D. vulgaris* cells exposed to 3.14mg/l DO at 2.5h and **(D)** EM of *D. vulgaris* cells exposed to 3.14mg/l DO at 24h 142

40. Model for possible electron flow with lactate or pyruvate and polyglucose as carbon sources.....156

LIST OF FIGURES-CONTINUED

Figure Page

S1. WT and SO3389 were spot inoculated on 0.3% motility agar inoculated from exponential phase aerobic cultures.. 167

S2. Growth of *D. vulgaris* planktonic cells at various DO concentrations with lactate as substrate in the presence of yeast extract.....168

S3. Sulfide levels of washed *D. vulgaris* planktonic cells grown with lactate and pyruvate as carbon sources at 60mM concentration at 0.3 and 0.78mg/l DO concentrations. Open circle and square represents WT *D. vulgaris* washed cells at 0.3 mg/l DO grown with lactate and pyruvate respectively. Closed circle and square represents WT *D. vulgaris* washed cells at 0.78 mg/l DO grown with lactate and pyruvate respectively.....169

S4. GlgA complemented strain grown with (A) lactate and (B) pyruvate as carbon and energy source. GlgA cells were exposed to 0.78, 1.5 and 1.86mg/l DO.. 170

S5. *D. vulgaris* biofilm cells (non-vortexed) grown in LS4D with 60mM lactate and 50mM sulfate in the presence of 1.86mg/L DO.. 171

ABSTRACT

Evolution of molecular oxygen and accumulation in Earth's atmosphere is considered to be the one of the most significant changes on Earth that impacted the evolution of life. Over the past 600 million years, there have been fluctuations in atmospheric oxygen concentrations that have driven the evolution of species in all three domains of life. Over the years, microbes and other life acquired different strategies to survive efficiently in the presence of oxygen through mutagenic evolutionary mechanisms.

This dissertation demonstrates how a facultative bacterium, *Shewanella oneidenisis* MR-1, through signaling mechanisms, senses oxygen as an external stimulus and regulates metabolism accordingly. These signaling molecules reside within open reading frames as small domains that sense/transmit signals based on stimulus and subsequently trigger a response within the cell. One such open reading frame, SO3389, containing multiple domains was characterized in the first two chapters of this dissertation.

Physiology and genetics based experiments were employed to address hypotheses that a putative sensory-box protein was involved in oxygen sensing. It was elucidated that this protein plays a role in sensing dissolved oxygen (DO) levels that affected both aerobic biofilm formation and transitions to anoxia. While oxygen can be an attractant in aerobic growth mode, it is considered to be toxic to strict anaerobes, such as *Desulfovibrio vulgaris* Hildenborough, a sulfate-reducing bacterium. Recent studies, however, reveal that even strict anaerobes can tolerate micromolar concentrations of oxygen. These organisms have evolved several protective mechanisms to combat oxidative stress and some may even possess oxygen-utilization machinery. Chapters 3 and 4 address the phenomenon of oxygen tolerance in *D.vulgaris* planktonic and biofilm cells and the variation in this response based on available carbon and energy sources. Physiology- and genetic-based approaches revealed that *D.vulgaris* cells grown on pyruvate exhibit increased tolerance towards DO but lactate-grown cells utilized oxygen for energy production at intermediate levels of DO. The substrate-dependent nature of oxygen response in *D. vulgaris* has not been previously reported, and could impact remediation strategies as well as possible implications for community interactions. The results demonstrated in this dissertation underscore two major findings with respect to oxygen responses: (i) the elucidation of unknown function for a conserved hypothetical protein, and (ii) the substrate-dependent nature of oxygen utilization in a "strict" anaerobe. The elucidation of function for these genes/proteins/organisms will further our fundamental understanding of microbial physiology, of the versatility that allows adaptation to constantly changing environments, and help improve future remediation strategies.

CHAPTER 1

INTRODUCTION

Evolution of Molecular Oxygen and Emergence of Life

Evolution of molecular oxygen in the Earth's atmosphere is by far one of the most significant changes that have affected life on Earth. The early Earth atmoshphere was deficient in oxygen and the accumulation of molecular oxygen occurred with evolution of oxygenic photosynthesis in cyanobacteria about 2.5 billion years ago. Atmoshpheric oxygen has gone through drastic fluctuations over the years (Payne *et al*., 2011). Prior to 2.4 billion years ago, it was thought to be 0.001% less than the present levels (PAL) which gradually increased to between 1 and 18% of the present levels around 800 million years ago. Figure 1 illustrates the fluctuations in oxygen levels over a span of 600 million years. Oxygen concentration peaked near 30% around 250 million years ago and was the least, ie, 12% of the atmosphere between 200 to120 million years ago (Berner, 2004).

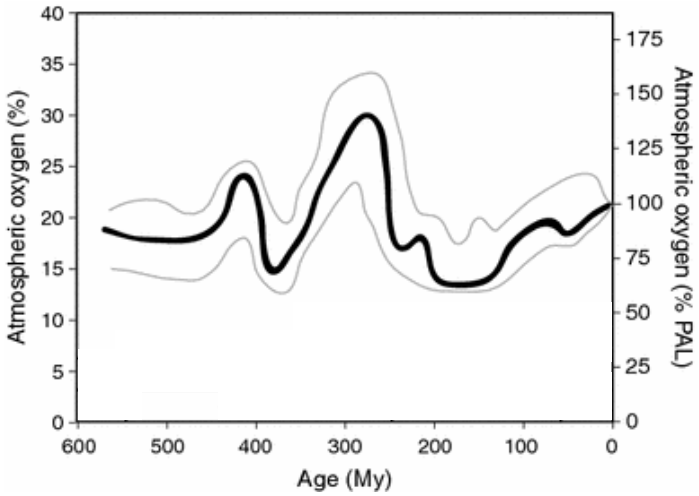


Figure 1: Phanerozoic (current eon on geological time scale where maximum animal life exists, 543 million years ago – present) history of atmospheric oxygen concentrations (modified, Payne *et al*., 2011).

The early Earth atmosphere was perceived to be composed of nitrogen, carbon di-oxide, molecular hydrogen and methane and was considered an anaerobic and reducing environment. Since anaerobic conditions were favourable for their evolution, methanogens and anaerobic bacteria were one of the earliest inhabitants of Earth (Walker *et al*., 1981; Kasting *et al*., 1993; Tian *et al*., 2005). Several theories have been proposed on how the build-up of molecular oxygen could have occurred. Some reports attribute tectonic processes to the emergence of oxygen (by burial of reduced carbon) (Des Marais *et al*., 1993) while another hypothesis suggests escape of hydrogen from atmosphere caused concomitant oxidation of Earth (Catling *et al*., 2001). The most popular theory for oxygen evolution stresses the involvement of oxygenic photosynthesis (by cyanobacteria) as the most significant player (Brocks *et al*., 1999).

Oxygen is often considered to be an attractant or a repellent by most organisms, even aerobic, due to the toxicity of elevated oxygen (Shioi *et al*., 1987). This dissertation

describes experiments that compare oxygen responses to two different microorganisms, a strictly anaerobic sulfate-reducer and a facultative iron-reducer.

Along with detoxifying mechanisms, facultative bacteria such as *Shewanella oneidensis* MR-1 exhibit the presence of sensory box proteins that are predicted to specialize in sensing external stimuli like oxygen. The first two chapters of this dissertation address how the deletion in a sensory box gene (with certain domain architecture) impacted growth transitions from oxic to anoxic conditions as well as biofilm growth. Oxygen is considered detrimental to the growth of strict anaerobes such as *Desulfovibrio vulgaris* Hildenborough. However, they are capable of tolerating μM or mM concentrations of oxygen, depending on the organism. The third and fourth chapters of this dissertation seek to elucidate physiological growth effects of dissolved oxygen on *D.vulgaris* Hildenborough planktonic and biofilm cells.

Shewanella and MR-1

Shewanella are gram negative, facultative aerobes that are classified as γ-*Proteobacteriaceae*. According to some reports, members from this genus have been implicated in food spoilage, and as well as being associated with humans and aquatic animals as opportunistic pathogens (Jorgensen and Huss, 1989; Brink *et al*., 1995). *Shewanella* have been considered to be involved in a variety of anaerobic processes and utilize a range of electron acceptors during anaerobic respiration including manganese and iron oxides, uranium, thiosulfate and elemental sulfur. *Shewanella* sp. have been

regarded as one of the most metabolically versatile organisms (Myers and Nealson, 1988; Lovely and Philips, 1988; Perry *et al*., 1993; Moser and Nealson, 1996).

Shewanella oneidensis MR-1 (formerly *Shewanella putrefaciens* strain MR-1) in particular is an apt fit into the group as it exhibits enormous plasticity for a range of electron acceptors during anaerobic respiration. MR-1 is capable of reducing oxidized metals like Mn(IV), Fe(III), Cr(VI), U(VI), fumarate, nitrate, trimethylamine N-oxide, dimethylsulfoxide, sulfite, thiosulfate and elemental sulfur (Nealson and Saffarini, 1994; Schwalb *et al*., 2002; Gon *et al*., 2002; Schwalb *et al*., 2003; Burns and DiChristina, 2009; Klonowska *et al*., 2005; Liu *et al*., 2002; Maier and Myers, 2004)

Genome analysis and pattern matching of the cytochrome c heme-binding site of MR-1 reveals the presence of 42 possible c-type cytochromes in MR-1 (Meyer *et al*., 2004). Presence of such a large number of cytochromes is an indirect function of its respiratory versatility. Functional characterization of certain large molecular weight cytochromes present in the outer membrane revealed that they could perform metal oxide reduction. Some of these include OmcA, OmcB, MtrC, CymA, MtrA, MtrB (Beliaev and Saffarini, 1998; Beliaev *et al*., 2001; Myers and Myers, 1997, 1998, 2000, 2001, 2001; Pitts *et al*., 2003).

Hypothetical and Conserved Hypothetical Proteins in MR-1

The *S.oneidensis* MR-1 genome was sequenced in 2002 and 4,467 open reading frames (ORF's) were predicted. Genome studies revealed that 60% of the genes in the MR-1 genome have annotated functions while approximately 40% (1,623 genes) have been categorized as hypothetical proteins that are both unique and conserved. (Heidelberg

et al., 2002). Hypothetical proteins are often categorized as 'known unknowns' and 'unknown unknowns'. Despite massive sequencing efforts (almost 6,000 complete and on-going microbial projects), approximately one-third of any given sequenced genome is typically annotated as hypothetical and conserved hypothetical genes. As defined by TIGR (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi), hypothetical (HyP) protein.s are those without significant sequence similarity (*i.e.*, homology) to any predicted proteins, while conserved hypothetical (CHyP) proteins are those that have significant similarity to a predicted protein in another species or strain, with no direct evidence of expression of the genes encoding these proteins. Many efforts have been made to annotate hypothetical proteins in many organisms including *S. oneidensis* MR-1 (Kolker *et al*., 2005; Yost *et al*., 2003); however despite significant advances in the acquisition of genomic information, -omics approaches have revealed extensive knowledge gaps in cellular physiology, and microbial physiology (*e.g.*, activity) is often a key parameter to understanding organismal biology and ecology. A more complete physiological knowledge of the "parts list" for a single cell will not only advance cellular biochemistry but population, community, and ecosystem ecology.

Many aspects of signaling and stress response most likely reside in this fraction of genes for a given organism and underlie the lack of understanding in physiological responses and control of metabolism. Many uncharacterized proteins are classified as sensory box proteins based upon conserved domains, but signals and cellular responses for most presumptive sensory box proteins are not known.

Signal Transduction in Bacteria

Environmental microorganisms exhibit very sophisticated systems for sensing environmental cues and signal transduction. Typically the environment microorganisms are found in dictates the complexity of signal transduction. Two-component regulatory systems are widely studied in biology (Hoch and Silhavy, 1995; Stock *et al*., 2000; Hoch, 2000). Typically in a two- component system, there is an input domain which is a sensor histidine kinase. An environmental cue is detected by this domain that results in activation via autophosphorylation. The activated phosphoryl group is then transferred to the receiver domain of the response regulator that triggers a cellular response that is usually transcriptional control in bacteria (Hoch and Silhavy, 1995; Stock *et al*., 2000; Hoch, 2000).

In addition to two component regulatory systems, bacteria and archea have what is known as one-component systems for signal transduction. These systems have been considered to be more primitive and more widely distributed among prokaryotes. They are widespread in the archeaeal domain which is thought to lack the two component regulatory systems (Ulrich et al., 2005).

In these systems, within a single protein, there exists a predicted input and output domain that function to receive an environmental signal and elicit a response as a result (Ulrich et al., 2005). As an example, *Bacillus subtilis* RocR has a PAS (defined below) domain which acts as the sensor and a HTH (helix-turn-helix) domain acts as the output domain (Calogero *et al*., 1994). RocR is predicted to encode for a polypeptide that belongs to the NtrC/NifA family of transcriptional regulators found in *Escherichia coli*

(Calogero *et al*., 1994). In addition to PAS domains, several sensory/signaling domains have been studied extensively in bacteria. Some of these include, GAF (Hurley, 2003), GGDEF, EAL (defined below), HD-GYP, and HMA (heavy metal associated domain). (Galperin *et al*., 2001; Anantharaman *et al*., 2001). These domains are also called small molecule binding domains (SMBDs), involved in different types of regulatory processes (Anantharaman *et al*., 2001). The SO3389 ORF in *S. oneidensis* MR-1 encodes for a polypeptide that has unique domain architecture in that it has both the sensor module (PAS) and the signaling module (EAL-GGDEF) contained within the same protein which is typically trademark of a two-component regulatory system. However, the putative protein does not contain a histidine kinase and appears to be monocistronic without a cognate response regulator.

Signaling Domains

PAS

PAS domains were first recognized in *Drosophila* and since they have been identified from all three domains of life. These were identified in proteins that are serine/threonine kinases, chemo and photo receptors, circadian clock proteins, response regulators, and cyclic nucleotide phosphodiesterases (Taylor and Zhulin, 1999). It was named after the protein it was initially identified, PER (clock protein in *Drosophila*), which also comprises of transcription factors, ARNT (aryl-hydrocarbon receptor nuclear translocator) and SIM (single-minded protein which is required for central nervous system functioning in mammals). PAS sensory modules participate in sensing oxygen

tension, redox potential and light intensity. They are also involved in protein-protein interactions and bind to small ligands (Vreede *et al*., 2003; Anantharaman *et al*., 2001).

GGDEF and EAL

GGDEF and EAL (so named after conserved amino acid residues) domains are very commonly and extensively observed in bacterial genomes. However, to date, the domains have not been observed in genomes of archeae or eukaryotes (Galperin, 2004) and this result suggests that GGDEF/EAL mediated regulation is unique to bacterial cells. More often than not, GGDEF and EAL domains are observed within a single protein with the GGDEF domain in N-terminal position from the EAL domain with a few exceptions. Both domains, in concert, are involved in maintaining the turnover of c-di-GMP (Bis-(3'-5)-cyclic dimeric guanosine monophosphate), a secondary messenger molecule. GGDEF domains are thought to be involved in c-di-GMP production as it exhibits very close relation to adenylate cyclase found in mammals (Galperin *et al*., 1999). This relation was later established via genetic data and biochemical experimentation in *Salmonella* (Paul *et al*., 2004; Simm *et al*., 2004). EAL domains on the other hand, were shown to be responsible for c-di-GMP degradation. Experiments with purified EAL domains from *E. coli* proteins, Dos and YahA showed that EAL hydrolysed c-di-GMP molecules into linear GMP (Delgado-Nixon *et al*., 2000; Schmidt *et al*., 2005). Sensor domains, such as PAS, N-terminal of GGDEF-EAL, can receive and transmit input signals (environmental stimuli) that can alter intracellular c-di-CMP concentration. This alteration in concentration results in a behavioral response by the cell (Romling *et al*., 2005) as depicted in Figure 2.

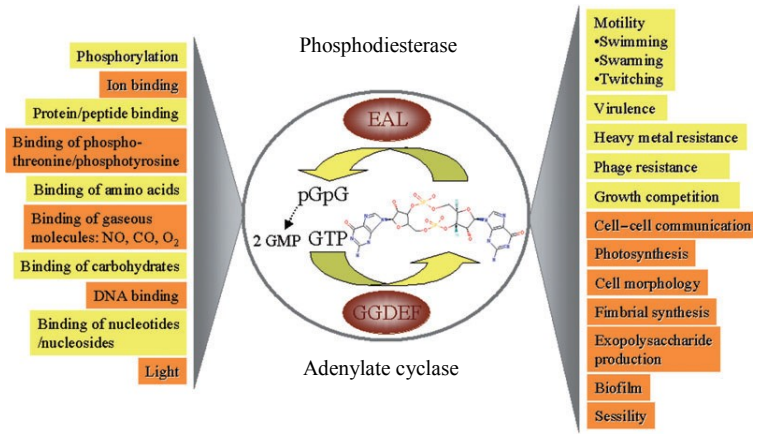


Figure 2: Input signals and output of c-di-GMP metabolism (modified, Romling *et al*., 2005) ΔSO3389, the mutant characterized in this thesis has a unique combination of sensor modules and signaling modules withing the open reading frame. The domains are a PAS, PAC, GGDEF and EAL. This mutant was characterized for phenotype defects to elucidate generalized functions for these domains.

Sulfate Reducing Bacteria

Discovery and History

In the 1860's, Louis Pasteur first discovered anaerobic bacteria and discussed fermentative organisms:- 'not only these infusoria live without air, but they are killed by air (Pasteur, 1861). Following the first description of anaerobes, another breakthrough was attained via the discovery of sulfate reducing bacteria (SRBs) in 1895 by Beijerinck (Beijerinck, 1895). Molecular oxygen is considered to be inhibitory to dissimilatory sulfate reduction and hence the growth of SRBs. Sulfate reducers were initially regarded as fermentative organisms based on their capacity to incompletely oxidize some substrates to acetate and CO_2. In 1954, a pigment in SRBs that was not identical to cytochrome *c* was first observed which was subsequently confirmed by studies from

another group. It was speculated that this cytochrome was involved in the metabolism of H_2 and perhaps played a role in sulfate-reduction. However efforts to demonstrate a role in sulfate reduction were unsuccessful (Postgate, 1954; Ishimoto *et al*., 1954). Later in 1959, it was demonstrated that *Desulfovibrio desulfuricans* sulfate reduction proceeded via a complex pathway in the presence of ATP and molecular hydrogen (Peck, 1959). In addition to incomplete oxidation in the presence of acetate and propionate, some SRB's isolated from the North Sea were found to be capable of utilizing other simple organic compounds as electron donors that were completely oxidized to CO_2 (Widdel and Pfenning, 1981; Widdel and Pfenning, 1982). Electrons generated via oxidation of a wide variety of electron donors are utilized towards the reduction of many electron acceptors. Although named after a single electron acceptor, several studies have indicated that besides sulfur compounds and fumarate (Miller and Wakerley, 1966), some SRBs are capable of reducing a wide range of electron acceptors. They have been reported to carry out dissimilatory nitrate reduction, carbonate respiration, iron, uranium and chromate, arsenate and Mn (IV) reduction (Widdel and Pfenning, 1982; Stackebrandt *et al*., 1997; Coleman *et al*., 1993; Lovley *et al*., 1993; Lovley and Phillips, 1992; Lovley and Phillips, 1994; Tebo and Obraztsova, 1998; Newman *et al*., 1997). SRBs are observed in diverse environments and due to their respiratory capabilities; they are often significant players in anaerobic biomineralization pathways that link the carbon and sulfur cycles (Barton and Hamilton, 2007).

Where Do SRBs Live?

SRBs are commonly found in sulfate-rich anoxic environments, both in fresh water and marine environments. These environments have varying concentrations of sulfate ranging from 1mM in fresh water to 30mM in marine sediments (Sass *et al*., 1992; Holmer and Storkholm, 2001). In addition, SRBs have also been isolated from more oxic environments. For instance, *Desulfovibrio oxyclinae* has been reported to inhabit cyanobacterial mats where drastic fluctuations in oxygen concentrations can occur (Krekler *et al*., 1998) while some species belonging to *Desulfococcus* and *Desulfobacter* occupy the deeper layers (anoxic) of bacterial mats. On the other hand, some representatives from *Desulfovibrio* thrive in the oxic-anoxic interfaces of the sediments where oxygen concentrations can reach up to1mM (Jonkers *et al*., 2005; Eschemann *et al*., 1999; Brune *et al*., 2000). Because of these observations, studies in the last twenty-five years have sought to understand how SRBS respond to oxygen. . Due to the inherent problems associated with production of potentially toxic sulfides, interpretation of experimental results can be difficult; however, the impact of oxygen on the physiology and metabolism of SRBs is important to understand with implications for ecosystem function from natural systems to metal corrosion to oil souring to bioremediation

Oxidative Stress Response in SRBs

SRBs have been perceived to be stringent anaerobes since their initial discovery and subsequent studies. However, observations of environmental distributions have prompted some researchers to question the assumption of essential anaerobiosis for

SRBs, and the genome content of different SRBs indicate they have developed several strategies to obtain maximal protection against the deleterious effects of oxygen exposure (Brioukhanov and Netrusov, 2006). Initially, SRBs were thought to remain dormant but not be killed when exposed to ambient atmosphere (Postgate, 1984). Later, it was established that some SRBs could not only tolerate oxygen but could also carry out aerobic respiration (Cypionka *et al*., 1985; Dilling and Cypionka, 1990; Johnson *et al*., 1997). Subsequent work in the last decade has shown that SRBs exhibit diverse behavioral responses to oxygen exposure. Some cells form aggregates or clump together when exposed to changing oxygen concentrations (Krekler *et al*., 1998), while other cells can migrate to minimize exposure to O_2 in response to changing O_2 concentration in the environment. . During daytime, under oxic conditions, SRB cell counts were 20-fold lower in the upper 3mm layer of the mat compared to night time (in the absence of oxygen) (Krekler *et al*., 1998). Aerotaxis is yet another phenomenon displayed by SRBs in oxic- anoxic interfaces where systems are highly stratified in terms of oxygen distribution. In these environments, SRBs exhibit positive and negative responses to oxygen. Some genes, involved in oxygen sensing play a role in aerotaxis (Eschemann *et al*., 1999). Sass *et al* showed oxygen exposure induced morphological changes in *Desulfovibrio litoralis* and *Desulfovibrio cuneatus* wherein cells attain a more atypical elongated form in the presence of oxygen (Sass *et al*., 1998).

Protective Mechanisms Towards Oxygen Exposure

Anaerobes were thus classified based upon the lack key enzymes and detoxifying systems (e.g.,, catalase, superoxide dismutase, etc) that are present in aerobes and facultatives that participate in alleviating and/or eliminating deleterious effects of oxidative stress. An iron-containing superoxide dismutase was described in *Desulfovibrio desulfuricans* in 1977 (Hatchikian, 1977) and further studies have revealed that SOD (superoxide dismutase) and catalase have been found in several species of *Desulfovibrio,* for example*, Desulfovibrio gigas* (Dos Santos *et al*., 2000). Both enzymes play a vital role in detoxification of oxygen by-products and SOD and catalase are constitutively expressed in *D. gigas* (Dos Santos *et al*., 2000). Catalase activity generates oxygen during detoxification step and hence most anaerobes use this system as a secondary system to eliminate peroxide. Reactive oxygen species are eradicated without re-generation of oxygen primarily by superoxide reductase and rubrerythrin/ rubredoxin system. First characterized in SRBs in 1994 by Moura *et al*, (Moura *et al*, 1994) Rbr and Rbo were described in *Desulfovibrio vulgaris* Hildenborough in the late 1990's. In addition, two non-heme reductases, neelaredoxin and nigererythin, were isolated from *Desulfovibrio* species (Lumppio *et al*., 1997; Lumppio *et al*., 2001; Moura *et al*., 1994; Fournier *et al*, 2003). An (Fe) hydrogenase in *Desulfovibrio vulgaris* Hildenborough was reported to be involved in protection of this organism against oxidative stress (Fournier *et al*., 2004). A few reports indicate that *Desulfovibrio* sp. are capable of respiring endogenous carbohydrates in the presence of oxygen (by reducing oxygen) as a protective mechanism (van Niel *et al*., 1996 and van Niel and Gottschal, 1998).

Thesis Motive

Through our hypothesis we attempt to resolve the mechanism by which facultative or 'stringent' anaerobes, respond to oxygen. While facultative organisms like *Shewanella oneidensis* MR-1 have evolved regulatory mechanisms (one-component systems) to sense oxygen, anaerobes like *Desulfovibrio vulgaris* Hildenborough have developed machinery to reduce or perhaps utilize molecular oxygen to alleviate the deleterious effects of this stressor.

Chapters 1 and 2 involve experiments that elucidate function to a sensory box protein with a unique domain architecture. The open reading frame characterized has PAS, PAS, GGDEF and EAL domains. Experiments were designed to elucidate the potential environmental stimulus as well as possible physiolgoical roles of this protein in MR-1.

Chapters 3 and 4 address substrate-based differential response of *Desulfovibrio vulgaris* Hildenborough (*D. vulgaris*) to dissolved oxygen (DO) since in natural environments; these organisms are constantly exposed to DO. Lactate and pyruvate were used as electron donors in this study as most of the documented work with *D. vulgaris* has been carried out with lactate and pyruvate as carbon/energy sources and *D. vulgaris* exhibits high growth yields with these electron donors.

References

Anantharaman, V., E. V. Koonin, and L. Aravind.2001. Regulatory potential, phyletic distribution and evolution of ancient, intracellular small-molecule-binding domains. J. Mol. Biol. 307:1271-1292.

Beijerinck, M. W.1895.Uber Spirillum desulfuricans als Ursache von Sulfate reduction. Zentbl. Bakt. Parasitkde (abs 2), 1st Edn., pp. 1-9, 45-59, 104-114.

Beliaev, A. S., D. A. Saffarini, J. L. McLaughlin, and D. Hunnicutt.2001. MtrC, an outer membrane decahaem c cytochrome required for metal reduction in *Shewanella putrefaciens*MR1. Mol. Microbiol. 39:722–730.

Beliaev,A.S., and D. A. Saffarini.1998. *Shewanella putrefaciens mtrB* encodes an outer membrane protein required for Fe(III) and Mn(IV) reduction. J. Bacteriol. 180: 6292–6297.

Berner, R. A. 2004.The Phanerozoic carbon cycle: CO_2 and O_2. Oxford University Press, New York. Biochemistry 39:2685-2691.

Brink, A. J., A. van Straten, and A. J. van Rensburg. 1995. *Shewanella (Pseudomonas pu trefaciens).* Bacteremia. Cl. Inf. Dis. 20: 1327-1332.

Brioukhanov, A. L., and A. I. Netrusov.2007. Aerotolerance of strictly anaerobic microorganisms and factors of defense against oxidative stress: A review. Appl. Biochem. Microbiol. 43:637-654.

Brocks, J. J., G. A. Logan, R. Buick, and R. E. Summons.1999. Archean molecular fossils and the early rise of eukaryotes. Science. 285:1033-1036.

Brune, A., P. Frenzel, and H. Cypionka.2000. Life at the oxic-anoxic interface: microbial activities and adaptations. FEMS Microbiol. Rev. 24:691-710.

Burns, J. L., and T. J. DiChristina.2009.Anaerobic respiration of elemental sulfur and thiosulfate by *Shewanella oneidensis* MR-1 requires psrA, a homolog of the phsA gene of *Salmonella enterica* Serovar Typhimurium LT2. Appl. Environ. Microbiol. 75: 5209-5217.

Calogero, S., R. Gardan, P. Glaser, J. Schweizer, G. Rapoport, and M. Debarbouille. 1994. RocR, a novel regulatory protein controlling arginine utilization in *Bacillus subtilis*, belongs to the NtrC/NifA family of transcriptional activators. J. Bacteriol. 176:1234-1241.

Catling, D. C., and M. W. Claire.2005. How earth's atmosphere evolved to an oxic state:a status report. Earth. Planet. Sci. Lett. 237:1-20.

Coleman, M. L., D. B. Hedrick, D. R. Lovley, D. C. White, and K. Pye.1993. Reduction of Fe(III) in sediments by sulfate-reducing bacteria. Nature. 361:436–38.

Cypionka, H., F. Widdel, N. Pfennig. 1985. Survival of sulfate-reducing bacteria after oxygen stress, and growth in sulfate free oxygen-sulfide gradients. FEMS Microbiol.Ecol. 31:39–45.

Delgado-Nixon, V. M., G. Gonzalez, and M.A. Gilles-Gonzalez. 2000. Dos, a heme-binding PAS protein from *Escherichia coli*, is a direct oxygen sensor. Biochemistry 39:2685-2691.

Des Marais, D. J., H. Strauss, R. E. Summons, and J. M. Hayes.1993.Carbon isotope evidence for the stepwise oxidation of the Proterozoic environment. Nature 362: 117-118.

Dilling, W., and H. Cypionka.1990. Aerobic respiration in sulfate reducing bacteria. FEMS Microbiol. Ecol. 73:123-128.

Dos Santos, W.G., I. Pacheco, M. Y. Liu, M. Texeira, A.V. Xavier, and J. DeGall.2000. Purification and characterization of an iron superoxide dismutanse and a catalase from the sulfate reducing bacterium *Desulfovibrio gigas*. J. Bacteriol. 182:796-804.

Eschemann, A., M. Kuhl, and H. Cypionka.1999. Aerotaxis in *Desulfovibrio.* Environ. Microbiol. 1:489-494.

Fournier, M., Y. Zhang, J. D. Wildschut, A. Dolla, J. K. Voordouw, D. C. Schriemer and G. Voordouw. 2003. Function of oxygen resistance proteins in the anaerobic sulfate reducing bacterium *Desulfovibrio vulgaris* Hildenborough. J. Bacteriol. 185:71-79.

Fournier, M., Z. Dermoun, M-C. Durand, and A. Dolla. 2004. A new function of the *Desulfovibrio vulgaris* Hildenborough (Fe) hydrogenase in the protection against oxidative stress. J. Biol. Chem. 279:1787-1793.

Galperin, M. Y., A. N. Nikolskaya, and E. Koonin.2001. Novel domains of the prokaryotic two-component signal transduction systems. FEMS. Microbiol. Lett. 203:11-21.

Galperin, M. Y., and E. V. Koonin.2004. 'Conserved hypothetical' proteins: prioritization of targets for experimental study. Nucleic. Acid. Res. 32:5452-5463.

Galperin, M. Y., D. A. Natale, L. Aravind and E. V. Koonin.1999. A specialized version of the HD hydrolase domain implicated in signal transduction. J. Mol. Microbiol. Biotechnol. 1:303-305.

Galperin, M. Y.2004 Bacterial signal transduction network in a genomic perspective. Environ. Microbiol. 6: 552-567.

Gon. S, J. C. Patte, J. P. Dos Santos, and V. Mejean.2002. Reconstitution of the trimethylamine oxide reductase regulatory elements of *Shewanella oneidensis* in *Escherichia coli*. J. Bacteriol. 184: 1262-1269.

Hatchikian, E., and Y. A. Henry.1977. An iron-containing superoxide dismutase from the strict anaerobe *Desulfovibrio desulfuricans* (Norway 4). Biochemie 59: 153-161.
Heidelberg, J. F., I.T. Paulsen, K. E. Nelson, E. J. Gaidos, and W. C. Nelson.2002. Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. Nat. Biotech. 20:1118-1123.

Hoch, J. A., and T. J. Silhavy eds.1995. Two-component signal transduction, ASM Press.

Hoch, J.A. 2000.Two-component and phosphorelay signal transduction. Curr. Opin. Microbiol. 3:165-170.

Holmer, M., and P. Storkholm.2001.Sulfate reduction and sulfur cycling in lake sediments: a review. Freshwater. Biol. 46:431-451.

Hurley, J. H.2003. GAF domains: cyclic nucleotides come full circle. Sci STKE. 2003. PE1.

Ishimoto, M., J. Koyama, and Y. Nagai.1954 Biochemical studies on sulfate-reducing bacteria. IV. The cytochrome system of sulfate-reducing bacteria. J. Biochem. 41:763-70.

Johnson, M. S., I. B. Zhulin, M. E. R. Gapuzan, and B. L. Taylor.1997. Oxygen-dependentgrowth of the obligate anaerobe *Desulfovibrio vulgaris* Hildenborough. J. Bacteriol. 179: 5598-5601.

Jonkers, H. M., I. O. Koh, P. Behrend, G. Muyzer, and D. de Beer.2005. Aerobic organic carbon mineralization by sulfate-reducing bacteria in the oxygen-saturated photic zone of a hypersaline microbial mat. Microbial. Ecol. 49:291-300.

Jørgensen, B. R and H. H. Huss.1989. Growth and activity of *Shewanella putrefaciens* isolated from spoiling fish. Int. J. Food. Microbiol. 9:51-62.

Kasting, J. F., D. P. Whitmire, and R. T. Reynolds.1993. Habitable zones around main sequence stars. Icarus 101:108-128.

Klonowska, A., T. Heuli, and A. Vermeglio.2005. Selenite and Tellurite reduction by *Shewanella oneidensis*. Appl. Environ. Microbiol. 71: 5607-5609.

Kolker, E., A. F. Picone, M. Y. Galperin, M. F. Romine, R. Higdon, K. S. Makarova, N. Kolker, G. A. Anderson, X. Y. Qiu, K. J. Auberry, G. Babnigg, A. S. Beliaev, P. Edlefsen, D. A. Elias, Y. A. Gorby, T. Holzman, J. A. Klappenbach, K. T. Konstantinidis , M. L. Land, M. S. Lipton, L. A. McCue, M. Monroe, L. Pasa-Tolic, G. Pinchuk, S. Purvine, M. H. Serres, S. Tsapin, B. A. Zakrajsek, J. H. Zhou, F. W. Larimer, C. E. Lawrence, M. Riley, F. R. Collart, J. R. Yates, R. D. Smith, C. S. Giometti, K. H. Nealson, J. K. Fredrickson, and J. M. Tiedje.2005. Global profiling of *Shewanella oneidensis* MR-1: Expression of hypothetical genes and improved functional annotations. PNAS. 102: 2099-2104.

Krekler. D., A. Teske, and H. Cypionka.1998. Strategies of sulfate-reducing bacteria to escape oxygen stress in a cyanobacterial mat. FEMS. Microbiol. Ecol. 25: 98-96.

Liu, C., Y. A. Gorby, J. M. John, J. K. Fredrickson, and C. F. Brown.2002.Reduction kinetics of Fe(III), Co(III), U(VI), Cr(VI), and Tc(VII) in cultures of dissimilatory metal-reducing bacteria. Biotechnol. Bioengg. 80: 637-649.

Lovley, D. R., and E. J. P. Phillips. 1992. Reduction of uranium by *Desulfovibrio desulfuricans*. Appl. Environ. Microbiol. 58:850–56.

Lovley, D. R., E. J. P. Phillips. 1994. Reduction of chromate by *Desulfovibrio vulgaris* and its c3 cytochrome. Appl. Environ.Microbiol. 60:726–28.

Lovley, D. R.E. E. Roden, E. J. P. Phillips, and J. C. Woodward. 1993. Enzymatic iron and uranium reduction by sulfate-reducing bacteria. Mar. Geol. 113:41–53.

Lovley,D. R and J. R. Phillips.1988. Novel mode of microbial energy metabolism: Organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl. Environ. Microbiol. 54: 1472-1480.

Lumppio, H. L., N. V. Shenvi, G. Voordouw, A. O. Summers, and D. M. Kurtz, Jr. 2001. Rubrerythrin and rubredoxin oxidoreductase in *Desulfovibrio vulgaris*: a novel oxidative stress protection system. J. Bacteriol. 183: 101-108.

Lumppio, H. L., N. V. Shenvi, R. P. Garg, A. O. Summers, and D. M. Kurtz, Jr. 1997. A rubrerythrin operon and nigerythrin gene in *Desulfovibrio vulgaris* (Hildenborough). J. Bacteriol. 179:4607–4615.

Maier, T. M., and C. R. Myers.2004.The outer membrane protein Omp35 affects the reduction of Fe(III), nitrate, and fumarate by *Shewanella oneidensis* MR-1. BMC. Microbiol. 4:23.

Meyer, T. E., A. I. Tsapin, I. Vandenberghe,L. Smet, D. Frishman, K. H. Nelason, M. A. Cusanovich, and J. J. VanBeeuman.2004. Identification of 42 possile cytochrome c genes in the *Shewanella oneidensis* genome and characterization of six soluble cytochromes. J. Int. Biol. 8: 57-77.

Miller, J. D. A., and D. S. Wakerley.1966. Growth of sulfate reducing bacteria by fumarate dismutation. J. Gen. Microbiol. 43: 101-7.

Moser, D. P and K. H. Nealson.1996. Growth of the facultative anaerobe *Shewanella putrefaciens* by elemental sulfur reduction. Appl. Environ Microbiol. 62: 2100–2105.

Moura, I., P. Tavares, and N. Ravi. 1994. Characterization of 3 proteins containing multiple iron sites—rubrerythrin, desulfoferrodoxin, and a protein containing a six-iron cluster. Methods. Enzymol. 243:216–240.

Myers, C. R and K. H. Nealson. 1988. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. Science. 240: 1319-1321.

Myers, C. R., and J. M. Myers.1997.Cloning and sequence of *cymA*, a gene encoding a tetraheme cytochrome c required for reduction of iron(III), fumarate, and nitrate by *Shewanella putrefaciens MR-1*. J. Bacteriol. 179: 1143–1152.

Myers, J. M., and C. R. Myers.1998.Isolation and sequence of *omcA*, a gene encoding a decaheme outer membrane cytochrome c of *Shewanella putrefaciens* MR-1, and detection of *omcA* homologs in other strains of *S. putrefaciens*. Biochim. Biophys. Acta 1373: 237–251.

Myers, J. M., and C. R. Myers.2000. Role of the tetraheme cytochrome CymA in anaerobic electron transport in cells of *Shewanella putrefaciens MR-1* with normal levels of menaquinone. J. Bacteriol. 182: 67–75.

Myers, J. M., and C. R. Myers.2001. Role for outer membrane cytochromes OmcA and OmcB of *Shewanella putrefaciens* MR-1 in reduction of manganese dioxide. Appl. Environ. Microbiol. 67: 260–269.

Myers, J. M., and C. R. Myers.2002. Genetic complementation of an outer membrane cytochrome *omcB* mutant of *Shewanella putrefaciens* MR-1 requires *omcB* plus downstream DNA. Appl. Environ. Microbiol. 68: 2781–2793.

Nealson, K. H and D. Saffarini.1994. Iron and manganese in anaerobic respiration: environmental significance, physiology and regulation. Annu. Rev. Microbiol. 48:311-343.

Newman, D. K., E. K. Kennedy, J. D. Coates, D. Ahmann, and D. J. Ellis.1997.

Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum* sp. nov. Arch. Microbiol. 168:380–88.

Pasteur, L. 1861. Animalcules infusoires vivant sans gaz oxigene libre et determinant des fermentations. Compt. Rendus. Acad. Sci. Paris, L II: 344-347.

Paul, R., S. Weiser, N. C. Amiot, C. Chan, T. Schirmer, and B. Geise.2004. Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. Genes. Dev. 18:715-727.

Payne, J. L., C. R. McClain, A. G. Boyer, J. H. Brown, S. Finnegan, M. Kowalewski, R. A. Krause, S. K. Lyons, D. W. McShea, P. M. Novack-Gottshall , F. A. Smith, P. Spaeth, J. A. Stempien, and S. C. Wang.2011. The evolutionary consequences of oxygenic photosynthesis: a body size perspective. Photosyn. Res. 107: 37-57.

Peck, H. D.1959. The ATP-dependent reduction of sulfate with hydrogen in extracts of *Desulfovibrio desulfuricans*. PNAS. 45: 701-708.

Pereira, P. M., Q. He, A. V. Xavier, J. Z. Zhou, I. A. C. Pereira, and R. O. Louro.2008. Transcriptional response of *Desulfovibrio vulgaris* Hildenborough to oxidative stress mimicking environmental conditions. Arch. Microbiol. 189: 451-461.

Perry, K. A., J. E. Kostka, G. W. Luther III and K. H. Nealson.1993.Mediation of sulfur speciation by a Black Sea facultative anaerobe. Science. 259: 801-803.

Pitts, K. E., P. S. Dobbin, F. Reyes-Ramirez, A. J. Thomson,D. J. Richardson, and H. E. Seward.2003. Characterization of the *Shewanella oneidensis* MR-1 Decaheme Cytochrome MtrA. J. Biol. Chem. 278: 27758–27765.

Postgage, J. R.1984. The sulfate-reducing bacteria, 2nd Edn, p.208 Cambridge University Press, Cambridge.

Postgate ,J. R.1954. Presence of cytochrome in an obligate anaerobe. Biochem. J. 56: 11-12.

Romling, U., M. Gomelsky, and M. Y. Galperin.2005. C-di-GMP: the dawning of a novel bacterial signaling system. Mol. Microbiol. 57:629-639.

Sass, H., J. Steuber, M. Kroder, P. M. Kroneck, and H. Cypionka.1992. Formation of thionates by fresh-water and marine strains of sulfate reducing bacteria. Arch. Microbio. 158:418-421.

Sass, H., M. Berchtold, J. Branke, H. Konig, H. Cypionka, and H. D. Babenzien.1998. Psychrotolerant sulfate-reducing bacteria from an oxic freshwater sediment, description

of *Desulfovibrio cuneatus* sp. nov. and *Desulfovibrio litoralis* sp. nov. Syst. Appl. Microbiol. 21: 212-219.

Schwalb, C, S. K. Chapman, and G. A. Reid.2003.The tetraheme cytochrome CymA is required for anaerobic respiration with dimethyl sulfoxide and nitrite in *Shewanella oneidensis*. Biochemistry. 31: 9491-9497.

Schwalb, C., S. K. Chapman, and G. A. Reid.2002. The membrane-bound tetrahaem c-type cytochrome CymA interacts directly with the soluble fumarate reductase in *Shewanella*. Biochem. Soc. Trans. 30: 658-662.

Shioi, J., C. V. Dang, and B. L. Taylor.1987. Oxygen as attractant and repellent in bacterial chemotaxis. J.Bacteriol. 169:3118-3123.

Simm. R., M. Morr, A. Kader, M. Nimtz and U. Romling.2004. GGDEF and EAL domains inversely regulate c-di-GMP levels and transitions from sessility to motility. Mol. Microbiol. 53:1123-1134.

Stackebrandt , E., C. Sproer, F. A. Rainey,J. Burghardt, O. P¨auke, and H. Hippe.1997. Phylogenetic analysis of the genus *Desulfotomaculum*:evidence for the misclassification of *Desulfotomaculum guttoideum* and description of *Desulfotomaculum orientis* as *Desulfosporosinus orientis* gen. nov., comb. nov. Int. J. Syst. Bacteriol.47:1134–39.

Stock, A.M., V. L. Robinson, and P. N. Goudreau.2000. Two component signal transduction. Annual Rev. Biochem. 69:183-215.

Taylor, B. L., and I. B. Zhulin.1999. PAS domains: Internal sensors of oxygen, redox potential and light. Microbiol. Mol. Bio. Rev. 63:479-506.

Tebo, B. M., and A. Y. Obraztsova. 1998. Sulfate reducing bacterium grows with Cr(VI),U(VI), Mn(IV), and Fe(III) as electron acceptors. FEMS Microbiol. Lett. 162:193–98.

Tian, F., O. B. Toon, A. A. Pavolv, and H. DeSterck.2005. A hydrogen-rich early Earth atmosphere. Science. 308:1014-1017.

Ulrich, L. E., E. V. Koonin, and Zhulin. I. B. 2005 One-component systems dominate signal transduction in prokaryotes. Trends. Microbiol. 13:52-56.

Vreede, J., M. A. van der Horst, K. J. Hellingwerf, W. Crielaard, and D. M. F. van Aalten.2003. PAS domains - Common structure and common flexibility. J. Biol. Chem. 278:18434-18439.

Walker, J. C. G., P. B. Hays, and J. F. Kasting.1981.A negative feedback mechanism for the long term stabilization of Earth's surface temperature. J. Geophys. Res. 86:9776-9782.

Yost, C., L. Hauser, F. Larimer, D. K. Thompson, A. S. Beliaev, J. Zhou, Y. Xu and D. Xu. 2003.A computational study of *Shewanella oneidensis* MR-1: Structural prediction and functional inference of hypothetical proteins. OMICS. 7:177-191.

CHAPTER 2

A *SHEWANELLA ONEIDENSIS* MR-1 SENSORY BOX 1 PROTEIN INVOLVED IN AEROBIC AND ANOXIC GROWTH

Contribution of Authors and Co-Authors

Manuscripts in Chapter 2

Chapter 2:

Author: Anitha Sundararajan

Contributions: Conducted most experiments, analyzed data and helped in manuscript writing

Co-author: Jacob Kurowski

Contributions: Started mutant characterization as an undergraduate researcher

Co-author: TingFen Yan, Dawn Klingeman, Marcin Joachimiak, Jizhong Zhou,

Contributions: Mutant construction, gene expression experiment and analysis.

Co-author: Jeff Gralnick, Belen Naranjo

Contributions: Genetic Complementation

Corresponding Author: Matthew Fields

Contributions: Principal Investigator

Manuscript Information Page

Anitha Sundararajan, Jacob Kurowski, TingFen Yan, Dawn. M. Klingeman, Marcin. P. Joachimiak, Jizhong Zhou, Jeff. D. Gralnick, Belen Naranjo and Matthew. W. Fields
Journal Name: Applied and Environmental Microbiology
Status of manuscript
___Prepared for submission to a peer-reviewed journal
__X_Officially submitted to a peer-reviewed journal
___Accepted by a peer-reviewed journal
___Published in a peer-reviewed journal
Published by American Society for Microbiology, High Wire Press
Date of submission: 22th December, 2010

Abstract

Shewanella oneidensis MR-1 is a facultative anaerobic bacterium that can utilize a wide assortment of electron acceptors for energy; however, little is known how energetically versatile organisms such as *S. oneidensis* sense and process environmental stimuli to optimize electron flow and metabolism. Although little is known of potential function for conserved signaling proteins, it is hypothesized that such proteins play roles in coordinating responses to environmental stimuli. In order to elucidate function for a putative sensory box protein, the physiological role of SO3389 in *S. oneidensis* MR-1 was characterized. The predicted ORF encodes a putative sensory box protein that has PAS, GGDEF, and EAL domains, and an in-frame, deletion mutant was constructed (△SO*3389*) with approximately 95% of the ORF deleted (*i.e.*, intact domains were not present). Under aerated conditions, wild-type and mutant cultures had similar growth rates, but the mutant culture had a slower growth rate in aerobic, static conditions. Oxygen consumption rates were slightly lower for mutant cultures (1.5-fold), and wild-type cultures also maintained lower dissolved oxygen levels under aerated growth conditions. When transferred to anoxic conditions, the mutant did not grow with fumarate, iron (III), or DMSO as electron acceptors. Biochemical assays demonstrated the expression of different *c*-type cytochromes as well as decreased fumarate reductase activity in the mutant transferred to anoxic growth conditions and transcriptomic studies showed the inability of the mutant to up-express and down-express particular *c*-type cytochromes. The complemented strain did not lag when transferred from aerobic to anoxic growth conditions with the tested electron acceptors, and had comparable growth rates under aerobic, static conditions to wild-type. Despite low peptide sequence identity (31% to 42%), the modeled structure for the SO3389 PAS domains was highly similar to the crystal structures of FAD-binding MmoS and NifL PAS domains that are known O_2/redox sensors. Based on physiological, genomic, and bioinformatic results, we suggest that the sensory box protein, SO3389, is an O_2/redox-sensor that is involved in optimization of aerobic growth and transitions to anoxia.

Introduction

In the post-genomics era, uncharacterized genes pose a major challenge in biology (Galperin 2004), and many sequenced genomes still contain a large fraction (approximately 30%) of genes without defined physiological roles. In fact, many aspects of signaling and stress response most likely reside in this fraction of genes for a given organism and underlie the lack of understanding in physiological responses and control of metabolism. Many uncharacterized genes/proteins are classified as sensory box proteins based upon conserved domains, but signals and cellular responses for most presumptive sensory box proteins are not known.

Shewanella oneidensis MR-1, a facultative anaerobe classified as a γ-Proteobacterium, can utilize numerous organic and inorganic compounds for energy including oxygen, nitrates, and metals. The *S. oneidensis* MR-1 genome sequence was determined (Heidelberg *et al*. 2002) and the most recent annotation of the 5.1-Mb genome estimated 4,467 genes of which 1,623 had hypothetical functions (Kolker *et al*. 2005). Many bacteria considered to be "environmental" organisms typically have diverse metabolic capacity, particularly facultative organisms such as *Shewanella*, and aside some well-studied systems (e.g., *Escherichia, Bacillus*, pathogens) little is known how these organisms sense and respond to changing environments.

An earlier study reported a correlation between the total number of PAS protein domains [*Drosophila* period clock, aryl hydrocarbon receptor, single-minded protein] (Ponting and Aravind 1997) and the number of respiratory and photosynthetic electron transport-associated proteins in completely sequenced microbial genomes (Zhulin and

Taylor 1998). The MR-1 genome has approximately 31 ORFs that are predicted to encode PAS domains and this compares to approximately 15 PAS domains in *Escherichia coli* K-12 MG1655 (www.microbesonline.org). PAS domains are usually part of larger polypeptides involved in signal transduction and previous studies have shown that PAS domains can sense changes in environmental stimuli, such as light, redox potential, oxygen, and energy state of the cell (Taylor and Zhulin 1999). As recently noted, many bacterial genomes contain a wide variety of presumptive signal transduction systems (Galperin 2004); however, the physiological role or the exact array of signals is not known. A high number of PAS proteins likely coincide with the fact that MR-1 can utilize a wide variety of electron acceptors and must be able to coordinate a diverse metabolic capacity with varying environmental conditions.

As of May 2010, the Pfam database (v24.0) reports 33,313 sequences classified in the PAS protein clan that contains 7 families, and the PAS and PAC (PAS associated domains) motifs adopt a single globular fold of approximately 100 residues now known as the PAS domain (Moglich *et al*. 2009). PAS domains are observed in all three domains of life, including metazoans, in which the domains can be part of hypoxia-inducible factors that play important roles in anaerobic metabolism, oxygen delivery, and angiogenesis (Scheurmann *et al*. 2007). The few PAS domain proteins that have been studied in a physiological context have been observed to bind different co-factors (*e.g.*, metabolites, ions, heme, and flavin nucleotides), and these interactions in turn control a catalytic output (*e.g.*, kinase, cyclase, esterase). However, physiological roles for the vast majority of putative PAS proteins are unknown; therefore, phenotypic

characterization would provide great insight into the relationship between biological stress response and environmental perturbations.

The fact that most sequenced genomes contain a high percentage of conserved hypothetical proteins with unknown physiological roles represents a knowledge gap across biology, and the current work set out to characterize the physiological role of a conserved sensory box protein. The mutant was affected in low-oxygen growth responses and transitions to anoxia, and these phenotypes could be at least partially explained by altered *c*-type cytochrome content, oxygen consumption, and reductase activity. In addition, the modeled PAS domain structure suggested high structural similarity to known FAD-based O_2/redox-sensors.

Materials and Methods

Sequence Comparisons

The Smart (Simple Modular Architecture Research Tool) database (v.6.0) and the Microbes On Line website were used for domain predictions and comparisons as previously described (Dehal *et al*., 2009; Letunic *et al*., 2008). Phylogenetic and molecular evolutionary analyses were conducted with MUSCLE (v3.7) (www.ebi.ac.uk/Tools/muscle/index.html) and within the Microbes On Line workbench (www.microbesonline.org). Maximum-likelihood trees were constructed with a Jones, Taylor and Thornton **(**JTT**)** model with no correction for across-site rate variation with the Microbes On Line workbench. The protein model for SO3389 was predicted with CPHmodels (v3.0) and I-Tasser (Nielsen *et al*., 2010; Roy *et al*., 2010). The CPHmodels

quality was checked with QMEAN (Benkert *et al*., 2009), ProSA (Wiedertstein and Sippl, 2007), and MolProbity (Davis *et al*., 2007). I-Tasser has an internal method to check model quality (Roy *et al*., 2010). Protein Structural Alignment (v3.04) at GANGSTA^{+}, DaliLite (v3.1), Phyre (v0.2), and I-Tasser were used for structural alignments with crystal structures of known PAS domains (Guindon and Gascuel, 2003; Holm *et al*., 2008; Kelley *et al*., 2009; Roy *et al*., 2010).

Mutant Construction

An approximately 2.4 kbp fragment was deleted from SO3389 (2639 bp) with PCR-based crossover amplification as described previously (Gao *et al*., 2006; Wan *et al*., 2004). The primer pairs used to generate two DNA fragments with 3' staggered ends were as follows: No-5'-CGCGAGCTCGTTCGGACCTTCGATAAATC-3' and Ni-5' TGT TTAAACTTAGTGGATGGGGCCCAATCTGTTAACTGCTT 3' and Co-5' CGC GAGCTCGCACCTTGACGCAAATGATC-3' and Ci-5'-CCCATCCACTAAGTTTAA ACAGCGGATGTTGAGGCCTTTTT-3' (non-complementary tag sequences are underlined) and the outside primers were used to amplify a fusion molecule. The crossover PCR product was purified and digested by SacI, cloned into treated suicide vector pDS3.0, and electroporated into *E. coli* S17-1/λ_{pir}. The suicide plasmid construct was moved into MR-1 as previously described (Gao *et al*., 2006; Wan *et al*., 2004), and the mutated SO*3389* gene was verified by PCR (5'-GAGTGGCATTCAGCACTAGA-3' and 5'-CATACGGTCGGCTTCATCAA-3') and sequence determination. The resulting mutant did not possess any of the predicted domains after completion of the in-frame

deletion. The mutation was an in-frame deletion that removed approximately 2400 bp of the gene.

△SO*3389* Complementation

Multiple vectors (pACYC184, pJB3Cm6, pBBR1MCS-5, and pBAD; see Table 1) were attempted for complementation, but construction of the correct, stable *E. coli* strain was not successful or expression was not detected in the case of pBAD. Therefore, pBBR1MCS-2 and pBBAD were used, and the following complementation primers were used to amplify genomic DNA: 3389FWDCOM (GAATTCTGTCTGCGGTAATATCTGCG) and 3389REVCOM (GGATCCCACCAAAAGCAATCATGTCG) (Table 1). The amplicon was cloned into a TOPO vector and digested with EcoR1 and BamH1. Post digestion, the insert was purified with a QIAquick Gel Extraction Kit as per instructions and subsequently cloned by ligation into vectors, pBBR1MCS-2 and pBBAD at 1:3 and 1:10 molar ratios (vector to insert) also digested using EcoR1 and BamH1. To increase transformation efficiency, ligations were transformed into *E. coli* UQ950 and then *E. coli* WM3064, a diaminopimelic (DAP) auxotroph. Transformants thus obtained were verified for inserts by PCR. The correct transformant was then mated with △SO*3389* onto a LB-DAP (0.3 mM) plate and incubated at 30°C for 24 hours. Transconjugants were selected by streaking mating mixtures onto LB-Kn_{50}.

Table 1. Strains and vectors.		
***Shewanella oneidensis* MR-1**		
S. oneidensis MR-1		Heidelberg *et al*.,2002.
ΔSO3389	in-frame deletion of SO3389	this study
ΔSO3389-pBBR1MCS-SO3389	complemented strain	this study
Escherichia coli		
S17-1/λ_{pir}	Mating strain	Wan *et al*., 2004.
UQ950	MDH5α/λ_{pir}	Saltikov and Newman, 2003.
WM3064	DAP autotroph	Saltikov and Newman, 2003.
Vectors		
pJB3Cm6	Expression vector	Blatny *et al*., 1997.
pBBR1MCS-2	Expression vector	Kovach *et al*., 1995.
pBBR1MCS-5	Expression vector	Gift from Y. Yang
pACYC184	Expression vector	Myers and Myers, 1997.
Pbad	Expression vector	Pinchuk *et al*., 2009
pBBAD	Expression vector	Sukchawalit *et al*., 1999.
pDS3.0	Pir-dependent suicide vector	Wan *et al*., 2004.

Growth

The mutant was grown in aerobic and anoxic defined, minimal media. The defined medium (HBa) contained the following ingredients: PIPES, 3 mM; NaOH, 7.5 mM; NH_4Cl, 28 mM; KCl, 1.3 mM; NaH_2PO_4, 4.4 mM; Na_2SO_4, 30 mM; minerals, 10 ml/l; vitamins, 10 ml/l; and lactate, 90 mM. Amino acids, $CaCl_2$ (0.7 mM), and electron acceptors (fumarate, Fe(III)-citrate, DMSO) were added after autoclaving. The vitamin mix contained (g/l): biotin, 0.002; folic acid, 0.002; pyridoxine HCl, 0.01; riboflavin, 0.005; thiamine, 0.005; nicotinic acid, 0.005; pantothenic acid, 0.005; B-12, 0.0001; *p*-aminobenzoic acid, 0.005; and thioctic acid, 0.005. The amino acid mix contained glutamic acid, arginine, and serine (2.0 g/l each). The mineral mix contained (g/l): nitrilotriacetic acid, 1.5; $MgSO_4$, 3.0; $MnSO_4 \bullet H_2O$, 0.5; NaCl, 1.0; $FeSO_4 \bullet$ 7 H_2O, 0.1;

$CaCl_2 \cdot 2\ H_2O$, 0.1; $CoCl_2 \cdot 6\ H_2O$, 0.1; $ZnCl_2$, 0.1; $CuSO_4 \cdot 5\ H_2O$, 0.01; $AlK(SO_4)_2 \cdot 12\ H_2O$, 0.01; H_3BO_3, 0.01; Na_2MoO_4, 0.03; $NiCl_2 \cdot 6\ H_2O$, 0.03; and $Na_2WO_4 \cdot 2\ H_2O$, 0.03. For aerobic growth, cultures (50 ml) were grown in 300 ml shake flasks (varying rpm at 30ºC). Anoxic medium was prepared by boiling under O_2-free N_2 gas, dispensed into sparged tubes (N_2) or bottles, and sealed with butyl stoppers and aluminum crimp seals. Anoxic growth was done at 30ºC. Growth was monitored via a spectrophotometer (600 nm).

Growth for Transcriptomic Analysis

Wild-type and mutant cells were grown as above or anoxically in 2 l media bottles, and then the cells were harvested, centrifuged at 4°C, and then snap-frozen in triplicate for RNA extraction. For wild-type growth, samples were collected during aerobic exponential-phase growth, 10 h post-inoculation in anoxic medium (anoxic, exponential-phase growth) and approximately 70 h into anoxic growth (anoxic, stationary-phase growth). Samples for mutant cell growth were harvested at similar time points, and the 10 h post-inoculation into anoxic medium represented the lag-phase for the mutant when transferred to anoxia. All samples were centrifuged, snap-frozen in triplicate, and RNA was extracted and used subsequently for microarray analysis.

Transcriptomics

Microarray fabrication, hybridization, probe labeling, image acquisition and processing were carried out as described previously (Beliaev *et al*., 2002; Gao *et al*., 2006). Gene expression analysis was performed using three biological replicates for each

microarray experiment, and each slide contained two replicates of two independent probes for each considered gene in the *S. oneidensis* MR-1 transcriptome (Liu *et al*., 2005). Microarray data analyses were performed using gene models from NCBI. All mRNA changes were assessed with total genomic DNA as a control. Log_2 ratios and z-scores were computed as previously described (Liu *et al*., 2005).

Dissolved Oxygen (DO) and
Oxidation-Reduction Potential (ORP) Measurements

DO was measured with a SG6 DO meter (Mettler Toledo) at 5 sec intervals for 4 min. ORP was measured via a HQ20 probe (Hach) at 5 sec intervals for 4 min. Measurements were made for wild-type and mutant cultures at similar cell densities (approximately 0.3 OD) during static incubation of an aerobic culture or during growth at a respective shake speed. It should be noted that cultures were pre-grown at the respective condition and the inoculums were in mid-exponential phase.

SDS-PAGE and Cytochrome Stain

In order to determine the *c*-type cytochrome content of wild-type and mutant cells, spheroplast- and periplasmic-fractions were obtained as previously described (Reyes-Ramirez *et al*., 2003). Prior to electrophoresis, samples were incubated with urea and SDS at room temperature (1 h) as previously described (Reyes-Ramirez *et al*., 2003), and separated on a gradient polyacrylamide gel (4 to 20%). The *c*-type cytochromes were observed via a heme-linked peroxidase stain with TMBZ as previously described (Reyes-Ramirez *et al*., 2003). Protein was quantified via the Lowry method and serum albumin as a standard (Lowry *et al*., 1951).

Fumarate Reductase

Cells were harvested and sonicated at a constant rate (1 sec bursts) for 12 sec on ice 4 times. After sonication, cells were centrifuged at 4°C for 15 min at 9,000xg. Protein concentrations were obtained via the Lowry method and approximately equal amounts of protein were loaded. Fumarate reductase activity in native gels was detected as a clear band as a result of oxidized methyl viologen after the addition of fumarate (250 mM). Gels were incubated in a round bottom flask with 10 mM methyl viologen in 0.05 M potassium phosphate (10 ml; pH 7.2; 1 mM sodium dithionite) that was gassed continuously with oxygen-free N_2 (Lund and DeMoss, 1976).

Fumarate reductase activity was quantified by the benzyl viologen-linked reductase assay as described previously (Sellars *et al*., 2002; Weingarten *et al*., 2009). Reagents (75 mM sodium phosphate buffer (pH6.8), 0.2 mM benzyl viologen and cell extract) were added to a 1 ml cuvette through the stopper while gassing continuously. Sodium dithionite (20 mM) was added to the cuvette until absorbance at 585 nm reached 0.8 to 0.9 (half-reduced benzyl viologen). Sodium fumarate (5 mM, final concentration) was then added to the mixture and oxidation was immediately recorded at 585 nm. Activity was expressed in mM of benzyl viologen oxidized min^{-1} ug^{-1}.

Quantitative PCR

Quantitative PCR (qPCR) was performed with RNA extracts from samples obtained from wild-type, mutant and suppressor cells under aerobic and anoxic conditions. cDNA was prepared by reverse transcription with 5 μg RNA. A mixture was prepared containing RNA and random primers which was incubated at 70°C for 10 min

and then cooled on ice. Reverse transcription was performed by adding 4 µl DTT, 1 µl dNTPs at 10 mM, 1µl RNase inhibitor and 1µl R reverse transcriptase (Invitrogen). cDNA was generated on incubation at 42°C for 2 h and 70°C for 15 min. On cooling, concentration of cDNA was measured at 260 nm. In order to perform quantitative PCR, cDNA was first diluted to 20 ng/µl and 2 µl of diluted sample was used per tube. Primers were added at 10 mM concentration along with SYBR Green Master Mix (Applied Biosystems) and nuclease-free water. Standards were prepared by carrying out serial dilution of genomic DNA starting with 10^8 copies/µl to 10^1 copies/µl. Negative control without cDNA was included along with all runs. qPCR was performed using Smart Cycler II (Cepheid) using following conditions: Stage1: 95°C for 9.5 min, Stage2: 95°C for 15 sec, 55°C for 30 sec and 60°C for 30 sec. Stage 2 was repeated to complete 45 cycles. Stage3: 60°C for 95 sec.

Total Heme Quantitation

Wild-type and mutant cells were harvested and sonicated at a constant rate for 15 sec for four times on ice. Cells were then centrifuged at 4°C for 15 min at 9,000 x g. Protein was quantified by the Lowry assay of the fractions obtained after sonication. Total heme was measured per µg of protein using the QuantiChrom Heme Assay Kit according to the manufacturer's instructions (BioAssay Systems).

Results and Discussion

SO3389 in Shewanella

The predicted monocistronic ORF (SO*3389*) encodes a putative 879 amino acid polypeptide. The SO*3388* gene (107 intergenic nt) encodes a putative ATP-dependent RNA helicase and the SO*3390* gene (75 intergenic nt) encodes a small hypothetical protein (36 amino acids). The predicted protein encoded by SO*3389* was assigned to COG5001 that contained an EAL (4.0e-111), a GGDEF (3.6e-62), GAF (4.8e+0.0), and PAS (5.5e+0.0; 2.4e-08) domains (Figure 3; E-values from SMART database). The respective Interpro families were IPR003018, IPR000014, IPR013656, and IPR013767 (http://www.ebi.ac.uk/interpro/). A signal sequence was not predicted via SignalP (v3.0; Emanuelsson *et al*., 2007) or TatP (v1.0; Bendtsen *et al*., 2005), although one hydrophobic α-helix was predicted from residue 137 to 162 with TMphred (Hofmann and Stoffel, 1993) and DAS (Davis *et al*., 2007) and weakly predicted with TMHMM (v2.0; Moller *et al*., 2001).

Phylogenetic Relationships

The proteins assigned to COG5001 are identified in 708 genomes from very diverse bacteria, and over 20 genomes contain 20 or more COG5001 proteins (data not shown). To date, 22 *Shewanella* genome sequences are available in public databases (www.img.jgi.doe.gov) and fifteen contained an ortholog to SO*3389* with peptide sequence identity between 52 and 85% (microbesonline.org). *S. putrefaciens* CN-32, *S. putrefaciens* 200, *S. woodyi*, *S. pealeana*, *S. violacea*, and *Shewanella* W3-18-1 did not

contain a predicted ortholog to SO3389. The genomic context for this locus varied between the different *Shewanella* genomes. The closely related sequences of SO*3389* were detected in *Shewanella* ANA-3, *Shewanella* MR-4, and *Shewanella* MR-7 (84-85% identity) as well as *Vibrio* (Figure 4). Presumptive sequences were also detected in *Vibrio*, *Pseudomonas*, *Oceanospirillum*, *Cyanothecae*, *Rhodoferax*, *Desulfuromonas*, *Magnetococcus*, and others. When the entire protein sequence of SO3389 was compared to sequenced genomes, the closest non-*Shewanella* relatives were putative sensory box proteins in *Vibrio* species. Based on a PAS domain sequence phylogeny (see Methods), the closest relatives to SO3389 were observed in *Shewanella* and *Vibrio*; however, physiological roles have not been assigned to these putative proteins (Figure 4).



Figure 3: Genome region view and domain architecture of SO*3389*. SO*3388* is annotated as a RNA helicase, SO*3390* is annotated as a small (36 aa) hypothetical protein, and SO*3391* is annotated as an ATP-dependent protease. The domains were predicted with SMART v6.0 (smart.embl-heidelberg.de/)

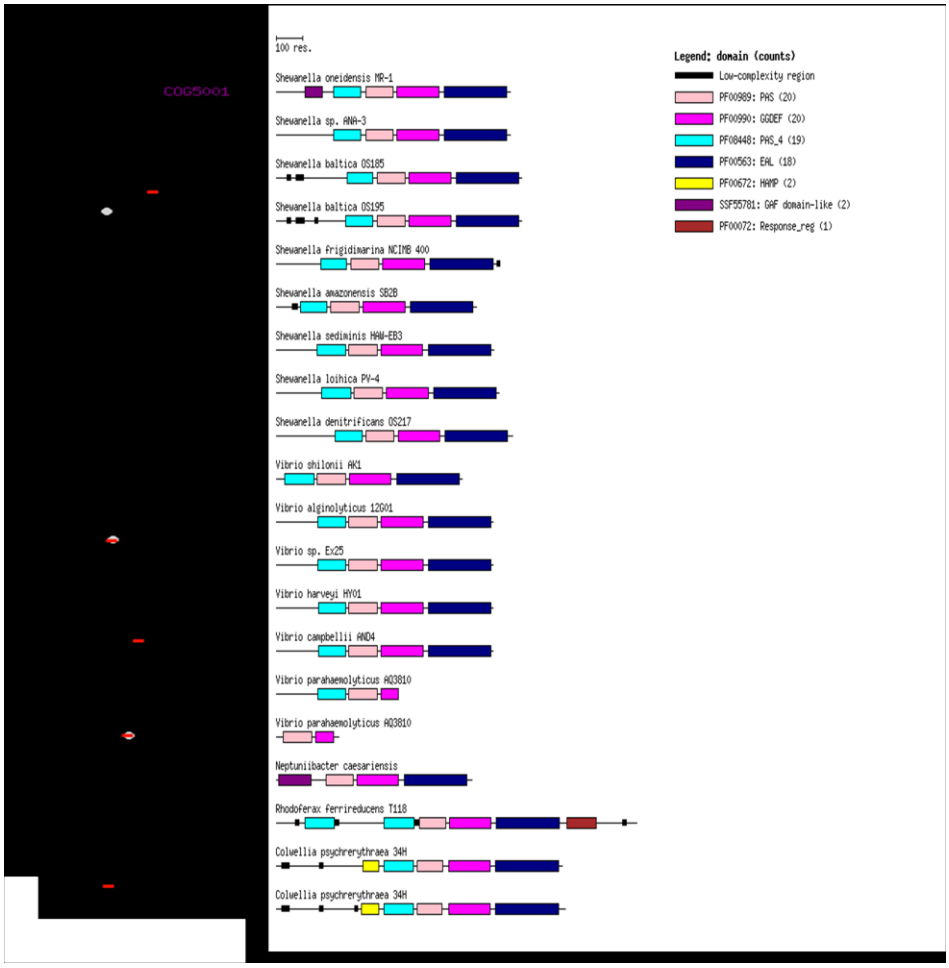


Figure 4**:** Phylogram based upon PAS domains of SO*3389* with a neighbor-joining method within the Microbes On Line workbench (microbesonline.org).

Growth Phenotypes

The mutant was tested for possible phenotypes in aerobic and anoxic defined, minimal medium. The cell cultures (wild-type and ΔSO*3389*) did not differ significantly in growth rate in shaken, aerobic medium (50 to 200 rpm), but the mutant culture had a slower growth rate in static cultures (decreased 1.6-fold; p=0.05) (Fig. 5 A and B).

A.



B.

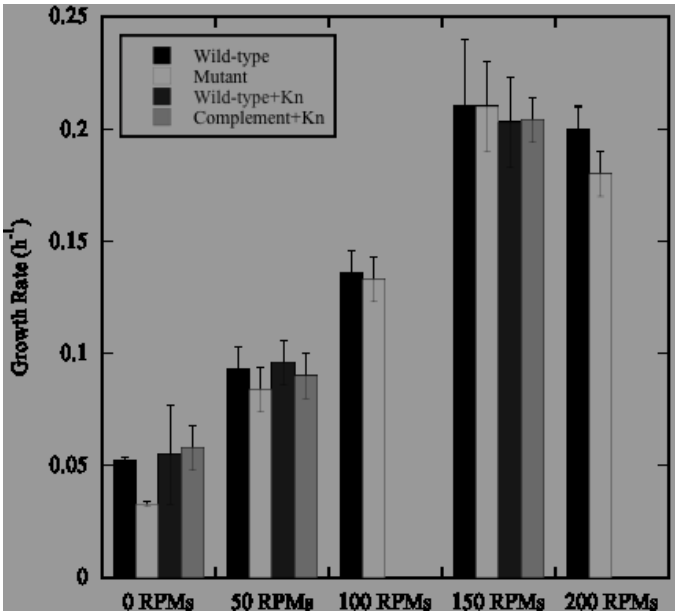


Figure 5: (**A**) Growth curves of wild-type MR-1 (●) and △SO3389 (○) cells in aerobic, defined, minimal medium with lactate (150 rpms). **(B)** Growth rates of wild-type (filled) and mutant (empty) planktonic cultures at different shake speeds (rpms). The static cultures were statistically different ($p < 0.05$). In addition, wild-type with empty vector (dark gray) and complement (light gray) strains were grown at respective shake speed in the presence of Kn.

Oxygen levels were depleted at a slower rate by mutant cells (first-order rate constants were approximately 1.5-fold lower) compared to wild-type cells when cultures growing at 150 rpm were incubated statically (Figure 6A). In addition, the

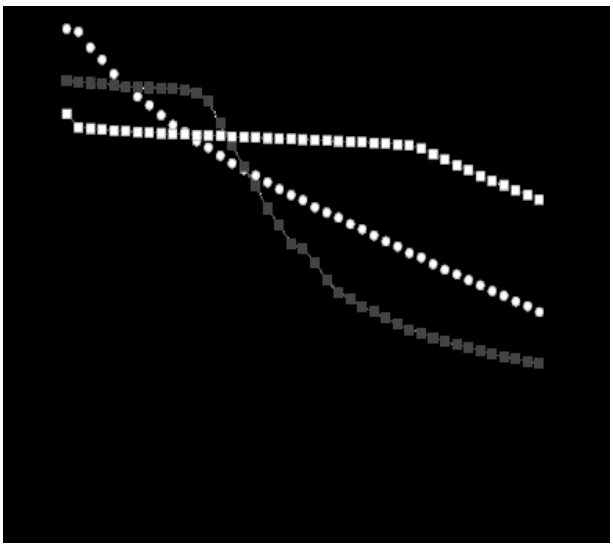
oxidative/reductive potential (ORP) declined at a slower rate for the mutant culture compared to wild-type (Figure 6A). When the DO levels were measured during shaking, the mutant cultures also had lower rates of O_2 consumption (approximately 1.5-fold), but also had higher steady-state DO levels (0.35 versus 1.7 mg/l and 2.8 versus 3.7 mg/l, respectively for wild-type and mutant cultures) (Figure 6B). These results indicated that wild-type cultures increased growth rates in response to increasing O_2 levels and that the mutant culture was deficient in O_2 regulation that resulted in less-efficient O_2 consumption at low and high O_2 levels.

When cells were transferred from aerobic to anoxic medium with lactate and fumarate, the △SO*3389* cells did not grow (Figure 7). Similar results were observed when the anoxic medium contained lactate as the electron-donor and DMSO or Fe (III)-citrate as the electron-acceptor (data not shown). These results indicated that the mutant was deficient in responses to the absence of O_2.

Complementation

Complementation was achieved with the pBBRMCS-2 vector and a full-length SO*3389* gene. The complemented strain, Δ*SO3389*:pBBR1MCS-2-SO3389, was able to grow when transferred to anoxic medium (Figure 8). In addition, the complemented strain grew more similar to wild-type (empty vector strain) under aerobic conditions (Figure 8). These results confirmed that the growth defect during growth at low DO levels and transition from aerobic to anoxia was due to the ΔSO*3389* mutation.

A.



B.

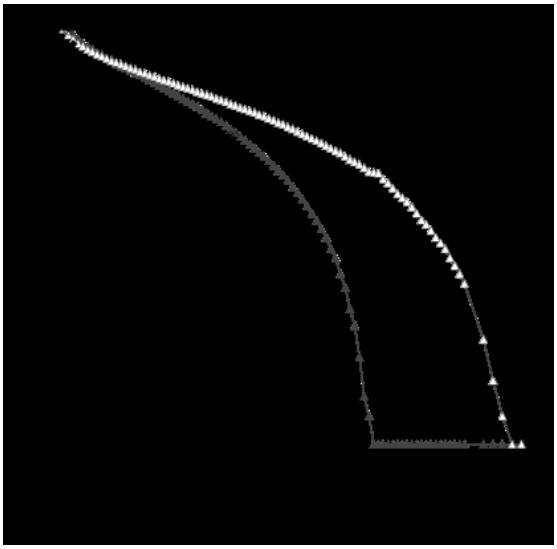


Figure 6**:** (**A**) Dissolved oxygen measurements for WT (●) and △SO3389 (○) and oxidative-reductive potential (mV) measurements for WT (■) and △SO3389 (□) versus time when shaking aerobic cultures were incubated statically (**A**) and (**B**) The decrease in DO (mg/l) was measured for cultures growing in lactate medium for wild-type (filled symbols) and mutant (empty symbols) at 0 rpm (▲,△) and 150 rpm (◆,◇).



Figure 7**:** Growth curves of wild-type MR-1 (●) and △SO3389 (○) cells in anaerobic, defined, minimal medium with lactate and fumarate. Aerobic cultures were grown in shake flasks and used to inoculate anoxic cultures to similar initial ODs. The arrows depict sampling time point for transcriptomic analyses.

qPCR was used to monitor the expression of SO*3389* and SO*3388* in wild-type and mutant cells under aerobic and anoxic conditions. Wild-type cells had higher expression levels of SO*3389* under aerobic and anoxic conditions compared to the mutant, and the SO*3388* gene had similar expression levels in both wild-type and mutant cells under aerobic and anoxic conditions (data not shown). These results indicated that the ΔSO*3389* mutation did not have a polar effect on SO*3388* expression.

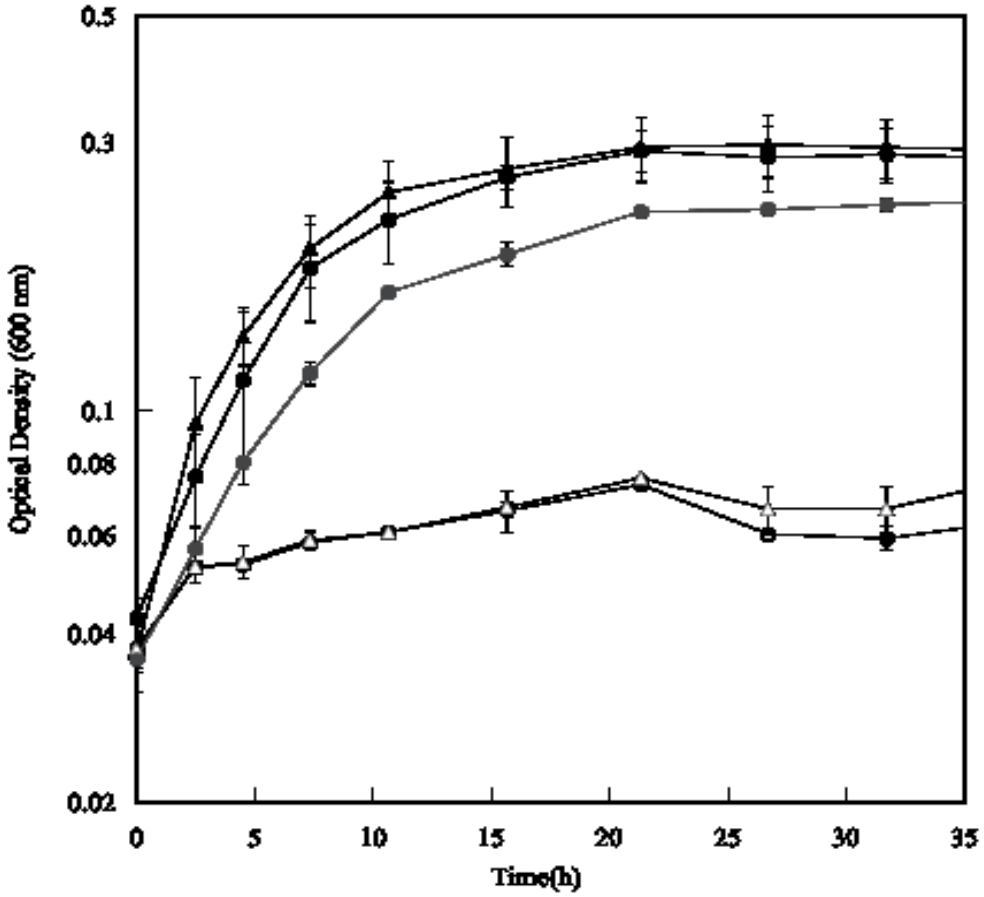


Figure 8: Growth curves of wild-type MR-1 (●), ΔSO3389 (○), wild-type pBBR1MCS-2 (▲), ΔSO3389 pBBR1MCS-2 (△) and Δ3389:: pBBR1MCS-2-SO3389 (●) in defined minimal medium with lactate as the electron donor and fumarate as the electron acceptor. Wild-type^{kn} and ΔSO3389^{kn} were constructed by mating with WM3064-empty plasmid pBBRMCS-2. Error bars represent standard deviation.

<u>*c*-type Cytochrome Content</u>

Due to the response to anoxic growth, spheroplast- and periplasmic-fractions were obtained as previously described in order to determine *c*-type cytochrome content (Reyes-Ramirez *et al*., 2003). Minor differences were observed in the spheroplast and periplasmic fractions for aerobically grown wild-type and mutant cells (*i.e.*, intensity

differences). When cells were allowed to transition from aerobic to anoxic conditions (5 to 10 h post-transfer), differences were observed in the spheroplast fraction, most noticeably for heme-containing polypeptides with a predicted molecular weight of 57, 33, and 20 kDa (Figure 9A and B). These results indicated that △SO*3389* was deficient in at least three *c*-type cytochromes during the transition period from aerobic to anoxic conditions.

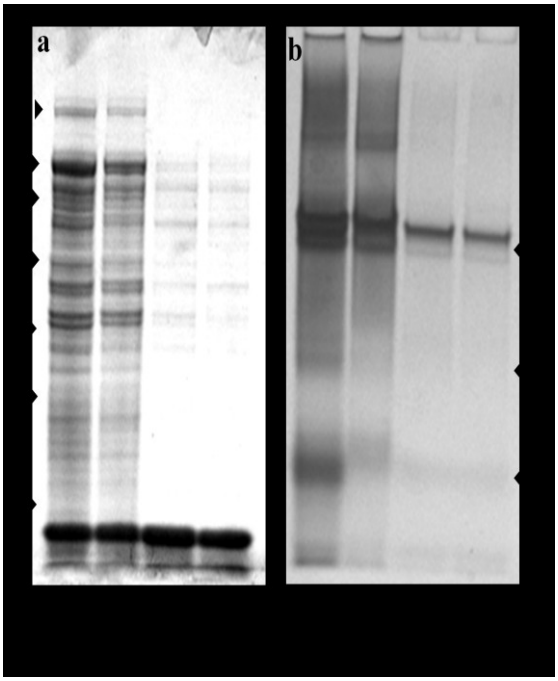


Figure 9 A and B: SDS-PAGE gels of spheroplast (lanes 1 and 2) and periplasmic-shock (lanes 3 and 4) fractions of wild-type and △SO3389 cells stained with Coomassie blue (**a**) or cytochrome-c heme stain (**b**). Equal amounts of total protein for wild-type (lanes 1 and 3) and △SO3389 (lanes 2 and 4) were loaded per well, and molecular weight markers were as follows (kDa): 150, 100, 75, 50, 35, 25, and 15.

In addition to the qualitative analysis of cytochromes, total heme content (soluble and membrane) was quantified for wild-type and mutant cells grown under aerobic conditions and transition to anoxia. There was not a significant difference in the heme

content between wild-type and mutant cell fractions grown aerobically (1.4±0.1 versus 1.5±0.1 ng/μg protein), and this data coincided with the similar cytochrome profile of wild-type and mutant cells grown under aerobic conditions. During the transition to anoxia, the mutant cells exhibited slightly elevated levels of heme in comparison to wild-type cells from membrane fractions (0.15±0.01 versus 0.27±0.06 ng/μg protein).

Gene Expression

A possible explanation is that the mutant elevated the expression of heme-containing proteins in order to compensate for the decline in oxygen affinity (at the cellular level) and/or the mutant failed to down-express certain cytochromes typically expressed during aerobic growth once transferred to anoxia. The later explanation is more likely, and coincides with the microarray data for numerous cytochromes that were down-expressed in wild-type but not mutant upon transfer to anoxia (*e.g.*, SO3285, SO3286, SO1427) (Table 2). SO1427 was previously shown to be a DMSO reductase subunit (Gralnick *et al*., 2005), and the expression data suggested that SO3389 could be involved in coordinating the expression of reductases in the absence of oxygen. These results also coincide with the observation that the mutant did not grow with DMSO under anoxic conditions. Interestingly, SO0054 displayed heightened expression in the wild-type compared to mutant cells upon transfer to anoxia (Table 2), and SO0054 is assigned to a family of conserved hypothetical proteins that contain a dinucleotide-binding motif.

Table 2. Comparison of $\log_2$ expression levels for selected genes when wild-type or mutant cells were compared between aerobic and anoxic conditions, respectively (z-scores are in parentheses). Genes in red or blue were up- or down-expressed, respectively more in the wild-type versus the mutant cultures (|z-score| >1.6). The time of sampling is denoted by arrow in Figure 5.

Gene	**Gene Annotation**	**Wild-type to Anoxia**	**Δ3389 to Anoxia**
SO0054	dinucleotide-binding oxidoreductase/dehydrogenase	1.55 (2.23)	0.06 (0.10)
SO0399 (*frd*B)	Succinate dehydrogenase/fumarate reductase	-1.35 (-2.09)	0.17 (0.31)
SO0583 (*bfd*)	bacterioferritin-associated ferredoxin	2.89 (3.15)	0.67 (1.01)
SO0768	Flavin reductase	2.90 (3.36)	0.63 (1.38)
SO1329 (*cya*A)	Adenylate cyclase	2.11 (2.88)	0.41 (0.73)
SO1427	Decaheme cytochromes *c* DMSO reductase subunit)	-3.30 (-2.64)	0.79 (1.16)
SO1519	L-Lactate dehydrogenase (subunit)	-0.67 (-1.63)	0.15 (0.40)
SO1521	D-Lactate dehydrogenase	-2.17 (-2.51)	-0.76 (-1.42)
SO1522	Lactate permease	-2.37 (-3.26)	-1.46 (-1.70)
SO1927 (*sdh*C)	Succinate dehydrogenase	1.29 (1.59)	0.24 (0.63)
SO1928 (*sdh*A)	Succinate dehydrogenase/Fumarate reductase	1.06 (1.12)	0.12 (0.22)
SO1929 (*sdh*B)	Succinate dehydrogenase/Fumarate reductase	0.77 (1.78)	0.34 (1.09)
SO2097 (*hyd*C)	Ni/Fe hydrogenase	-3.22 (-2.41)	-0.53 (-0.89)
SO2098 (*hya*B)	Ni/Fe hydrogenase	-3.46 (-2.49)	1.22 (1.04)
SO3285 (*cyd*B)	Cytochrome oxidase	-3.24 (-2.97)	-1.14 (-1.68)
SO3286 (*cyd*A)	Cytochrome oxidase	-2.69 (-3.02)	-0.68 (-1.17)
SO4047	Monoheme cytochrome c	0.86 (2.03)	0.45 (1.29)
SO4048	Monoheme cytochrome c	0.91 (2.16)	0.20 (0.70)

S. oneidensis MR-1 has over 40 putative cytochrome *c* genes, and two putative monoheme cytochrome *c* genes (SO4047 and SO4048) located in an operon were up-expressed in wild-type cells but not mutant cells once cultures were transferred to anoxic medium (Table 2). The predicted molecular weights are 38 and 21 kDa, respectively. A putative decaheme cytochrome *c* gene (SO4360) has a predicted molecular weight of 33.1 kDa, and expression for SO4360 (Carousolle and Gralnick, 2010) was lower in the mutant culture transferred to anoxia as compared to wild-type (logR=-0.91 with z=-2.10).

Recent work demonstrated that *S. oneidensis* MR-1 can utilize both L- and D-lactate, and the SO1518-1520 genes encode a L-lactate dehydrogenase and SO1521 encodes a D-lactate dehydrogenase (Pinchuk *et al*., 2009). In our study, these genes were down-expressed in wild-type cells compared between aerobic cells and cells transitioning to anoxia. In contrast, these genes were not significantly down-expressed in the mutant culture when cells were transferred from aerobic to anoxic growth medium. The Pinchuk *et al*. (Pinchuk *et al*., 2009) study reported that these genes functioned under both aerobic and anaerobic conditions, and the decreased expression observed in our study might be a result of a decline in lactate consumption under anoxic conditions compared to aerobic conditions.

The *fcc*A (SO0970) gene in *S. oneidensis* has been shown to be a major, soluble fumarate reductase during anaerobic growth (Pealing *et al*., 1992), but *fcc*A expression was not significantly altered in wild-type or mutant cultures under the tested conditions. However, recent work has shown that FccA might be involved in multiple periplasmic electron transfer networks (Schuetz *et al*., 2009). It should be noted that gene expression

was done with cultures that had been grown aerobically and then transferred to anoxic conditions, and therefore, gene expression for some genes may differ to previous studies that have used cultures grown in pre-reduced, anaerobic medium for longer time periods. The current study was focused on the transition from aerobic to anoxic conditions for which the mutant was deficient.

Wild-type cells had slightly elevated expression levels for a succinate dehydrogenase/fumarate reductase operon (*sdh*; SO1927-1929) compared to the mutant, and the mutant also did not up-express a presumptive bacterioferritin-associated ferredoxin (SO0583), a flavin reductase (SO0768), and a presumptive adenylate cyclase (SO1329) (Table 2). The class III adenylate cyclase was recently shown to regulate anaerobic respiration in *S. oneidensis* MR-1 and a separate study showed that cAMP levels were involved in the regulation of anaerobic respiration (Charania *et al*., 2009; Saffarini *et al*., 2003). Hence, SO3389 may regulate directly and indirectly via different modulators for the up- and down-expression of cytochromes, reductases, Fe-responsive gene products, and other regulators.

Fumarate Reductase Activity

Because the anoxic cultures were grown on lactate and fumarate, fumarate reductase activities were measured. During the transition, wild-type cells increased fumarate reductase activity more than mutant cells as detected with activity gels and this further corroborated the observation that reductase activity necessary for anoxic growth was not up-expressed in mutant cells during the transition to anoxia. A small amount of activity was detected in the mutant cultures, but this activity was similar to the levels

observed in aerobic, wild-type cultures. In addition, fumarate reductase activity was quantified in wild-type and mutant cultures spectrophotometrically (585 nm) during the transition to anoxia. Activity was not detected for the mutant compared to 0.76±0.09 mM benzyl viologen min^{-1} ug^{-1} for wild-type cultures.

Structural Similarity to PAS O_2/Redox-Sensors

A role in O_2/redox sensing for SO3389 coincides with the strong evidence that PAS domains can be sensors for gases (*e.g.*, O_2) and/or intracellular redox potentials (Taylor and Zhulin, 1999). In addition, IPR003018 GAF domains have been identified in proteins that respond to O_2 and have also been shown to be involved in protein-protein interactions (http://www.ebi.ac.uk/interpro/), although it should be noted that the putative GAF domain assignment in SO3389 has a weak e-value (4.8e+0.0; SMART database v6.0). For example, the PAS protein, NifA, has been shown to respond to O_2 via a flavin adenine dinucleotide (FAD) (Bueno *et al*., 2010), and the *E. coli* Dos protein (EcDos) is a heme-containing gas-sensor that has N-terminal PAS domains and a C-terminal catalytic domain (Delgado-Nixon *et al*., 2000). PAS domain families comprise a protein clan with divergent sequences (Finn *et al*., 2006), however, a recent study characterized the structures of PAS domains with known and unknown co-factors and showed a broadly conserved structure that is comprised of a five-stranded antiparallel β-sheet and several α-helices for all PAS domains characterized to date (Moglich *et al*., 2009).

The NifL sensor has been shown to be a reversible, FAD-based O_2/redox sensor that helps control the expression of nitrogen-fixation genes in *Azotobacter* (Hill *et al*., 1996; Key *et al*., 2007). More recently, the MmoS in *Methylococcus capsulatus* (strain

Bath) has been shown to be a FAD-based PAS sensor that coordinates responses to Cu via changes in redox (Ukaegbu and Rosenzweig, 2009). The SO3389 PAS1 domain did not have significant (e-value >0.1) peptide sequence identity with characterized PAS domains, but had 50% peptide sequence identity (e-value=4.7e-12; SMART database) with a hypothetical protein (VP0092) in *Vibrio parahemolyticus*. The SO3389 PAS2 domain had the highest peptide sequence identity (42%; e-value=4e-23) with the MmoS PAS1 from *M. capsulatus* (Table 3). Important residues for FAD-binding were previously predicted from the crystal structure of MmoS, and included H132, R133, N136, K142, M148, W149, N164, and S193 (Ukaegbu and Rosenzweig, 2009). The SO3389-PAS2 peptide sequence shared 6 of these MmoS residues and also 5 of the 10 important residues for FAD-binding in the *A. vinelandii* NifL (Figure 10).

Table 3. Peptide sequence identity between PAS2 domain of SO3389 and PAS domains of FAD-based redox sensors in *Methylococcus capsulatus* (Mmo), *Azotobacter vinelandii* (Az), *Escherichia coli* (Ec). The heme-based redox sensor Dos, from *E. coli*, is shown for comparison

	MmoS-PAS1	**MmoS-PAS2**	**AzNifL-PAS**	**EcAer-PAS**	**EcDos-PAS**
SO3389-PAS2	42%(4e-23)	28%(1e-13)	31%(2e-20)	38%(3e-12)	23%(4e-06)

In order to compare SO3389 to known PAS domain structures, models were predicted with CHPModels and I-Tasser tools. Both models were similar and the I-Tasser model was used for analysis. The I-Tasser model for the SO3389PAS1-2 domain had a C-score of 0.8, a TM-score of 0.7±0.1, and an experimental RMSD of 5.6±3.5, and positive C-scores and TM-scores >0.5 indicate models with correct topology (Roy *et al*., 2010). Moglich *et al*. (Moglich *et al*., 2009) used z-scores from structural superpositions

to construct a dendrogram based upon the degree of structural diversity between PAS domains, and PAS domains with known co-factors (i.e., FMN-, FAD-, and heme-) formed separate clusters. Therefore, we compared the predicted model of SO3389-PAS1-2 to known PAS sensors to gain insight into possible functions of SO3389 in conjunction with the presented phenotypic data.

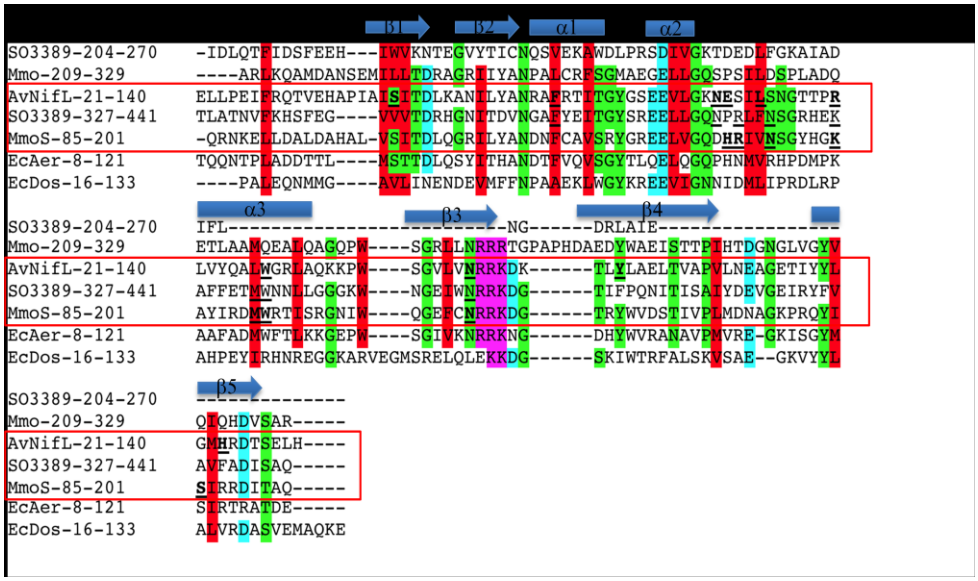


Figure 10 : Alignment of *M. capsulatus* MmoS PAS1 (85-201), *M. capsulatus* MmoS PAS2 (209-329), *A. vinelandii* NifL PAS (21-140), *E. coli* Dos PAS (16-133), *B. japonicum* FixL PAS (151-257), SO3389 PAS1 (204-270), and SO3389 PAS2 (327-441). Hydrophobic or small residues are colored red, acidic residues are colored cyan, basic residues are colored magenta, and residues with hydroxyl or amine are colored green. Important residues for FAD ligand interactions in MmoS and NifL are denoted in underline-bold, and similar residues are underlined in SO3389 PAS2. Approximate location of b-sheets and a-helices are denoted based upon MmoS structure (3EWK; Wiederstein *et al*. 2007).

The I-Tasser model (http://zhanglab.ccmb.med.umich.edu/I-TASSER) predicted the closest match with 3EWK from *M. capsulatus* (91% coverage; z=4.91) (Figure 11).

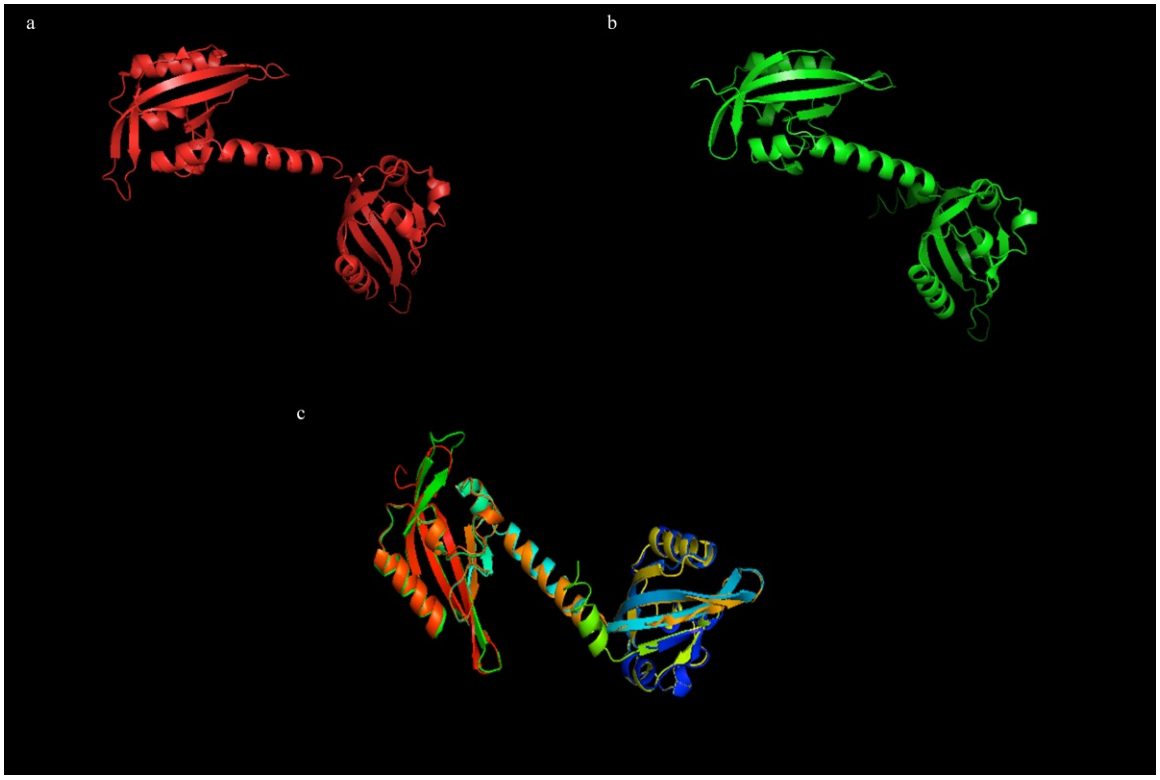


Figure 11: Structure of the *M. capsulatus* MmoS (a) (PDB:3EWK) and SO3389 (b) PAS domains and the structural superposition (c). The SO3389 PAS domain model was predicted with I-Tasser and the structural alignment was visualized with PyMol (www.pymol.org).

When the DALI Protein Structure Database (v3.0; http://ekhidna.biocenter.helsinki.fi/dali_server/start) was used, the closest matches with the SO3389PAS1-2 model was the FAD-binding MmoS PAS1 from *M. capsulatus* (3EWK) and the NifL PAS1 (2GJ3) from *A. vinelandii* (RMSD of 0.6Å (z=24.9) and 1.4Å (z=15.4), respectively. In addition, the Phyre server (http://www.sbg.bio.ic.ac.uk/~phyre/index.cgi) predicted that 2GJ3 was the closest match based upon protein sequence and structure (E-value=2.3e-15; 30% sequence identity).

Both 2GJ3 and 3EWK are known FAD-based redox PAS-sensors (Key *et al*., 2007; Slavny *et al*., 2009; Ukaegbu and Rosenzweig, 2009)

The SO*3389* ORF also contains putative GGDEF and EAL domains (originally named for the conserved amino acid residues) (Galperin, 2004). The two domains are thought to interact with cyclic-diguanosine monophosphate (c-di-GMP), and c-di-GMP was recently proposed to be a global secondary messenger involved in regulating cell-cell and cell-surface interactions (Christen *et al*., 2006). The GGDEF domain was recently shown to be associated with the synthesis of c-di-GMP via di-guanylate cyclase (DGC) and the EAL domain to contain phosphodiesterase (PDE) activity in *Yersinia*, *Caulobacter*, and *Salmonella* (Christen *et al*., 2006; Galperin, 2004; Kirillina *et al*., 2004; Jenal, 2004; Paul *et al*., 2004) and more recently in *Shewanella* (Thormann *et al*., 2006). In addition, the involvement of the DGC and PDE domains in cell metabolism and cell behavior has recently been shown in *Burkholderia*, *Xanthomonas*, *Streptococcus*, *Vibrio*, and *Pseudomonas* (for example Borlee *et al*., 2010; Krasteva *et al*., 2010). Interestingly, sequence comparisons suggested that SO3389 was a phosphodiesterase and not a cyclase based upon conserved amino acid residues predicted for active phosphodiesterases (data not shown; Schuetz *et al*., 2009).

Our data indicated that SO3389 was involved in sensing DO levels that affected both aerobic and transitions to anoxia. The resultant phenotypes were a consequence of the inability to regulate genes that included cytochromes and reductases. The sequence and structure comparisons suggested that SO3389 could be a FAD-based O_2/redox sensor, and future work includes the characterization of ligand interactions for SO3389.

It is interesting to speculate that the orthologs identified in *Vibrio* and *Pseudomonas* (Figure 4) could function in a similar manner to help cells control growth associated with O_2, anoxia, and virulence.

The most similar proteins to SO3389 were annotated as conserved hypothetical and sensory box proteins, and thus SO3389 is a novel protein for which a function has not been previously described. Numerous systems biology studies have demonstrated the often realized but unknown importance of hypothetical and conserved hypothetical proteins in microorganisms (Elias *et al*., 2009), and the elucidation of these proteins will provide insight into the evolution of sensing molecules as well as the ability of microorganisms to sense external stimuli and optimize metabolism.

References

Beliaev, A.S., D. K. Thompson, M. W. Fields, L. Wu, K. H. Nealson, and J. Zhou. 2002. Microarray transcription profiling of a *Shewanella oneidensis* etrA mutant. J. Bacteriol. 184:4612-4616.

Bendtsen, J. D., H. Nielsen, D. Widdick, T. Palmer, and S. Brunak. 2005. Prediction of twin-arginine signal peptides. BMC Bioinformatics 6: 167.

Benkert, P., M. Künzli, and T. Schwede. 2009. QMEAN server for protein model quality estimation . Nucleic Acids Res. 37:W510-4.

Blatny, J.M., T. Brautaset, and H. C. Winther-503 Larsen. 1997. Construction and use of a versatile set of broad-host-range cloning and expression vectors based on the RK2 replicon. Appl. Environ. Microbiol. 63:370-379.

Bueno, E., S. Mesa, C. Sanchez, E. J. Bedmar, and M. J. Delgado. 2010. NifA is required for maximal expression of denitrification genes in *Bradyrhizobium japonicumemi*. Environ. Microbiol. 12:393-400.

Borlee, B.R., A. D. Goldman, K. Murakami, R. Samudrala, D. J. Wozniak, and M. R. Parsek. 2010. *Pseudomonas aeruginosa* uses a cyclic-di-GMP511 regulated adhesin to reinforce the biofilm extracellular matrix. Mol. Microbiol. 75: 827 – 842.

Carousolle, D., and J. A. Gralnick. 2010. Modularity of the Mtr respiratory pathway of *Shewanella oneidensis* strain MR-1. Mol Microbiol. 77: 995-1008

Charania, M.A., K. L. Brockman, Y. Zhang, A. Banerjee, G.E Pinchuk, J. K. Fredrickson, A. S. Beliaev, and D. A. Saffarini. 2009. Involvement of a membrane-bound class III /85/88denylate cyclase in regulation of anaerobic respiration in *Shewanella oneidensis* MR-1. J. Bacteriol. 191: 4298–4306.

Christen, B., M. Christen, R. Paul, F. Schmid, M. Folcher, P. Jenoe, M. Meuwly, U. Jenal. 2006. Allosteric control of cyclic di-GMP signaling. J. Biol. Chem. 281:32015-32024.

Davis, I .W., A. Leaver-Fay, V. B. Chen, J. N. Block, G. J. Kapral, X. Wang, L. W. Murray, W. B. Arendall III, J. Snoeyink, J. S. Richardson, and D. C. Richardson . 2007. MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. Nucleic Acids Res. 35:W375-W383

Dehal, P.S., M. P. Joachimiak, M. N. 526 Price, J. T. Bates, J. K. Baumohl, D.Chivian, G. D. Friedland, K. H. Huang, K. Keller, P. S. Novichkov, I. L. Dubchak, E. J. Alm, and A. P. Arkin. 2009. MicrobesOnline: an integrated portal for comparative and functional genomics. Nucleic Acids Res. doi:10.1093/nar/gkp919.

Delgado-Nixon, V.M., G. Gonzalez, and M.A. Gilles-Gonzalez. 2000. Dos, a heme-binding PAS protein from *Escherichia coli*, is a direct oxygen sensor. Biochemistry 39:2685-2691.

Elias, D.A., E. C. Drury, A. M. Redding, A. Mukhopadyay, C. B. Yen, M. W. Fields, T. C. Hazen, A. P. Arkin, J. D. Keasling, and J. D. Wall. 2009. Expression profiling of hypothetical genes in *Desulfovibrio vulgaris* leads to improved functional annotation. Nucleic Acids Res. 37:2926–2939.

Emanuelsson, O., S. Brunak, G. vonHeijne, and H. Nielsen. 2007. Locating proteins in the cell using TargetP, SignalP, and related tools. Nature Protocols 2:953-971

Finn, R.D., J. Mistry, B. Schuster-Bockler, S. Griffiths-Jones, V. Hollich, T. Lassmann, S. Moxon, M. Marshall, A. Khanna, R. Durbin, S. R. Eddy, E. L. Sonnhammer, and A. Bateman. 2006. Pfam: clans, web tools and services. Nucleic Acids Res. 34:D247–D251.

Galperin, M. Y. 2004. Bacterial signal transduction network in a genomic perspective. Environ. Microbiol. 6:552-567.

Gao, W., Y. Liu, C. S. Giometti, S. L. Tollaksen, T. Khare, L. Wu, D. M. Klingeman, M. W. Fields, and J. Zhou. 2006. Knock-out of a prohibitin-like protein results in alteration of iron metabolism, increased 549 spontaneous mutation and hydrogen peroxide sensitivity in the bacterium *Shewanella oneidensis*. BMC Genomics 7:76.

Gralnick, J.A., C. T. Brown, and D. K. Newman. 2005. Anaerobic regulation by an atypical Arc system in *Shewanella oneidensis*. Mol Microbiol. 56: 1347-1357.

Guindon, S., and O. Gascuel. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biol. 52:696-704.

Heidelberg, J.F., I.T. Paulsen, K. E. Nelson, E. J. Gaidos, and W. C. Nelson. 2002. Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. Nat. Biotech. 20:1118-1123.

Hill, S., S. Austin, T. Eydmann, T. Jones, and R. Dixon. 1996. *Azotobacter vinelandii* NIFL is a flavoprotein that modulates transcriptional activation of nitrogen-fixation genes via a redox-sensitive switch. Proc. Nat. Acad. Sci. USA 93: 2143-2148.

Hofmann, K., and W. Stoffel. 1993. TMbase-A database of membrane spanning proteins segments. Biol. Chem. 374:166

Holm, L., S. Kaariainen, P. Rosenstrom, and A. Schenkel. 2008. Searching protein structure databases with DaliLite v.3. Bioinformatics 24:2780-2781.

Jenal, U. 2004. Cyclic di-guanosine monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria. Curr. Opin. Microbiol. 7:185-191.

Kelley, L.A., and M. J. E. Sternberg. 2009. Protein 572 structure prediction on the web: a case study using the Phyre server. Nature Protocols. 4:363 – 371.

Key, J., M. Hefti, E. B. Purcell, and K. Moffat. 2007. Structure of the redox sensor domain of *Azotobacter* v*inelandii* NifL at atomic resolution: signaling, dimerization, and mechanism. Biochemistry 46*:*3614-3623.

Kirillina, O., J. D. Fetherston, A. G. Bobrov, J. Abney, and R. Perry. 2004. HmsP, a putative phosphodiesterase, and HmsT, a putative diguanylate cyclase, control Hms-dependent biofilm formation in *Yersinia pestis*. Mol Microbiol. 54:75-88.

Kolker, E., A. F. Picone, M. Y. Galperin, M. F. Romine, and R. Higdon. 2005. Global profiling of *Shewanella oneidensis* MR-1: expression of hypothetical genes and improved functional annotations. Proc. Natl. Acad. Sci. USA 102: 2099-2104.

Kovach, M.E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, and K. M. Peterson. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene. 166:175-6.

Krasteva, P.V., J. C. N. Fong, N. J. Shikuma, S. Beyhan, A. S. Navarro, F. H. Yildiz, and H. Sondermann. 2010. *Vibrio cholerae* VpsT regulates matrix production and motility by directly sensing cyclic di-GMP. Science 327: 866 – 868.

Letunic, I., T. Doerks, and P. Bork. 2008. SMART 6: recent updates and new developments. Nucleic Acids Res. 37:D229-32.

Liu, Y.Q., W. M. Gao, Y. Wang, L. Y. Wu, X. 595 D. Liu, T. F. Yan, E. J. Alm, A. P. Arkin, D. K. Thompson, M. W. Fields, and J. Zhou. 2005. Transcriptome analysis of *Shewanella oneidensis* MR-1 in response to elevated salt conditions. J. Bacteriol. 187: 2501-2507.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. R andall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-75

Lund, K., and J. A. DeMoss. 1976. Association-dissociation behavior and subunit structure of heat released nitrate reductase from *Escherichia coli*. J.Biol.Chem. 251:2207-2216

Moglich, A., R. A. Ayers, and K. Moffat. 2009. Structure and signaling mechanism of Per-ARNT-Sim domains. Cell Struct. 17:1282-1294.

Moller, S., M. D. R. Croning, and R. Apweiler. 2001. Evaluation of methods for the prediction of membrane spanning regions. Bioinformatics. 17:646-653.

Myers, C.R., and J.M Myers. 1997. Replication of plasmids with the p15A origin in Shewanella putrefaciens MR-1. Letts. Appl. Microbiol. 24:221-225.

Nielsen, M., C. Lundegaard, O. Lund, and T. N. Petersen. 2010. CPHmodels-3.0 Remote homology modeling using structure guided sequence profiles. Nucleic Acids Res. (In press).

Paul, R., S. Weiser, N. C. Amiot, C. Chan, T. Schirmer, B. Giese, and U. Jenal. 2004. Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. Genes Dev. 18:715-727.

Pealing, S.L., A. C. Black, F. D. C. Manson, F. B. Ward, S. K. Chapman, G. Reid. 1992. Sequence of the gene encoding flavocytochrome *c* from *Shewanella putrefaciens:* a tetraheme flavoenzyme that is a soluble fumarate reductase related to the membrane-bound enzymes 618 from other bacteria. Biochemistry. 31: 12132-12140.

Pinchuk, G.E., D. A. Rodionov, C. Yang, X. Li, A. L. Osterman, E. Dervyn, O. V. Geydebrekht, S. B. Reed, M. F. Romaine, F. R. Collart, J. H. Scott, J. K. Fredrickson, and A. S. Beliaev. 2009. Genomic reconstruction of *Shewanella oneidensis* MR-1 reveals a previously uncharacterized machinery for lactate utilization. Proc. Natl. Acad. Sci USA. 106:2874-2879

Ponting, C.P., and L. Aravind. 1997. PAS: a multifunctional domain family comes to light. Curr. Biol. 7:R674-R677.

Reyes-Ramirez, F., P. Dobbin, G. Sawers, and D. J. Richardson. 2003. Characterization of transcriptional regulation of *Shewanella frigidimarina* Fe(III)-induced flavocytochrome *c* reveals a novel iron-responsive gene regulation system. J. Bacteriol. 185:4564-4571.

Roy, A., A. Kucukural, and Y. Zhang. 2010. I-TASSER: a unified platform for automated protein structure and function prediction. Nature Protocols. 5:725-738.

Saffarini, D. A., R. Schultz, and A. S. Beliaev. 2003. Involvement of cyclic AMP (cAMP) and cAMP receptor protein in anaerobic respiration of *Shewanella oneidensis*. J. Bacteriol. 185: 3668-3671.

Saltikov, C.W., and D. K. Newman . 2003. Genetic identification of a respiratory arsenate reductase. Proc. Natl. Acad. Sci USA. 100:10983-10988.

Schuetz, B., M. Schicklberger, J. Kuermann, A. M. Spormann, and J. Gescher. 2009. Periplasmic electron transfer via the c-type cytochromes MtrA and FccA of *Shewanella oneidensis* MR-640 1. Appl. Environ. Microbiol. 75: 7789–7796.

Scheuermann, T.H., J. S. Yang, L. Zhan, K. H. Gardner, and R. K. Bruick. 2007. Hypoxia-inducible factors P-ER/ARNT/S-IM domains: structure and function. Methods in Enzymol. 435:3

Schmidt, A.J., D. A. Ryjenkov, and M. Gomelsky. 2005. The ubiquitous protein domain EAL is a cyclic diguanylate-specific phospodiesterase: enzymatically active and inactive EAL domains. J. Bacteriol. 187: 4774-4781.

Sellars, M.J., S.J. Hall, and D. J Kelly. 2002. Growth of *Campylobacter jejuni* supported by respiration of fumarate, nitrate, nitrite, trimethylamine-N-oxide, or dimethyl sulfoxide requires oxygen. J.Bacteriol. 184:4187-4196

Shi, L., J. T. Lin, L. M. Markillie, T. C. Squier, and B. S. Hooker. 2005. Overexpression of multi-heme c-type cytochromes. BioTechniques. 38:297-299.

Slavny, P., L. Richard, P. Salinas, T. A. Clarke, and R. Dixon. 2009. Quaternary structure changes in a second Per-Arnt-Sim domain mediate intramolecular redox signal relay in the NifL regulatory protein. Mol. Microbiol. 75: 61 – 75.

Sukchawalit, R., P. Vattanaviboon, R. Sallabhan, and S. Mongkolsuk. 1999. Construction and characterization of regulated L-arabinose-inducible braod host range expression vectors in *Xanthomonas*. FEMS Microbiol. Letts. 181:217-223.

Taylor, B.L., and I. B. Zhulin. 1999. PAS domains: Internal sensors of oxygen, redox potential, and light. Microbiol. Molc. Biol. Rev. 63:479-506.

Thormann, K.M., S. Duttler, R. M. Saville, 662 M. Hyodo, S. Shukla, Y. Hayakawa, and A. M. Spormann. 2006. Control of formation and cellular detachment from *Shewanella oneidensis* MR-1 biofilms by cyclic di-GMP. J. Bacteriol. 188:2681:2691.

Ukaegbu, U.E., and A. C. Rosenzweig. 2009. Structure of the redox sensor domain of *Methylococcus capsulatus* (Bath) MmoS. Biochemistry. 48:2207–2215.

Wan, X. F., N. C. VerBerkmoes, L. A. McCue, D. Stanek, H. Connelly, L. J. Hauser, L. Wu, X. Liu, T. F. Yan, A. Leaphart, R. L. Hettich, J. Zhou, and D. K. Thompson. 2004. Transcriptomic and proteomic characterization of the Fur modulon in the metal-reducing bacterium *Shewanella oneidensis*. J. Bacteriol. 186:8385-8400.

Weingarten, R.A., M. E. Taveirne, and J. W. Olson. 2009. The dual675 functioning fumarate reductase is the sole succinate:quinine reductase in *Campylobacter jejuni* and is required for full host colonization. J. Bacteriol. 191:5293-5300.

Wiederstein, M., and M. J. Sippl. 2007. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res. 35:W407-W410.

Zhulin, I.B., and B. L. Taylor. 1998. Correlation of PAS domains with electron transport-associated proteins in completely sequenced microbial genomes. Mol. Microbiol. 29:1522-1523. (Erratum, 31:741, 1999)

CHAPTER 3

DELETION OF A MULTI-DOMAIN PAS PROTEIN CAUSES PLEIOTROPIC EFFECTS IN *SHEWANELLA ONEIDENSIS* MR-1

Abstract

Shewanella oneidensis MR-1 can utilize a variety of electron donors and acceptors for energy, including oxygen and heavy metals. Understanding the physiological responses of MR-1 to environmental stresses is important in assessing the potential impact of such perturbations on metal-reducing activity. The presence of conserved hypothetical proteins and sensory box proteins help organisms modulate their physiology to respond to environmental perturbations; however stimuli for most sensory box proteins are not known for individual organisms. Here the possible physiological roles of a conserved hypothetical protein, SO3389 were characterized. The ORF contains PAS, PAC, EAL and GGDEF domains, and these domains have been implicated in multiple phenotypes. The exact physiological role(s) of proteins that contain these domains, however, have not been fully established. In addition, the possible role of a protein with this domain composition and architecture has not been previously described. Initial studies revealed that the in-frame deletion mutant lagged for 35 - 40 h when transferred from aerobic to anoxic medium, but growth rate was similar to wild-type once growth was initiated (Chapter 2). Interestingly, the mutant culture reverted after a prolonged lag period of approximately 40h and the lag was greatly diminished after a subsequent transfer to anoxic medium. However, motility and biofilm formation were still impaired. This suggested that phenotypes could be decoupled. Enumeration of the revertant population suggested the presence of a low-frequency suppressor mutation and not an overall physiological adaptation of the mutant population. Multiple suppressor strains displayed a similar combination of phenotypes (i.e., anoxic growth and motility but biofilm impediment). Results suggested that the SO3389 sensory box protein was involved in the coordination of anoxic growth, motility and biofilm formation. Further work is needed to elucidate the respective signal(s) and the mechanism(s) of signal transduction.

Introduction

Shewanella oneidensis MR-1 has a versatile metabolism but the optimization of physiological states in response to external electron acceptors is not well understood. With increased availability of sequenced microbial genomes since the 'dark ages' (pre genomic era) ample data is now available for a better understanding of cell metabolism, cell organization, and evolution of bacteria. Microorganisms regulate their metabolism according to a dynamic environment, and both simple and complex regulatory mechanisms exist in unicellular organisms that enable them to respond to environmental stimuli to optimize metabolism (Koonin and Galperin, 1997; Galperin, 2005). Proteins such as histidine kinases, response regulators, methyl accepting chemotaxis proteins and several conserved hypothetical proteins contain signal transduction domains that participate in multiple regulatory mechanisms (Hecht and Newton, 1995; Merkel *et al*., 1998; Slater *et al*., 2000; Tal *et al*., 1998; Pei and Grishin, 2001; Taylor and Zhulin, 1999).

PAS domains are usually part of larger polypeptides involved in signal transduction and recent data suggests that PAS domains can sense changes in environmental stimuli, such as light, redox potential, oxygen, and energy state of the cell as discussed in Chapter 1 (Taylor and Zhulin, 1999). Two other major domains commonly observed in conserved hypothetical ORFs from bacterial genomes are the GGDEF and EAL domains (named for the conserved amino acid residues) (Galperin, 2004). The two domains are thought to interact with cyclic- di-guanosine monophosphate (c-di-GMP), and c-di-GMP was recently proposed to be a global

secondary messenger involved in regulating cell-cell and cell-surface interactions (Christen *et al*., 2006). The GGDEF domain was recently shown to be associated with the synthesis of c-di-GMP via di-guanylate cyclase (DGC) and the EAL domain to contain phosphodiesterase (PDE) activity in *Yersinia*, *Caulobacter*, and *Salmonella* (Christen *et al*., 2006; Garcia *et al*., 2004; Jenal, 2004; Kirillina *et al*., 2004; Paul *et al*., 2004). Many bacterial genomes contain a wide variety of presumptive signal transduction systems (Galperin, 2004); however, the physiological role or the exact array of signals for these multi-domain proteins is not known. The putative PAS-PAC-GGDEF-EAL protein, SO3389, was selected to elucidate possible roles in cellular physiology for *S. oneidensis* MR-1. Because PAS domains can be involved in oxygen sensing, the mutant was tested for growth transitions from aerobic to anoxic conditions (Chapter 2). The mutant was also affected in aerobic biofilm formation and motility, and these phenotypes as well as suppressor mutants (that enable rescue of growth phenotype) are characterized in Chapter 3.

Materials and Methods

Growth

The mutant(from Chapter 2) was grown in aerobic and anoxic defined, minimal media. The defined medium (HBa) contained the following ingredients: PIPES, 3 mM; NaOH, 7.5 mM; NH_4Cl, 28 mM; KCl, 1.3 mM; NaH_2PO_4, 4.4 mM; Na_2SO_4, 30 mM; minerals, 10 ml/l; vitamins, 10 ml/l; and lactate, 90 mM. Amino acids, $CaCl_2$ (0.7 mM), and electron acceptors (fumarate (120mM, 40mM), Fe(III)-citrate(15mM), DMSO(10mM)) were added after autoclaving. The vitamin mix contained (g/l): biotin, 0.002; folic acid, 0.002; pyridoxine HCl, 0.01; riboflavin, 0.005; thiamine, 0.005; nicotinic acid, 0.005; pantothenic acid, 0.005; B-12, 0.0001; *p*-aminobenzoic acid, 0.005; and thioctic acid, 0.005. The amino acid mix contained glutamic acid, arginine, and serine (2.0 g/l each). The mineral mix contained (g/l): nitrilotriacetic acid, 1.5; $MgSO_4$, 3.0; $MnSO_4.H_2O$, 0.5; NaCl, 1.0; $FeSO_4.7\ H_2O$, 0.1; $CaCl_2 \cdot 2\ H_2O$, 0.1; $CoCl_2.\ 6\ H_2O$, 0.1; $ZnCl_2$, 0.1; $CuSO_4.5\ H_2O$, 0.01; $AlK(SO_4)_2.12\ H_2O$, 0.01; H_3BO_3, 0.01; Na_2MoO_4, 0.03; $NiCl_2.6\ H_2O$, 0.03; and $Na_2WO_4.2\ H_2O$, 0.03. For aerobic growth, cultures (50 ml) were grown in 300 ml shake flasks (varying rpm at 30ºC). Anoxic medium was prepared by boiling under N_2 gas, dispensed into sparged tubes (N_2) or bottles, and sealed with butyl stoppers and aluminum crimp seals. Anoxic growth was done at 30ºC. Growth was monitored via a spectrophotometer (600 nm).

Mutant Construction

An approximately 2.4 kbp fragment was deleted from SO3389 (2639 bp) with PCR-based crossover amplification as described previously (Gao *et al*., 2006; Wan *et al*., 2004). The primer pairs used to generate two DNA fragments with 3' staggered ends were as follows: No-5'-CGCGAGCTCGTTCGGACCTTCGATAAATC-3' and Ni-5' TGT TTAAACTTAGTGGATGGGGCCCAATCTGTTAACTGCTT 3' and Co-5' CGC GAGCTCGCACCTTGACGCAAATGATC-3' and Ci-5'-CCCATCCACTAAGTTTAA ACAGCGGATGTTGAGGCCTTTTT-3' (non-complementary tag sequences are underlined) and the outside primers were used to amplify a fusion molecule. The crossover PCR product was purified and digested by SacI, cloned into treated suicide vector pDS3.0, and electroporated into *E. coli* S17-1/λ_{pir}. The suicide plasmid construct was moved into MR-1 as previously described (Gao *et al*., 2006; Wan *et al*., 2004), and the mutated SO*3389* gene was verified by PCR (5'-GAGTGGCATTCAGCACTAGA-3' and 5'-CATACGGTCGGCTTCATCAA-3') and sequence determination. The resulting mutant did not possess any of the predicted domains after completion of the in-frame deletion. The mutation was an in-frame deletion that removed approximately 2400 bp of the gene.

△SO*3389* Complementation

Multiple vectors (pACYC184, pJB3Cm6, pBBR1MCS-5, and pBAD; see Table 1) were attempted for complementation, but construction of the correct, stable *E. coli* strain was not successful or expression was not detected in the case of pBAD. Therefore, pBBR1MCS-2 and pBBAD were used, and the following complementation primers were

used to amplify genomic DNA: 3389FWDCOM (GAATTCTGTCTGCGGTAATATCTGCG) and 3389REVCOM (GGATCCCACCAAAAGCAATCATGTCG) (Table 1). The amplicon was cloned into a TOPO vector and digested with EcoR1 and BamH1. Post digestion, the insert was purified with a QIAquick Gel Extraction Kit as per instructions and subsequently cloned by ligation into vectors, pBBR1MCS-2 and pBBAD at 1:3 and 1:10 molar ratios (vector to insert) also digested using EcoR1 and BamH1. To increase transformation efficiency, ligations were transformed into *E. coli* UQ950 and then *E. coli* WM3064, a diaminopimelic (DAP) auxotroph. Transformants thus obtained were verified for inserts by PCR. The correct transformant was then mated with △SO*3389* onto a LB-DAP (0.3 mM) plate and incubated at 30°C for 24 hours. Transconjugants were selected by streaking mating mixtures onto LB-Kn_{50}.

Iron Reduction Assay

Fe (II) was measured using FAS (ferrous ethylenediammonium sulfate) as a standard solution. Ferrozine was dissolved in 50mM HEPES buffer pH 7.0 to make a 0.2% solution. Sample (0.1ml culture) was added into a tube containing 0.5M HCl to make an acid extract. This step was done in the glove bag to maintain anaerobic conditions. After 30 min (or overnight), 0.1ml of this extract was added into another tube containing 3ml ferrozine reagent and vortexed thoroughly. Absorbance was measured at 562nm using Schimadzu Spectrophotometer.

Biofilm Analysis

Cells were grown in Erlenmeyer flasks in the defined medium at 150 rpm to late exponential phase, as measured by optical density measurements. The supernatant culture was decanted, and the flask was rinsed with saline (0.7% w/v) to remove planktonic cells. The adhered biomass was stained with 0.1% (w/v) crystal violet for 15 min. The excess stain was decanted and the adhered biofilm on the flask was washed with distilled water and then destained with 50 ml of an ethanol:acetone solution (80:20). The absorbance of the ethanol:acetone solution was measured via spectroscopy (580 nm) and a ratio was determined with the optical density at 600 nm of the original planktonic culture.

Motility Assay

For motility analysis, 0.01mL of aerobic precultures of wild-type MR-1and mutant were spot inoculated on 0.3% HBa minimal medium plates and incubated at 30°C until prominent colonies appeared. Colony migration on the agar from point-of-inoculation was measured which corresponded to motility of the strain.

MPN for Determining Suppressor

Most probable number was employed to determine frequency of suppressor population. Serial dilution was carried out up to 10^{-12}. Inoculum was obtained during the prolonged lag phase observed during transition from aerobic to anoxic conditions. Subsequent to serial dilution, tubes were inoculated at 30°C. MPN/ml was calculated

using online software (http://members.ync.net/mcuriale/mpn/mpncalc.zip). The viable cells that emerged from the 'lag phase' were termed as suppressor population.

Protein Estimation

Protein concentrations were obtained by Lowry's method. Reagents include Copper reagent: Lowry 1(20g/L Na_2CO_3 in 0.1M NaOH), 2%$CuSO_4$, 4% w/v Na tartarate and phenol reagent (2N Folin Ciocalteau solution). Bovine serum albumin was used to generate a standard curve. Samples were boiled with 0.2N NaOH for 15 min and copper reagent was added, mixed immediately and incubated for 45 min at 39°C. On cooling, 1ml phenol reagent was added and incubated at room temperature for 30 min. Reaction was measured at 660nm (Lowry *et al*, 1951).

Activity Gels

Cells were harvested and sonicated with a sonicator at a constant rate (1 sec bursts) for 12 sec on ice 4 times. After sonication, cells were centrifuged at 4°C for 15 min at 9,000xg. Protein concentrations were obtained via the Lowry method and approximately equal amounts of protein were loaded on native SDS gels (4-20% gradient). Fumarate reductase activity in native gels was detected as a clear band as a result of oxidized methyl viologen after the addition of fumarate (250 mM). Gels were incubated in a round bottom flask with 10 mM methyl viologen in 0.05 M potassium phosphate (10 ml; pH 7.2; 1 mM sodium dithionite) that was gassed continuously with N_2 (Lund and DeMoss, 1976).

Scanning Electron Microscopy

For SEM, glass cover slips were partially submerged in HBa medium and cells grown in tubes on a shaking incubator. Upon removal of the culture medium, the cover slips were treated with 2.5% gluteraldehyde in 0.05 M sodium cacodylate, sequentially dehydrated in increasing ethanol concentrations (25%-100%), and critical point dried. Samples were gold-coated and visualized with a JEOL T200 scanning electron microscope.

Epifluorescence Microscopy

Biofilm samples were also analyzed for carbohydrate by fluorescent stains and epifluorescent microscopy with the following procedures. Biofilms were grown on glass slides and rinsed as described above. Biofilms were stained with calcofluor white solution (10 mg ml-1) to determine the presence of β - 1,6-linked polymers, with concanavalin A, Alexa Fluor 488 conjugate (5.0 mg ml-1) that targeted non-reducing mannosyl groups, and with Congo red (0.1% w/v) for β-1,4 glucans. The calcofluor white solution was domed on top of the biofilm-covered glass slide. The slide was incubated at room temperature in the dark for 15 min. During the last 5 min, approximately 200 µl of an acridine orange solution (10 mg ml-1) was added to the slide. The biofilm was then rinsed three times with PBS and coverslips were placed over the biofilm for microscopy. Concanavalin staining was performed following protocol outlined by Magnuson and colleagues (2004). Briefly, biofilm slides were domed with the concanavalin solution along with acridine orange (2.0 mg ml-1) and incubated at room temperature for 1 h.

Slides were rinsed twice with PBS and viewed. For staining with Congo red, the biofilm-covered glass slide was covered with the solution and incubated at room temperature for 40 min. Slides were then washed twice with PBS and observed. Biofilms were viewed with an Olympus AX-70 Multimode Microscopy System and appropriate filters.

Results and Discussion

ΔSO3389 was tested for possible phenotypes in aerobic and anoxic defined, minimal medium. The mutant did not differ significantly as measured by growth rate, compared to WT when grown aerobically in the presence of lactate. However, when transferred from aerobic to anoxic media, the mutant was defective in growth (chapter 2). When cells were transferred from aerobic to anoxic medium with lactate and fumarate, the △SO*3389* cells did not grow for approximately 35 to 40 h compared to wild-type cells (Figure 12). However, final ODs and growth rates were similar in exponential phase once the mutant initiated growth after the extended lag period. Similar results were observed when the anoxic medium contained lactate as the electron-donor and DMSO (data not shown) or Fe (III)-citrate as the electron-acceptor (Figure 13). When the anoxic culture of the mutant cells was inoculated into fresh anoxic medium, the culture did not lag. In addition, when the mutant cells were transferred back to the aerobic medium, a lag was not observed (Figure 18 and Figure 19). These results indicated that (i) a cell-dependent inhibitor did not likely cause the initial lag in suppressor cells, and (ii) that mutant cells were affected in the transition from aerobic to anoxic conditions and not anaerobic growth *per se*. These results also suggest that the mutant could grow

aerobically, but the mutant cells did not respond to the absence of O_2 due to the extended lack of growth when transferred to anoxic medium.

When wild-type cells were cultivated in aerobic shake flasks, cultures produced biofilms in 40 to 45 h (Figure 14), and the amount of biofilm formation increased with the degree of shaking (Figure 15). Wild-type and mutant cells formed similar amounts of biofilm in static cultures and cultures grown at 50 rpm (Figure 15), but wild-type cells produced 2.5-fold more biofilm than the mutant at 150 rpm based upon crystal violet staining (Figure 14).

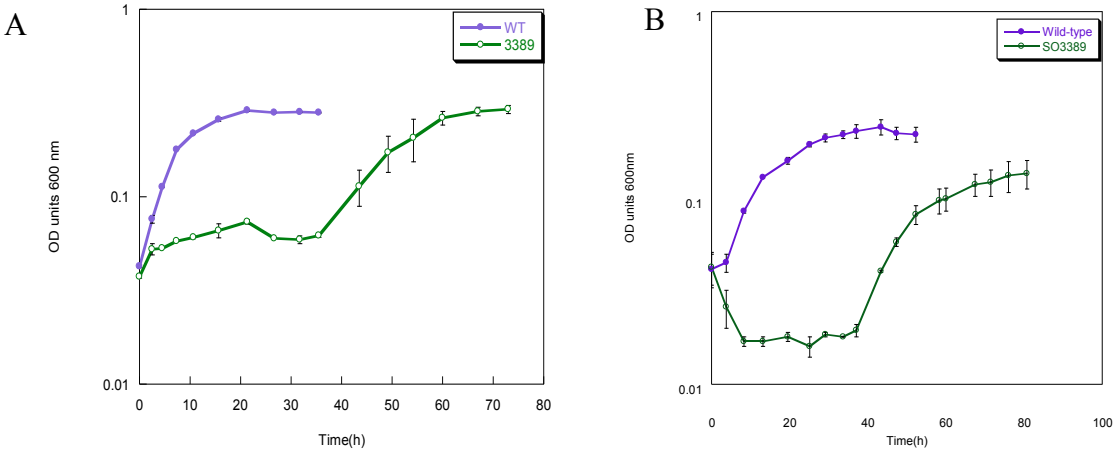


Figure 12: Anaerobic growth of WT and ΔSO3389 upon transfer from aerobic culture. A) Growth on 120mM lactate as electron donor and 120mM fumarate as electron acceptor. B) Growth on 20mM lactate and 40mM fumarate. n=3

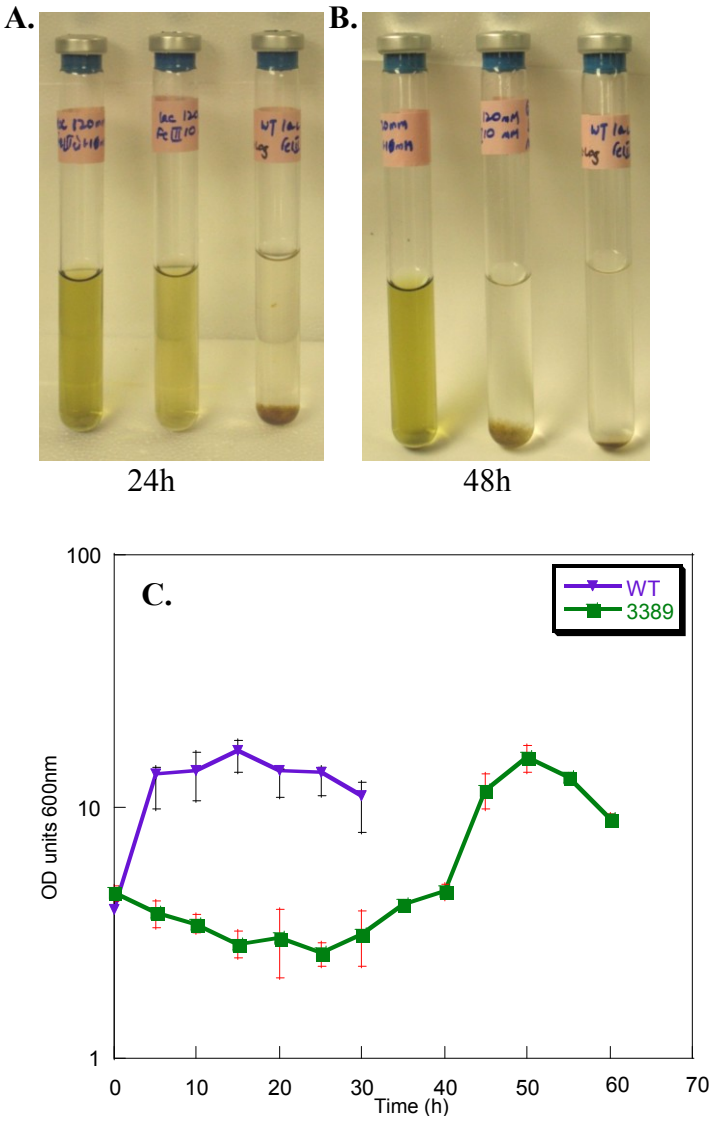


Figure 13: Growth of MR-1 WT and ΔSO3389 mutant in medium containing Fe(III) as electron acceptor. A) Growth of MR-1 and ΔSO3389 at 24h. B) Growth of MR-1 and ΔSO3389 at 48h. Disappearance of greenish-yellow color indicates reduction of Fe(III) (C) Fe(III) reduction quantified by Ferrozine assay in MR-1 WT (purple) and ΔSO3389 (green) over a 60h time period.

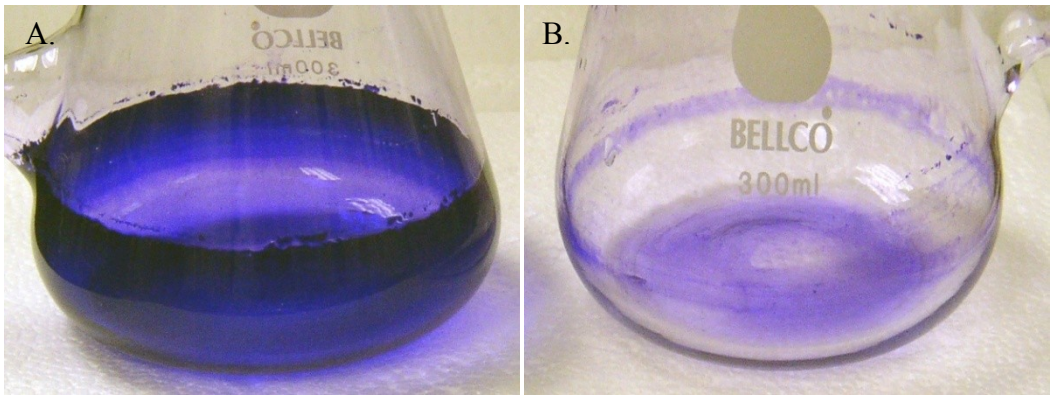


Figure 14: Crystal violet stained biofilms formed at 150rpm at approximately 35-40h by A) MR-1 WT and B) ΔSO3389.

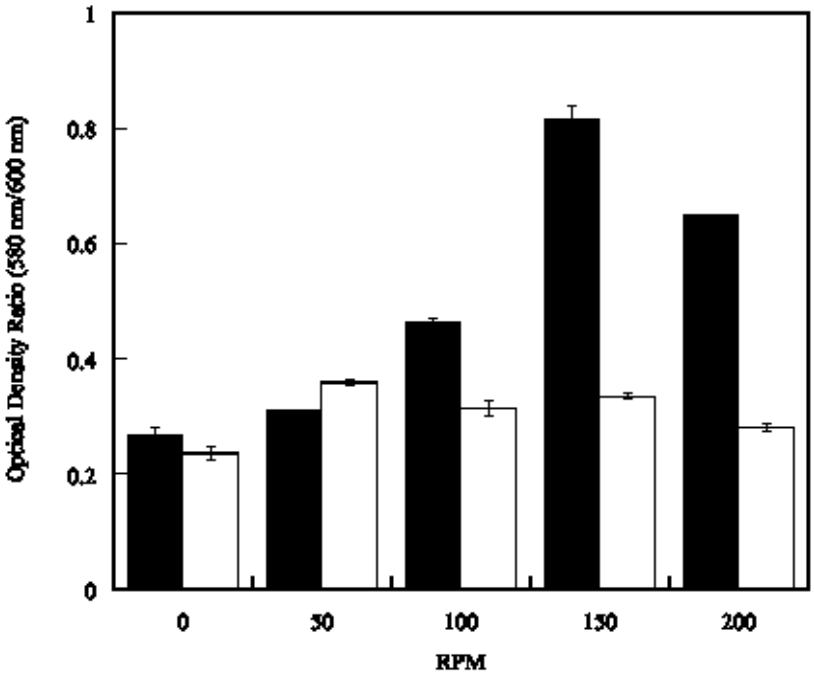


Figure 15: Comparison of biofilm formation between wild-type (filled) and mutant (empty) cultures at varying shake speeds (rpm). Speeds employed were 0, 50, 100, 150 and 200 rpm. 580/600nm was calculated to quantify biofilm formation. n=3

When the culture protein levels were measured directly (at maximal OD, 150rpm), the planktonic biomass did not differ significantly between wild-type and mutant cells (10.7±1.0 vs. 9.4±0.9 μg/μl), but the wild-type biofilm had 5.3-fold more protein than the mutant biofilm (1.23±0.2 vs. 0.23±0.0 μg/μl). It should be noted that the mutant culture did not increase biofilm formation after longer periods of incubation, and this result indicated that the decreased biofilm formation was not a consequence of slower growth. The difference in total biomass yield (planktonic plus biofilm) coincided with the hypothesis that the mutant was less efficient in oxygen utilization and the decreased utilization was directly and/or indirectly involved in the coordination of aerobic biofilm growth.

The SEM observations of the respective biofilms showed that the wild-type cells formed complex, multi-cellular layers, but the mutant cells were sparsely adhered in a monolayer at 20 h (data not shown). Moreover, it appeared that the mutant biofilm produced less extracellular matrix than the wild-type biofilm when observed at 40 h (Figure 16 B and D). The comparison of protein and carbohydrate content of wild type and mutant biofilms indicated that the later produced 30% less total carbohydrates compared to wild-type when normalized for protein content (data not shown). Finally, when wild type and mutant biofilms were stained with acridine orange (AO) and calcofluor white (CW), both biofilms contained cells as indicated by staining with AO. However, when observed with the CW filter, only wild-type biofilm had the characteristic fluorescent stain of extracellular carbohydrate (Figure 16 A and C). The

results indicated that △SO3389 was affected in biofilm formation, and that the capacity to form an extracellular carbohydrate matrix was involved.

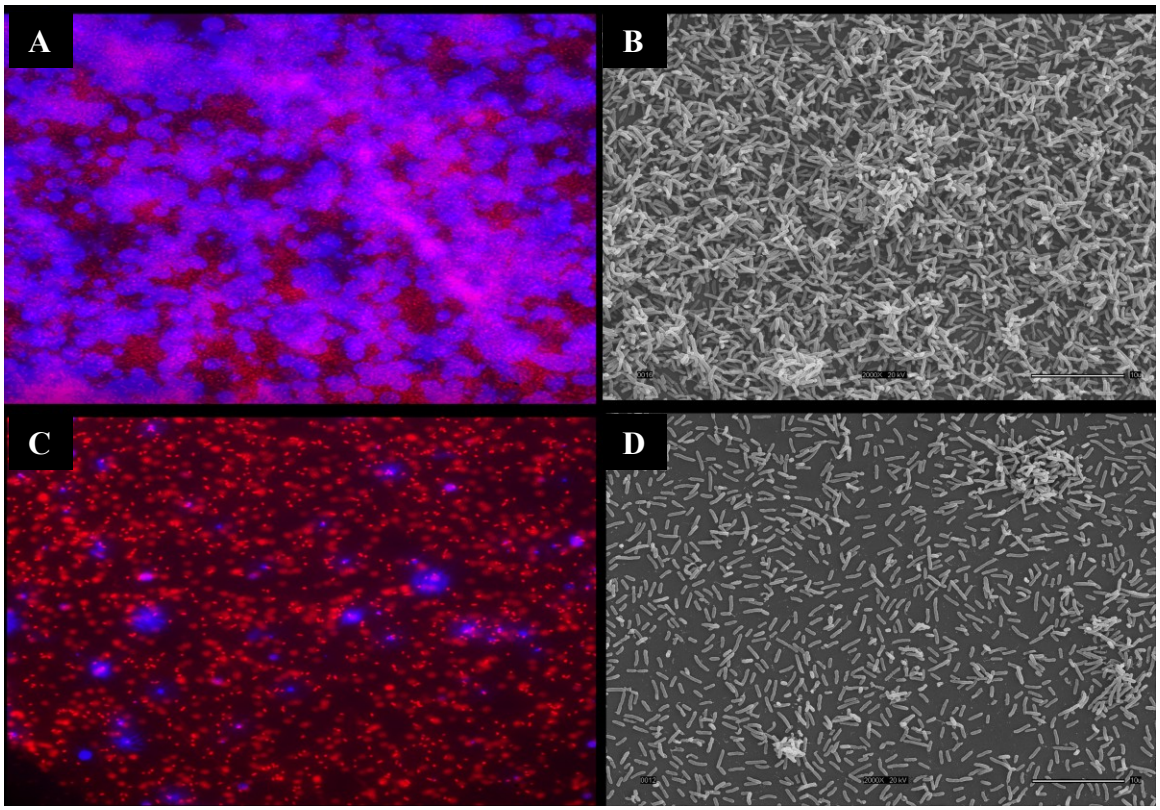


Figure 16: Epifluorescent microscopy of MR-1WT (A) and △SO3389 (C) biofilms grown in defined, minimal medium with lactate for 40 h. Acridine orange (stains nucleic acid) was used to stain cells (red) and calcofluor white was used to detect extracellular carbohydrate (blue). Scanning electron micrographs of wild-type (B) and △SO3389 (D) biofilms grown in defined, minimal medium with lactate for 40 h. Cells were grown at 150rpm. The magnification and bar size is 2000x and 10 μm, respectively.

Aerobic cultures were spot inoculated onto 0.3% agar medium and colony diameter observed over time. Assays indicated that the mutant was deficient in motility on 0.3% agar in aerobic conditions compared to wild-type cells (colony diameter of 26.5±3.5 vs. 15.5±0.5 mm) (Figure 17A and B), and TEM indicated that the mutant cells had intact flagella (data not shown). The mutant exhibited a similar degree of motility under anoxic conditions when compared to wild-type cells (data not shown), and these results indicated that the mutant was defective in motility under aerobic conditions and not flagella production. When the same culture was spotted on 1.0% agar plates from aerobic cultures, the wild-type and mutant colonies had similar diameters (data not shown).

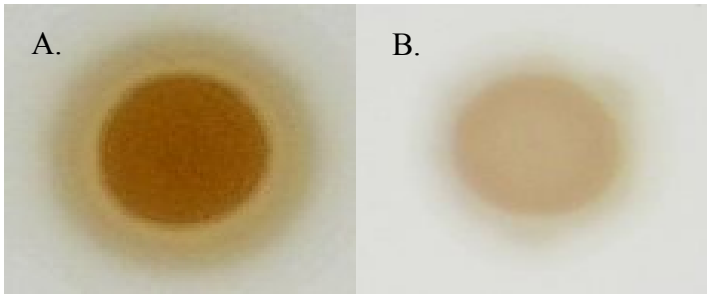


Figure 17: A colony of (A) wild-type or (B) △SO3389 cells on 0.3% motility agar inoculated from exponential phase aerobic cultures.

Thormann *et al;* 2004 documented the initial stages of biofilm formation in *S. oneidensis* MR-1 on glass, and showed that non-swimming mutants were deficient in biofilm formation. In addition, the study showed that swimming motility (and flagellum rotation) does not affect initial attachment but rather biofilm architecture. Similar to these results, △SO3389 was deficient in motility and biofilm formation, and flagella were observed via TEM (data not shown). These observations corroborated the

observation that the mutant could form the initial stages of a biofilm, but did not develop a mature biofilm with significant extra-cellular carbohydrate compared to wild-type cells.

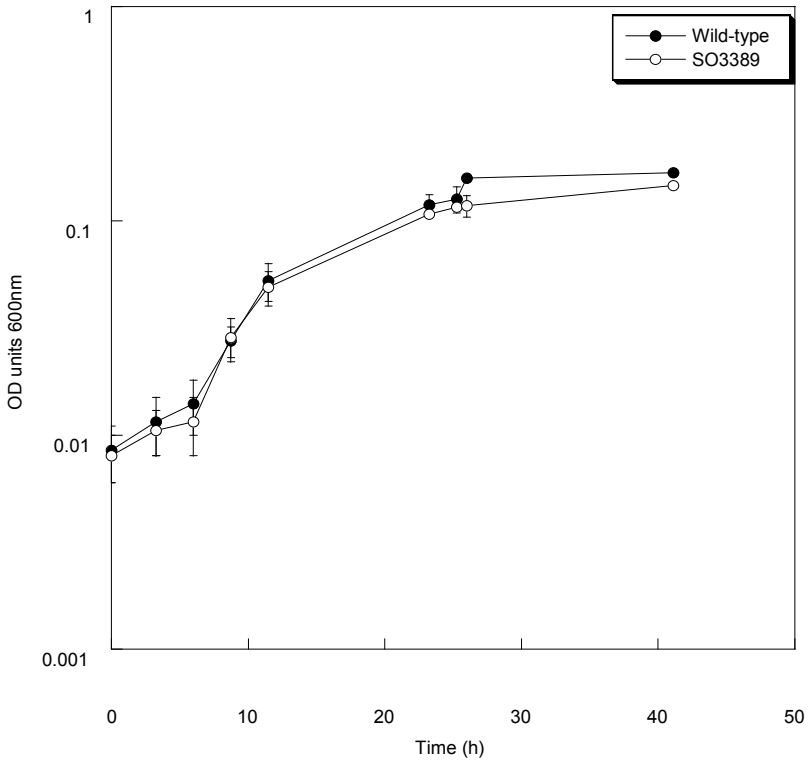


Figure 18: Anoxic growth of WT and ΔSO3389 in the presence of fumarate as electron acceptor. Growth experiment performed under anoxic conditions after four consecutive aerobic transfers. n=3

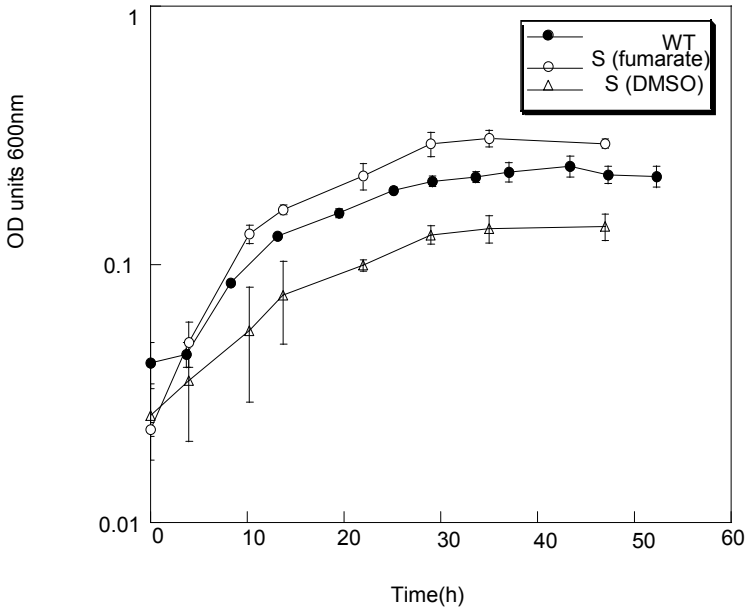


Figure 19: Anoxic growth of WT (with fumarate) and mutant suppressor strains (S) in the presence of fumarate and DMSO as electron acceptors (Experiment performed with isolated suppressor strains). n=3

Because the cells were grown on lactate and fumarate and the cytochrome stains and genomic data indicated that fumarate reductase may have been affected, fumarate reductase activities were measured via activity gels. During the transition, wild-type cells had up-expressed fumarate reductase activity as seen by zymograms, but the mutant did not. This further corroborated the idea that cytochromes necessary for fumarate reductase activity were not up-expressed in mutant cells (Figure 20A). The mutant did have a relatively small amount of activity that was either inadequate for growth, or other factors prevented optimal growth of the mutant. Once suppressor strains had grown anoxically, the fumarate reductase activity was elevated compared to wild-type cells (Figure 20B).

These results suggested that the mutant had altered the regulation of fumarate reductase activity although different proteins with similar activity could be responsible.

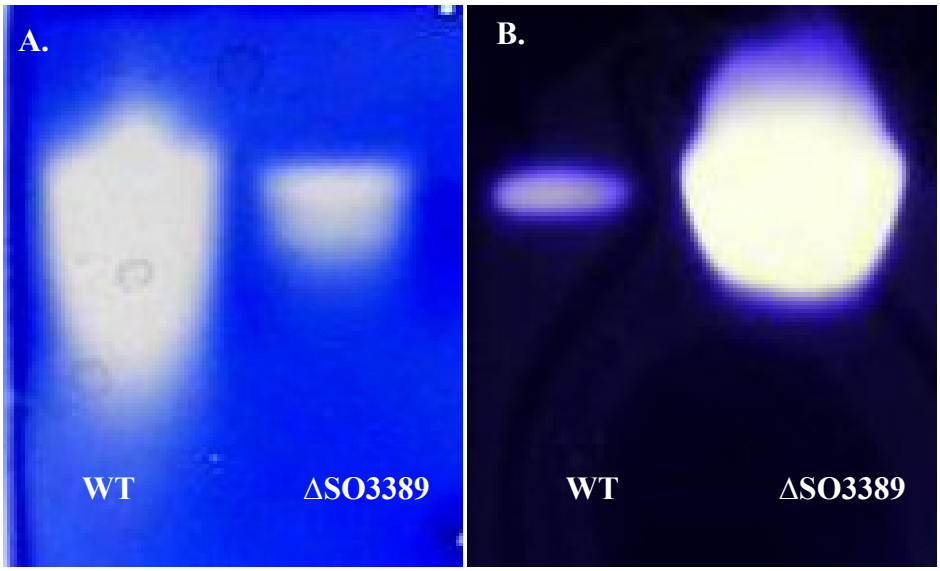


Figure 20: Fumarate reductase activity gel of wild-type and △SO3389 cells during transition (**A**) and anoxic conditions (**B**) in defined medium. Equal amounts of protein were loaded per lane under anoxic and transition conditions.

After the initial transfer and lag in anoxic medium, a small percentage of the population grew and the frequency was approximately 4.6 x 10^{-6}. These results indicated that suppression ($SO3389^{S}$) of the original mutation had most likely occurred and that the anoxic growth in the original mutant was not simply an adaptation of the existing population. It should be noted that the mutant was still deficient in biofilm formation (crystal violet stain = 5.9 versus 0.5, Figure 21, Table 4) and motility (Table 5, Supplemental Figure 1) and these results suggested that the altered signal pathway decoupled aerobic metabolism from biofilm formation.

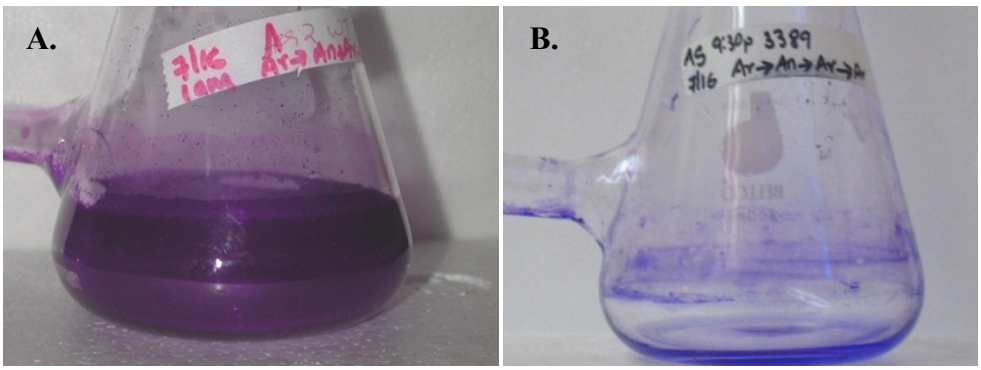


Figure 21: Crystal violet stained biofilms formed by A) WT and B) ΔSO3389. Shake flasks were examined for biofilm formation at all four transfers. Above represents CV staining of transfer #2.

Table 4. 580/600nm ratios which signify biofilm quantitation in WT and mutant during four consecutive aerobic transfers after which culture was transferred to anoxia. n=3		
Transfer #	580/600 WT	580/600 ΔSO3389
1	4.20	0.459
2	5.90	0.478
3	4.13	0.811
4	2.65	0.186

Table 5: Motility assay of WT and mutant at 4 aerobic transfers before culture was transferred to anoxia. Numbers represent diameter of colonies that were spot inoculated on 0.3% HBa agar. n=3		
Transfer #	Diameter WT (cm)	Diameter ΔSO3389 (cm)
1	1.9 ± 0.3	1.2 ± 0.2
2	1.8 ± 0.2	1.3 ± 0.3
3	2.02 ± 0.2	1.0 ± 0.2
4	1.9 ± 0.2	0.8 ± 0.2

In addition, after repeated transfers in aerobic medium, the suppressor strains maintained elevated levels of fumarate reductase activity compared to wild-type (Figure 22). These results also suggested that SO3389^{S} had altered signaling pathways and had a more constitutive expression of fumarate reductase activity to overcome the lack of up-expression in the △SO3389 background. It is feasible that multiple pathways respond to overlapping stimuli in MR-1, but the fact that fumarate reductase activity was not simply re-gained under a regulated mechanism suggested that the signaling mechanism(s) was in a constitutive mode rather than new control by an over-lapping system. Further work is needed to elucidate the cognate regulator(s) and respective regulon(s) of the SO3389 sensory box protein.

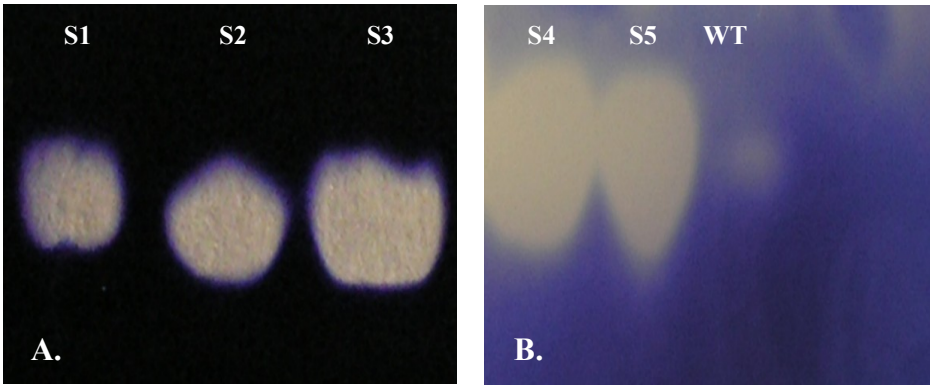


Figure 22: Fumarate reductase activity for wild-type and five separate suppressor strains, S1, S2, S3, S4 and S5 and WT during anoxic growth (Experiment performed for anoxic cultures after 4 aerobic transfers).

Our data indicated that SO3389 was involved in responses to declining and increasing oxygen levels and this result fits the working model for biofilm response to oxygen in MR-1. Such a scenario would explain the multiple phenotypes observed for △SO3389, and SO3389 could interact with multiple response regulators that would affect biofilms and anaerobic metabolism. Because the mutant did not form significant biofilms even in the presence of increasing oxygen levels and had a significant lag when transferred to anoxic growth conditions, the putative sensor appeared to respond to changes in oxygen and/or ORP (oxidation reduction potential) levels. The amount of biofilm biomass correlated with O_2 consumption first-order rate constants (R=0.91 and 0.94, wild-type and mutant, respectively) and might explain the inability of the mutant to form significant biofilms (*i.e.*, lower O_2 consumption rates). However, as expected, the oxidation-reduction potential of the growth medium also changed at different rates as wild-type and mutant cultures grew aerobically and could also be a signal that helps control cellular metabolism.

In conjunction with the biofilm deficient phenotype, the complement strain did not consume O_2 at wild-type levels although growth was similar when DO levels were higher (data not shown). The complement strain actually had a lower O_2 consumption rate than the mutant, and the inability of the complement strain to restore the biofilm phenotype might be a result of the inability to consume O_2 at faster rates under aerobic conditions. Multicopy expression of SO*3389* from the complementation vector may interfere with the appropriate timing of regulation and/or abundance of the protein that results in partial complementation of only some of the phenotypes associated with the

loss of SO*3389*. The non-optimal protein levels could be particularly relevant for proteins that interact with intracellular signaling metabolites such as c-di-GMP (discussed below) in which small fluctuations might have large physiological effects. A recent study with CckA (PAS-like domain) in *Caulobacter crescentus* showed that the spatiotemporal distribution of the protein associated with chromosome-based expression was important for full function (Angelastro et al., 2010**)** Sequence conservation predicts that SO3389 is a phosphodiesterase, and expression from the vector promoter may cause abnormal depletion of c-di-GMP levels. However, further work is needed to delineate activity of SO3389 and its effects on cellular pools of c-di-GMP.

qPCR as reported in chapter 2, was used to monitor the expression of SO*3389* and SO*3388* in wild-type and mutant cells under aerobic and anoxic conditions. As expected, wild-type cells had higher expression levels of SO*3389* under aerobic and anoxic conditions compared to the mutant. The SO*3388* gene, the ORF down-stream of SO*3389*, had similar expression levels in both wild-type and mutant cells under aerobic and anoxic conditions. These results indicated that the ΔSO*3389* mutation did not have a polar effect on SO*3388* expression that contributed to the observed phenotypes.

Summarizing chapters 2 and 3, our results with △SO3389 suggested that a combination of oxygen consumption and a direct sensing of oxygen levels played a role in the ability of MR-1 to coordinate aerobic growth and biofilm formation. A role in oxygen-sensing coincides with the strong evidence that PAS domains are typically sensors for oxygen and/or intracellular redox potentials (Simm *et al*., 2004). Previous results indicated that a decline in oxygen levels caused MR-1 biofilms to detach and that

c-di-GMP played a role in regulation of biofilm stability (Thormann *et al*., 2006; Thormann *et al*., 2005). It is interesting to speculate how cells sense and control stimuli responses to optimize metabolism and physiological states. Based upon recent results, the Spormann group put forth a model that explained biofilm formation and detachment in response to stimuli, such as oxygen, via c-di-GMP pools (Thormann *et al*., 2006; Thormann *et al*., 2005). Further work is needed to understand how c-di-GMP pools mediate control over multiple cellular processes, but SO3389 likely plays a role based upon predicted domains and the observed phenotypes.

References

Angelastro, P.S., O. Sliusarenko, and C. Jacobs-Wagner. 2010. Polar localization of the CckA histidine kinase and cell cycle periodicity of the essential master regulator ctrA in *Caulobacter crescentus*. J. Bacteriol. 192: 539–552.

Christen, B., M. Christen, R. Paul, F. Schmid, M. Folcher, P. Jenoe, M. Meuwly, and U. Jenal. 2006. Allosteric control of cyclic di-GMP signaling. J. Biol. Chem. 281:32015-32024.

Galperin, M. Y. 2004. Bacterial signal transduction network in a genomic perspective. Environ. Microbiol. 6:552-567.

Galperin, M. Y.2005.A census of membrane-bound and intracellular signal transduction proteins in bacteria: Bacterial IQ, extroverts and introverts. BMC Microbiol. 5: 35.

García, B., C. Latasa, C. Solano, F. García-del Portillo, C. Gamazo, and I. Lasa. 2004. Role of the GGDEF protein family in *Salmonella* cellulose biosynthesis and biofilm formation. Mol. Microbiol. 54:264–277.

Hecht, G. B., and A. Newton.1995Identification of a novel response regulator required for the swarmer-to-stalker cell transition in *Caulobacter cresentus*. J. Bacteriol. 177:6223-6229.

Jenal, U. 2004. Cyclic di-guanosine monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria? Curr. Opin. Microbiol. 7:185-191.

Kirillina, O., J. D. Fetherston, A. G. Bobrov, J. Abney, and R. D. Perry. 2004. HmsP, a putative phosphodiesterase, and HmsT, a putative diguanylate cyclase, control Hms-dependent biofilm formation in *Yersinia pestis*. Mol Microbiol. 54:75-88.

Koonin, E. V., and M. Y. Galperin.1997. Prokaryotic genomes: the emerging paradigm of genome-based microbiology.Curr. Opin. Genet. Develop. 7:757-763.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-75.
Lund, K., and J. DeMoss. 1976. Association-dissociation behavior and subunit structure of heat-released nitrate reductase from *Escherichia coli*. J. Biol. Chem. 251:2207-2216.

Magnuson, T. S., A. L. Neal, and G. G. Geesey. 2004. Combining in situ reverse transcriptase polymerase chain reaction, optical microscopy, and X-ray photoelectron

spectroscopy to investigate mineral surface-associated microbial activities. Microbiol. Ecol. 48:578-88.

Merkel, T. J., C. Barros and S. Stibitz.1998Characterization of the bvgR locus of *Bordetella pertussis*. J. Bacteriol. 180:1682-1690.

Paul, R., S. Weiser, N. C. Amiot,C.Chan, T. Schirmer, B. Giese, and U. Jenal. 2004. Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. Genes. Dev. 18:715-727.

Pei, J., and N. V. Grishin.2001. GGDEF domain is homologous to adenylyl cyclase. Proteins 42: 210-216.

Simm, R., M. Morr, A. Kader, M. Nimtz, and U. Romling. 2004. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility and motility. Mol. Microbiol. 53:1123-1134.

Slater, H., A. Alvarez-Morales, C. C. Barber, M. J. Daniels, and J. M. Dow.2000. A two-component system involving an HD-GYP domain protein links cell-cell signaling to pathogenicity gene expression in *Xanthomonas campestris*. Mol. Microbiol. 38:986-1003.

Tal, R., H. C. Wong,R. Calhoon, D. Gelfand,, A. L. Fear, G. Volman,R. Mayer, P. Ross, D. Amikam, H. Weinhouse, A. Cohen,S. P. Ohana, and M. Benziman.1998.Three cdg operons control cellular turnover of cyclic-di-GMP in *Acetobacter xylinum*: genetic organization and occurance of conserved domains in isoenzymes. J. Bacteriol. 180:4416-4425.

Taylor, B. L., and I. B. Zhulin. 1999. PAS domains: Internal sensors of oxygen, redox potential, and light. Microbiol. Molc. Biol. Rev. 63:479-506.

Thormann, K. M., R. M. Saville, S. Shukla, and A. M. Spormann. 2005. Induction of rapid detachment in *Shewanella oneidensis* MR-1 biofilms. J. Bacteriol. 187:1014-1021.

Thormann, K. M., R. M. Saville, S. Shukla, D. A. Pelletier, and A. M. Spormann. 2004. Initial phases of biofilm formation in *Shewanella oneidensis* MR-1. J. Bacteriol. 186:8096–8104.

Thormann, K. M., S. Duttler, R. M. Saville, M. Hyodo, S. Shukla, Y. Hayakawa, and A. M. Spormann. 2006. Control of formation and cellular detachment from *Shewanella oneidensis* MR-1 biofilms by cyclic di-GMP. J. Bacteriol. 188:2681:2691.

CHAPTER 4

SUBSTRATE-BASED DIFFERENTIAL RESPONSE OF *DESULFOVIBRIO VULGARIS* HILDENBOROUGH PLANKTONIC CELLS TO DISSOLVED OXYGEN

Abstract

Desulfovibrio vulgaris Hildenborough, although considered a strictly anaerobic, sulfate-reducing bacterium, is capable of surviving in environments that are exposed to mM levels of O_2. In this study, *D. vulgaris* was grown in the presence of lactate or pyruvate as carbon/energy sources and exposed to various concentrations of dissolved oxygen in a defined medium. Data suggested that lactate cells were more sensitive to DO exposure compared to pyruvate cells. Lactate-cells were inhibited at 1.9 ppm while pyruvate-cells could grow at 1.9 ppm DO after a lag phase. Even though lactate cells appeared to be more sensitive, cells demonstrated increased growth rates at intermediate levels of DO (0.3 to 1.5 ppm). Pyruvate-grown cells did not increase growth rate with intermediate levels of DO, but could tolerate higher DO levels. In addition, lactate-grown cells had 10-fold increase in ATP levels compared to cells at lower DO as well as pyruvate-grown cells (20-fold). These results indicated the lactate-grown cells utilized oxygen for energy production at intermediate levels of DO and coincided with increased growth rates. Lactate-grown cells had higher total carbohydrate levels than pyruvate-grown cells, but the intracellular carbohydrate levels were similar (0.04 vs. 0.035 ug carbohydrate/ug protein, respectively), and these results indicated that differences in the glycogen reserves could not solely explain the observed differences in oxygen tolerance between lactate- and pyruvate-grown cells. A mutant in the glycogen synthase (*glg*A) had decreased glycogen amounts in both lactate- and pyruvate-grown cells (approximately 3-fold). Lactate-grown Δ*glg*A cells were more sensitive to DO (inhibition at 1.5 ppm vs. 1.9 ppm for wild-type), and did not increase growth rate at intermediate DO levels compared to wild-type. Pyruvate-grown Δ*glg*A cells were only slightly affected in DO tolerance, and could still grow at levels up to 1.9 ppm. These results indicated that reducing power from glycogen was used to reduce oxygen for ATP production in the presence of lactate but not pyruvate, and that glycogen reserves were not essential for tolerance to 1.5 ppm in lactate cells or 1.9 ppm in pyruvate cells. A Δ*per*R mutant was tested in order to determine the potential role of oxygen detoxification via PerR regulated genes. For lactate-grown cells, Δ*per*R did not grow with 1.5 ppm DO similar to the Δ*glg*A mutant. However, Δ*per*R cells still had increased growth rates with 0.8 ppm DO. Unlike the pyruvate-grown Δ*glg*A cells, the Δ*per*R cells had decreased tolerance and did not grow with 1.9 ppm DO. These data suggested that glycogen is used in a substrate-dependent manner for oxygen consumption and energy production, and that *per*R played more a role in oxygen detoxification and was not essential for survival at intermediate DO levels.

Introduction

Molecular oxygen is considered to be inhibitory to dissimilatory sulfate reduction and hence to the growth of sulfate-reducing bacteria (SRBs). Although perceived as stringent anaerobes, SRBs have evolved numerous mechanisms to combat oxidative stress and have acquired several strategies not only for oxygen tolerance but also utilization, and over the past three decades, numerous studies have demonstrated the aerotolerance of SRBs (Postgate, 1984; Cypionka *et al*., 1985; Dilling and Cypionka, 1990; Johnson *et al*., 1997). Initially, SRBs were thought to remain dormant but not be killed when exposed to ambient atmosphere (Postgate, 1984). Later, it was established that some SRBs could not only tolerate oxygen but could also carry out oxygen-dependent respiration (Cypionka *et al*., 1985; Dilling and Cypionka, 1990; Johnson *et al*., 1997).

The role of oxygen in SRB population behavior has been demonstrated in several studies. Krekler *et al* (1998) showed that SRBs in cyanobacterial mats migrate due to diurnal changes in oxygen levels of the mat, and Eschemann *et al* (1999) demonstrated that some SRBs display aerotaxis to certain concentrations of oxygen (Krekler *et al*., 1998; Eschemann *et al*., 1999). These results coincide with multiple biochemical studies and more recent genomic data that show different SRBs contain annotated oxygen-tolerance systems as well as some gene products predicted to be involved with oxygen utilization (Hatchikian, 1977; Dos Santos *et al*., 2000; Moura *et al*, 1994; Fournier at al., 2004; Lemos *et al*., 2001; Heidelberg *et al*., 2004; Rabus *et al*., 2004). These results

suggest that the types of environments and micro-environments for SRBs must be reconsidered in the context of available electron-donors and -acceptors.

Speculation of possible oxygen utilization came from the capacity of some SRBs' ability to reduce oxygen. Dannenberg *et al*. (1992) reported oxygen reduction in up to 14 strains of SRBs with different substrates as electron donors, and respiration rates were comparable to rates in some aerobic organisms. *Desulfovibrio desulfuricans* CSN was reported to reduce 5 mM oxygen with hydrogen as the electron donor; however, aerobic respiration did not appear to be linked to growth in spite of ATP production (Dilling and Cypionka, 1990). Most work to date suggests that oxygen reduction by SRBs is more of a protective mechanism against oxidative stress rather than an energy-coupled conservation mechanism that promotes growth. However, Johnson *et al*. (1997) indicated oxygen-dependent growth in *Desulfovibrio vulgaris* Hildenborough in a rich medium (*i.e*., yeast extract) under sulfate-limiting conditions (Johnson., *et al*., 1997).

Other studies have shown that oxygen-reduction is linked to glycogen (polyglucose) utilization. Studies with *Desulfovibrio gigas* and *D. salexigens* have revealed that the oxygen reduction rate is directly proportional to the polyglucose levels (van Niel *et al*., 1996; van Niel and Gottschal, 1998; Santos *et al*., 1993) and proposed that the transfer of reducing power derived from polyglucose to oxygen is linked to NADH oxidase activity and this could enable organisms to survive under oxic conditions (Fareleira *et al*., 1997). However, it is important to note that most studies have been done in complex media with only lactate as the carbon and energy source. In the present work,

responses of *Desulfovibrio vulgaris* Hildenborough to dissolved oxygen (DO) were evaluated in the presence of the different electron donors, lactate and pyruvate.

Materials and Methods

Strains and Growth

Desulfovibrio vulgaris Hildenborough ATCC 29579 obtained from the Americal Type Culture Collection, VA, USA. *Desulfovibrio vulgaris* Δ*glg*A (DVU2244) and Δ*per*R (DVU3095) was kindly provided by Dr. Judy Wall.

Growth Conditions

Growth of *Desulfovibrio vulgaris* Hildenborough ATCC 29579 was carried out in LS4D (Clark *et al*., 2006; Brandis, 1981) minimal mineral medium with 60mM lactate and in PS4D minimal mineral medium with 60mM pyruvate as the electron donor. LS4D contains 50mM sulfate as the electron acceptor. LS4D also contains ammonium chloride(20mM), magnesium chloride(8mM), potassium phosphate (2.2mM), PIPES(30mM), calcium chloride(0.6mM), resazurin(0.064 as the redox indicator, 80X mineral solution, 100 X vitamin solutions. PS4D contains 50mM sulfate as the electron acceptor, ammonium chloride(20mM), magnesium chloride(8mM), potassium phosphate (2.2mM), PIPES(30mM), calcium chloride(0.6mM), resazurin(0.064 as the redox indicator, 80X mineral solution, 100 X vitamin solutions (Clark *et al*., 2006). Growth experiments were performed in 10ml anaerobic tubes with nitrogen as head space.

GlgA DVU2244 Deletion

Approximately 800bp sequence upstream and approximately 600bp sequence downstream of *glgA* (DVU2244) was cloned on either side of a kanamycin resistance cassette. The three segments were amplified by PCR and then ligated by a fourth PCR. This mutagenic cassette was introduced to the cell where a double recombination event replaced the target gene with the *kan* resistance gene. In order to be able to differentiate among other deletion isolates when grown simultaneously, the *kan* resistance cassette contained a sequence common to all deletion mutataions and a 'barcode' on each end (the common sequence and the barcode were included during primer design for the amplification of the kan resistance gene). The *kan* cassette and, upstream and downstream regions were PCR amplified using Herculase polymerase (Stratagene) and ligated by another round of PCR amplification. The product was separated on a 0.8% agarose gel, and the appropriate band was excised and cleaned using Wizard SV Gel and PCR Clean-up system (Promega). This product was captured in cloning vector pCR8-TOPO (Invitrogen) generating pMO920-2244, transformed into *E.coli* Top-10 cells (Invitrigen) and plated on LC medium (5g/L NaCl, 5g/L yeast extract, 10g/L tryptone) with 100ug/ml spectinomycin (Sigma). Transformants were inoculated into LC medium with 50ug/ml kanamycin. pMO0920-2244 was isolated from kan and spec resistant *E coli* transformants using a Qiagen Miniprep (Qiagen) , sequenced for verification and electroporated into *D. vulgaris*. Electroporation was carried out using a BTX electroporation pulse generator, ECM630 (Genetronix, San Jose, CA). Parameters for elctroporation were 1.75kV, 25uF, and 250 Ω. Following transformation, cells were

recovered in 1 ml LS4D medium (Mukhopadhyay *et al*., 2006) supplemented with 0.2% yeast extract. Transformants were plated on modified LS4D (Mukhopadhyay *et al*., 2006). Colonies that grew after 3 to 5 days of incubation at 30 C were replica plated onto LS4D medium containing 100ug/ml Spec and 400ug/ml G418 (RPI Corp.) G418 resistant isolates were subcultured into medium with lactate/sulfite and G418. The next day, genomic DNA was prepared and was digested with FspI restriction enzyme (New England Biolabs) along with WT *D. vulgaris* DNA and Southern blots were performed to verify that DVU2244 was replaced by the *kan* resistance gene. Primers used for this study are listed in the table below (Table 6)

Primer 1 (DVU2244-1b): 19bp	CGGGCGAGGCGACATCATT
Primer 2 (DVU2244-2b): 47bp	**AAGACTGTAGCCGTACCTCGAATCTA** GATGGCTGTCTCCACGGATGC
Primer 3 (DVU2244-3b): 45bp	**AATCCGCTCACTAAGTTCATAGACCG** GACCATTCCGTGCAGCCGT
Primer 4 (DVU2244-4b): 23bp	AGCAGTTCAAGGTGAGTGAAGCC
Primer 5 (bc0064f): 64bp	**TAGATTCGAGGTACGGCTACAGTCTT AGGTCGTTAAGAGGCAATAC CCCCAGAGTCCCGCTCAG**
Primer 6 (bc0064r): 66bp	**CGGTCTATGAACTTAGTGAGCGGATT ACGTTAATAAGAACTCGCCC GAGGTAGCTTGCAGTGGGCT**

Table 6: Primers used for mutagenesis of Δ*glgA*. Black represents *D.vulgaris* genomic sequence. **Black** represents common sequence. Blue represents unique barcode and orange represents Kan sequence.

PerR DVU3095 Deletion

perR deletion was performed similar to a method described for *fur* mutagenesis (Bender *et al*., 2007). Briefly, plasmid pMO707 (Bender *et al*., 2007) (kanamycin resistance gene) was transferred to wild-type *D. vulgaris* by electroporating 5ug of knockout vector (pMO707) along with positive (pSC27) and negative (lambda DNA) controls, Electroporation was carried out using a BTX electroporation pulse generator, model ECM630 (Genetronix, San Jose, CA). Parameters for elctroporation were 1.75kV, 25uF, and 250 Ω. Following transformation, cells were recovered in 1ml LS4D medium supplemented with 0.2% yeast extract. . Transformants were selected by plating on modified LS4D (with yeast extract and 400ug/ml G418). Colonies from pMO707 transformation were subcultured into LS4D supplemented with yeast extractand a second single-colony isolation was made. Deletion was then verified by running PCR product on a 0.8% agarose gel by checking for WT *perR* gene product and a gene product in 'deletion strain' that corresponds to the size of *kan* cassette (Bender *et al*., 2007).

Medium Preparation

LS4D and PS4D media were prepared typically under anoxic conditions in contact with a headspace of N_2. Upon cooling (post-autoclaving), different volumes of air (2 to10ml) were introduced into 10 ml balsch tubes (Bellco Glass, NJ, USA). An equal volume of N_2 headspace was recovered from the tubes with a sterile syringe before introduction of air. Tubes were allowed to stand for a few minutes to equilibrate before DO concentration in the medium was measured using a DO probe (Mettler Toledo). Air (21% oxygen) was introduced into 10ml anaerobic tubes (degassed with N_2) as described

in methods section. At least 10 measurements were made using a Mettler-Toledo DO probe with each volume of air injected to obtain standard deviations with each treatment. These values were plotted on a graph against volume of air introduced to obtain a standard curve with R=0.99581. For all experiments involving effect of DO, 2 ml air corresponded to 0.78± 0.1mg/l DO, 5 ml air corresponded to 1.5± 0.19mg/l DO, 8 ml air corresponded to 1.86± 0.1mg/l DO and 10 ml air corresponded to 3.19±0.109mg/l DO. Untreated media tubes, i.e., tubes without added air had 0.3± 0.1 mg/l DO.

Inoculation Procedure

Planktonic cells of *D. vulgaris* were grown in LS4D and PS4D until their Optical Density (OD) measured at 600nm reached 0.8, which represents exponential growth phase. Cells were harvested by centrifugation under aseptic anaerobic conditions in short balsch tubes and washed with sterile anaerobic S4D to remove excess sulfide. S4D minimal mineral medium contains all LS4D/PS4D media constituents except the electron donor, *i.e*., lactate and pyruvate. The cell pellet was used to inoculate tubes with pre-determined concentrations of dissolved oxygen to achieve an initial OD_{600nm} of 0.07 in all experiments.

Growth Experiments

D. vulgaris WT, Δ*glg*A, Δ*glg*A complement, Δ*per*R, Δ*per*R complement cells were grown planktonically in LS4D/PS4D medium in the presence of different concentrations of dissolved oxygen. *D. vulgaris* WT cells were inoculated into 10ml tubes to an initial OD of 0.07-0.075 in LS4D and PS4D with 0.78, 1.5, 1.86 and 3.14

mg/l DO and growth was monitored over time. Δ*glg*A and Δ*per*R were characterized with 0.78, 1.5, 1.86 and 3.14 mg/l DO, similar to WT planktonic cells and growth curves were conducted to monitor responses towards DO by recording optical density readings at 600nm. All growth experiments are representatives of at least two biological replicates and each biological replicate had two or three technical replicates.

Viability Experiments

Viability was carried out by a serial dilution method where 1ml of culture was transferred to 10^{-1} tube and subsequent, serial diluted to 10^{-9}. Tubes were inoculated in duplicate and incubated at 30°C for 1 week. Viability was assessed by growth or no growth based on measuring tubes using a spectrophotometer for maximum OD, and MPN/ml was obtained using a web-based MPN calculator (http://members.ync.net/mcuriale/mpn/mpncalc.zip)

Protein and Carbohydrate Measurements

Protein concentrations were made to normalize internal carbohydrate levels of wild-type and Δ*glg*A *D. vulgaris* cells in LS4D and PS4D to biomass. Protein was also measured to normalize ATP levels in the presence of 0.3 and 0.78 mg/l DO of WT and Δ*glg*A *D. vulgaris* cells grown in LS4D and PS4D to biomass produced. Protein concentration was done by the Lowry method as previously described (Lowry, 1951), and bovine serum albumin was used as the standard. Samples were boiled with 0.2N NaOH for 15 min and copper reagent was added, mixed immediately and incubated for 45 min

at 39°C. After cooling, 1ml phenol reagent was added and incubated at room temperature for 30 min. Reaction was measured at 660 nm.

Carbohydrates were quantified via the cysteine-sulfuric acid method. Samples were heated to 100°C for 3 min with 700 mg/l L-cysteine hydrochloride prepared in 86% ice-cold sulfuric acid. The mixture was rapidly cooled to room temperature and absorbance was measured at 415 nm (Chaplin, 1986).

Extraction of Internal/External Carbohydrates

Cell cultures (approximately 30 ml) were centrifuged at 8,000 x g for 8 min at 4°C and the supernatant was removed. The pellet was re-suspended in 30 ml dH_2O, vortexed vigorously for 1 min and centrifuged at 14,000 x g for 10 min. The supernatant was removed, and the pellet was re-suspended in 2 ml dH_2O and centrifuged at 8,000 x g for 8 min (Zhang, 1999). Carbohydrates were quantified by the method described above (Chaplin, 1986).

Sulfide Assay

Sulfide concentrations were measured via preparation of varying concentrations of sulfide (Na_2S) standards and the addition of copper solution (5M HCl, $CuSO_4$) and measurements were made immediately at 480 nm (Ruwisch, 1985). Anoxic water was used as a blank and 50 µl samples were treated similarly with copper sulfate reagent and absorbance was measured at 480 nm. Sulfide concentrations were either normalized to protein concentrations or OD_{600} readings.

ATP Measurement

ATP levels of *D. vulgaris* wild-type and Δ*glg*A cells at 0.3 and 0.78 mg/l DO in LS4D and PS4D media were measured using the ATP Bioluminescence Assay kit (Roche, part #11699709001). ATP standards were prepared as described and samples were subjected to heat treatment with Tris-EDTA buffer (pH 7.6) to release intracellular ATP. Samples (50 μl) was subsequently used for the assay. Wild-type planktonic cells were grown with desired DO levels (*i.e.*, 0.3mg/l or 0.78mg/l DO) in the presence of lactate or pyruvate. Samples were collected at 0.3 OD units and 0.8 OD units (early and late-exponential growth phases). The assay was carried out in a white 96-well plate to reduce signal loss from clear plates. Luminometer (Synergy HT, BioTek) emission filter was set at 560 nm and ATP concentrations were normalized to μg/μl protein. ATP levels were similarly measured for Δ*glg*A cells grown in LS4D and PS4D at 0.3 and 0.78mg/l DO.

Results

D. vulgaris Growth with Lactate and Pyruvate

D. vulgaris grew with lactate and pyruvate as carbon/energy source, and the growth rates of both washed and unwashed cells were calculated. For lactate-grown cells, the unwashed and washed cells had similar growth rates ($k=0.13h^{-1}$ and $0.14h^{-1}$, respectively). The pyruvate-grown cells exhibited similar growth rates for washed and unwashed cells ($k=0.13\ h^{-1}$ and $0.14\ h^{-1}$) (Figure 23A and B). For both substrates, the washed cells had a short lag period (1 to 2 h). Final sulfide levels for lactate-grown cells was 3-fold greater than pyruvate-grown cells (*i.e.*, sulfide levels in lacate-grown cells was

13.3±0.52 mM S_2^- /OD and pyruvate-grown cells had 4.06±0.20 mM S_2^-/OD. These results indicated that cells grew better when transferred with sulfide, and that pyruvate-grown cells had produced lesser sulfide than lactate-grown cells.

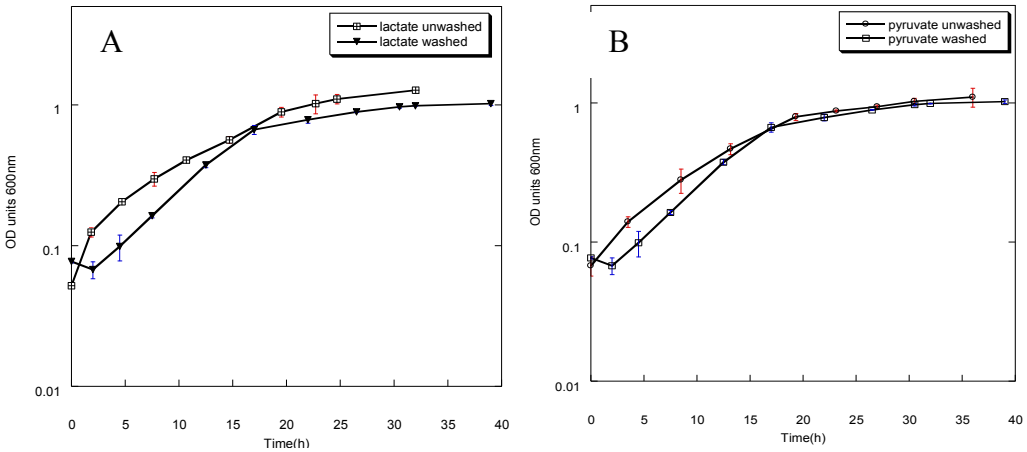


Figure 23: Growth of *Desulfovibrio vulgaris* Hildenborough washed and unwashed cells in the presence of 60mM (A) lactate and (B) pyruvate as carbon and energy sources and 50mM sulfate as the electron acceptor.

Effect of DO on Planktonic, Lactate-Grown Cells.

D. vulgairs planktonic cells were grown in the presence of 0.78, 1.5, 1.9 mg/l DO and growth was monitored via optical density. Cells were washed with media before inoculation to minimize sulfide effects upon re-inoculation as described above, and all tubes were inoculated to 0.07-0.08 OD_{600}. At 0.78 mg/l DO, the cells did not exhibit a lag phase and displayed a significantly greater growth rate of 0.27±0.06 h^{-1} compared to control cells (0.3 mg/l DO) at 0.14±0.0 h^{-1}. A similar response was also observed at 1.5 mg/l DO exposure; however, cells had a short lag of 2 to 3 hours post-inoculation (Figure 24 and 25). *D. vulgaris* growth was inhibited at 1.9 mg/l DO (Figure 26).

Although growth was completely inhibited at 1.9 mg/l DO, a fraction of the cells remained viable up to 24 hrs post-exposure (25% viability). However, viability decreased 3- log (0.1% viability) after 1 week compared to control cells without DO and lactate (Table 7). Previous results addressing effects of oxygen on *Desulfovibrio* spp. were conducted in the medium with yeast extract (Johnson *et al*., 1997). In order to validate increased tolerance to a stressor such as DO in the presence of yeast extract, growth effects were monitored in LS4D with 1 g/l yeast extract (S2). Results indicated that *D. vulgaris* cells grown with yeast extract tolerated the highest concentration of DO used for these experiments (*i.e.*, 3.1 mg/l).

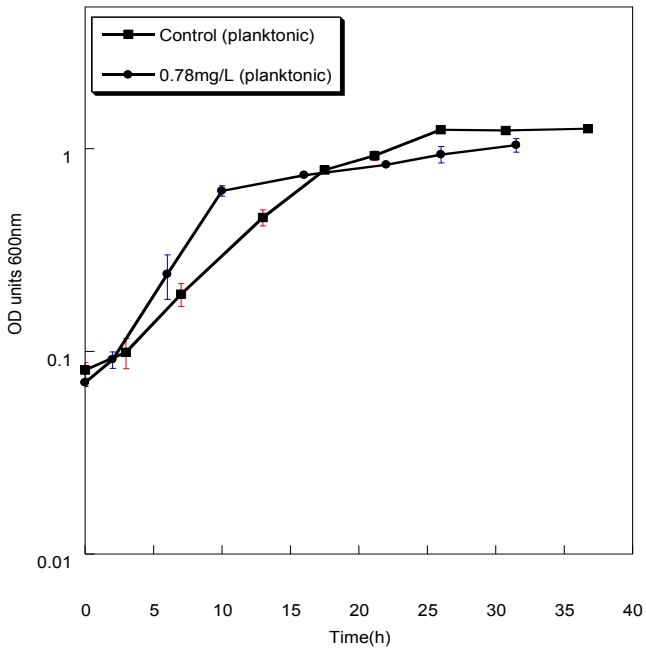


Figure 24: Growth of *D. vulgaris* planktonic cells in the presence of 0.78mg/l DO with lactate as substrate

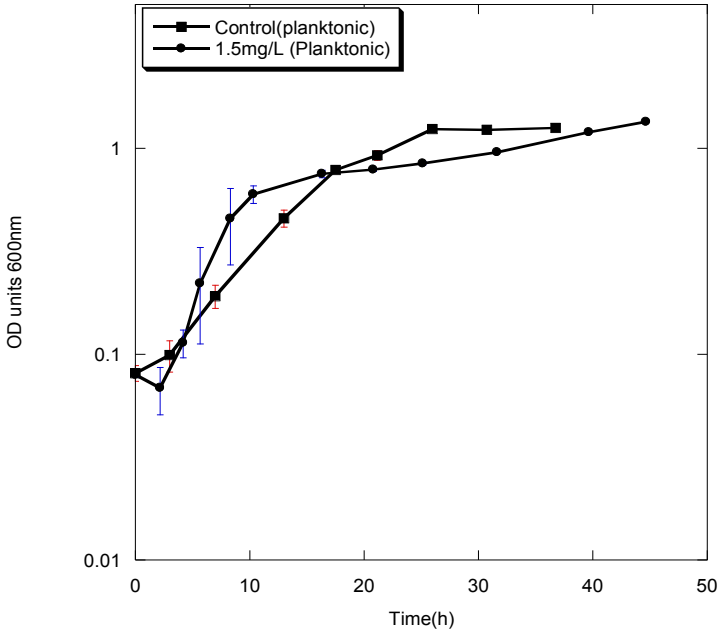


Figure 25: Growth of *D. vulgaris* planktonic cells in the presence of 1.5mg/l DO with lactate as substrate.

Table 7. Viability of *D. vulgaris* planktonic cells grown with lactate at 1.86mg/l DO determined by most probable number (MPN).

Condition	0 h	2.5 h	24 h	1 week	2 week
1.86(plank)	$1.3x10^8$	$7x10^8$	**$6x10^7$**	**$7x10^3$**	$2.4x10^3$
No lac con	$2.4x10^8$	$2.4x10^8$	**$2.4x10^8$**	**$6.2x10^6$**	$2.3x10^3$

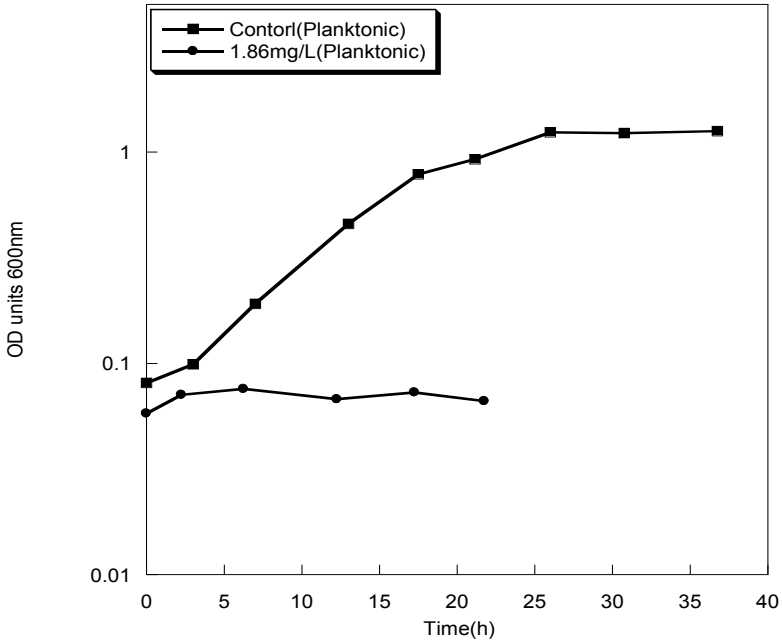


Figure 26: Growth of *D. vulgaris* planktonic cells in the presence of 3.14mg/l DO with lactate as substrate.

Effect of DO on Planktonic, Pyruvate-Grown Cells

D. vulgaris was grown at different concentrations of DO with pyruvate as the electron donor. Results indicated that at 0.8 mg/l DO, cells behaved similar to control cells (0.3 mg/l DO) and did not increase the growth rate (Figure 27). When the concentration of DO was increased to 1.5 mg/l, cells exhibited a short lag phase (approximately 5 h), but the growth rate was not increased. A similar lag period was observed when cells were exposed to 1.9 mg/l DO. Cells also exhibited increased tolerance (as observed by growth response) to DO when grown in the presence of pyruvate and were not inhibited until exposed to 3.1 mg/l DO (Figure 28). Growth rates of *D. vulgaris* planktonic cells (lactate or pyruvate) in the presence of different concentrations DO levels are shown in Table 8. While lactate- and pyruvate-grown cells

have similar growth rates with 0.3 mg/l DO, lactate-grown cells had increased growth rates with intermediate levels of DO but were more sensitive to DO levels above 1.5 mg/l (*i.e.*, decreased viability). Pyruvate-grown cells did not increase growth rates with increasing DO levles, but did have increased tolerance to higher DO levels.

Sulfide levels were measured over time for lactate- and pyruvate-grown cells in the presence of 0.3 and 0.8 mg/l DO. At 0.3 mg/l DO, lactate-grown cells produced significantly higher levels of sulfide compared to pyruvate-grown cells. At max OD (0.8), lactate-grown cells produced at least 3-fold more sulfide than pyruvate-grown cells at the control DO level (0.3 mg/l). However, in the presence of higher DO (*i.e.*, 0.78 mg/l), sulfide levels were strikingly similar at max OD_{600} (2.1 ± 0.5 for lactate vs. 2.2 ± 0.5 mM for pyruvate cells; Figure S3). Based on these results, the faster growth rate of lactate-grown cells coincided with lower sulfide levels, and higher sulfide levels could not explain the increased tolerance of pyruvate-grown cells.

Table 8. Growth rates of *D. vulgaris* planktonic cells grown in the presence of different concentrations of DO with lactate and pyruvate as substrates**.**

DO(mg/l)	Planktonic cells (Lactate) (h^{-1})	Planktonic cells (Pyruvate) (h^{-1})
Control (0.36)	0.14 ± 0	0.155 ± 0.01
0.78	0.27 ± 0.06	0.157 ± 0.004
1.5	0.24 ± 0.05 (lag)	0.15 ± 0.01
1.86	-	0.12 ± 0.03 (long lag)
3.14	-	-

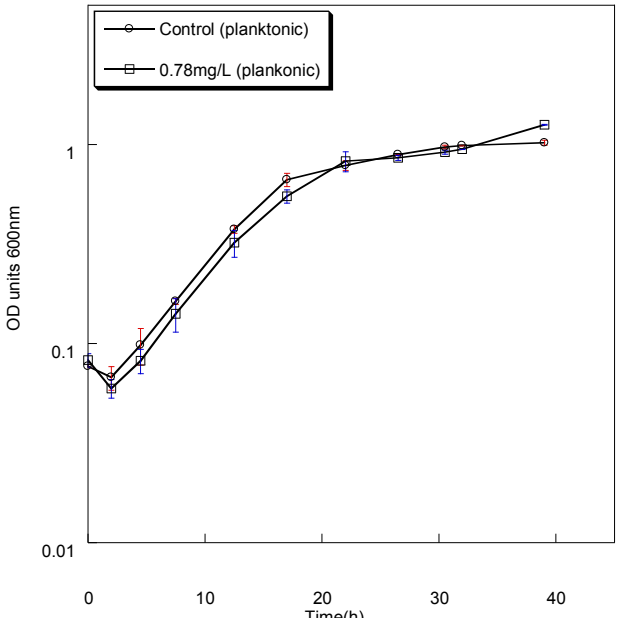


Figure 27: Growth of *D. vulgaris* planktonic cells in the presence of 0.78mg/l DO with pyruvate as substrate.

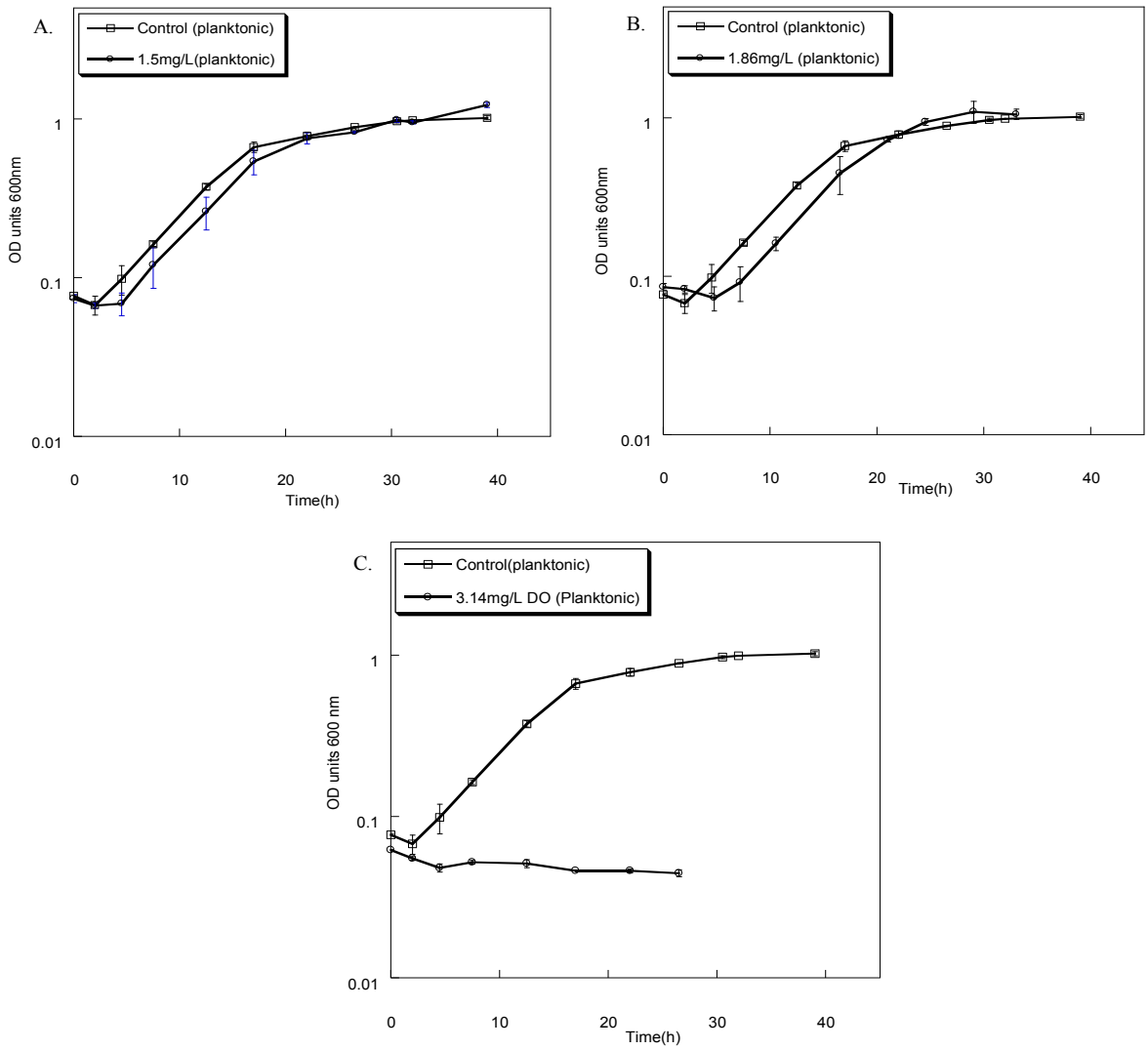


Figure 28: Growth of *D. vulgaris* planktonic cells in the presence of A) 1.5, B) 1.86 and C) 3.14 mg/l DO with pyruvate as substrate.

Total and Internal Carbohydrate Levels.

Total and internal carbohydrate levels were measured in wild-type cells grown with the different substrates at 0.3 mg/l DO. At 20 h (exponential-phase growth), total carbohydrate levels were higher in lactate-grown cells compared to pyruvate-grown cells (0.06 vs. 0.04 μg carb/μg protein). At 40 h (stationary-phase growth), lactate-grown cells still maintained high levels of carbohydrate to protein ratios compared to pyruvate-grown

cells (0.05 vs. 0.02). In order to determine if the differential response to DO by lactate- and pyruvate-grown planktonic cells was due to differences in internal carbohydrate levels, internal carbohydrate was measured at 20 h and 40 h post-inoculation. At 20 h and 40 h, both lactate- and pyruvate-grown cells had similar levels of internal carbohydrate (0.035 and 0.037 μg carb/μg protein and 0.023 and 0.026 μg carb/μg protein, respectively; Figure 29). These results indicated that even though total carbohydrate levels were higher for lactate-grown cells, the internal carbohydrate levels (*i.e.*, glycogen) were similar. In addition, the presence of elevated glycogen levels could not solely explain the observed differences between lactate- and pyruvate-grown cells in response to DO.

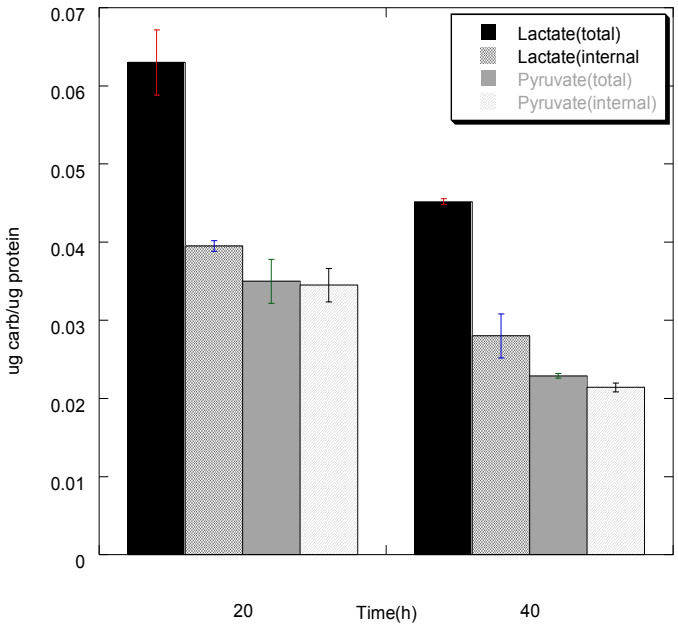


Figure 29: Total vs. internal carbohydrate levels for *D. vulgaris* planktonic cells at 20 and 40h in the presence of lactate and pyruvate

ATP Measurements of Lactate- and Pyruvate-Grown Cells

ATP concentrations were measured in lactate- and pyruvate-grown cells in the presence of 0.3 and 0.8 mg/l D with an ATP bioluminescence assay kit. Samples were collected when cells reached OD_{600} 0.3 and 0.7 to measure differences in ATP levels at different growth stages, and the samples were normalized to protein concentrations (Table 9). The data indicated that lactate-grown cells in general had higher ATP concentrations compared to pyruvate-grown cells at both growth stages (2 to 2.5-fold higher; Table 9). At DO of 0.8 mg/l, lactate-grown cells had 20-fold higher ATP levels compared to pyruvate-grown cells. When lactate-grown cells were compared between 0.3 and 0.8 mg/l DO, the increased DO levels coincided with a 10-fold increase in ATP levels (Table 9). These results indicated that lactate-grown cells utilized oxygen with concomitant ATP production that resulted in increased growth rates. Previous work has shown that glycogen can be utilized for oxygen reduction (van Niel *et al*., 1996; van Niel and Gottschal, 1998; Santos *et al*., 1993); however, the question arose that despite similar glycogen levels, did pyruvate-grown cells couple oxygen reduction with ATP production.

Table 9. ATP concentrations of lactate and pyruvate *D. vulgaris* planktonic cells at 0.3 and 0.8mg/l DO at early log (0.3 OD) and late log (0.8) phase.

Dissolved Oxygen mg/l	Substrate	Approximate OD (600nm)	n mol ATP/ng of protein
0.3	Lactate	0.3	0.470 ± 0.114
0.3	Pyruvate	0.3	0.177 ± 0.027
0.8	Lactate	0.3	5.03 ± 0.792
0.8	Pyruvate	0.3	0.247 ± 0.05
0.3	Lactate	0.7	0.316 ± 0.008
0.3	Pyruvate	0.7	0.17 ± 0.008
0.8	Lactate	0.7	0.583 ± 0.084
0.8	Pyruvate	0.7	0.105 ± 0.02

Δ*glg*A in the Presence of DO

Transcriptomic data suggests that *glg*A was up-expressed, z value > 1.5, within 20 min exposure to air in the presence of lactate. These observations lead to testing the response of Δ*glg*A to the described DO concentrations to investigate if this protein played a role in the differential response of lactate and pyruvate grown cells to DO.

*glg*A Growth

Transcriptomic data suggested that *glg*A was up-expressed (z value > 1.5) within 20 min exposure to air in the presence of lactate (microbesonline database). Based upon our results and these observations, we tested the growth of Δ*glg*A with the different DO levels when grown with lactate or pyruvate. First, glycogen levels were confirmed in the mutant. Both lactate- and pyruvate-grown cells had lower total carbohydrate levels (normalized to protein), and internal carbohydrate levels were less than half of wild-type levels for both lactate- and pyruvate-grown cells (Figure 31). The internal carbohydrate levels were similar for lactate- and pyruvate-grown cells (approximately 0.02 μg carb/μg protein), and these results corroborated that the Δ*glg*A mutant had lower glycogen levels for cells grown on both substrates.

Effect of DO on Δ*glg*A Planktonic, Lactate-Grown Cells

The Δ*glg*A mutant was grown in the presence of lactate as substrate at various DO concentrations. Lactate-grown cells were inhibited at 1.5 mg/l DO as opposed to wild-type cells that were inhibited at 1.9 mg/l DO. In addition, there was not an increase in growth rate at 0.8 mg/l DO (0.16 vs. 0.27 h^{-1}, mutant and wild-type respectively) (Figure

30A). Growth experiments with complemented Δ*glg*A cells revealed an increased growth rate in lactate-grown cells at 0.8 and 1.5 mg/l (Figure S4).

Effect of DO on Δ*glg*A Planktonic, Pyruvate-Grown Cells

In the presence of pyruvate, Δ*glg*A cells were more tolerant when exposed to higher DO concentrations than with lactate, and displayed similar tolerance to wild-type cells (i.e., mutant cells were inhibited at 3.14 mg/l DO) (Figure 30B). The complemented Δ*glg*A cells displayed increased tolerance to DO in a similar fashion to wild-type cells. Cells could tolerate 1.9 mg/l DO and were inhibited at 3.1 mg/l DO (Figure S4).

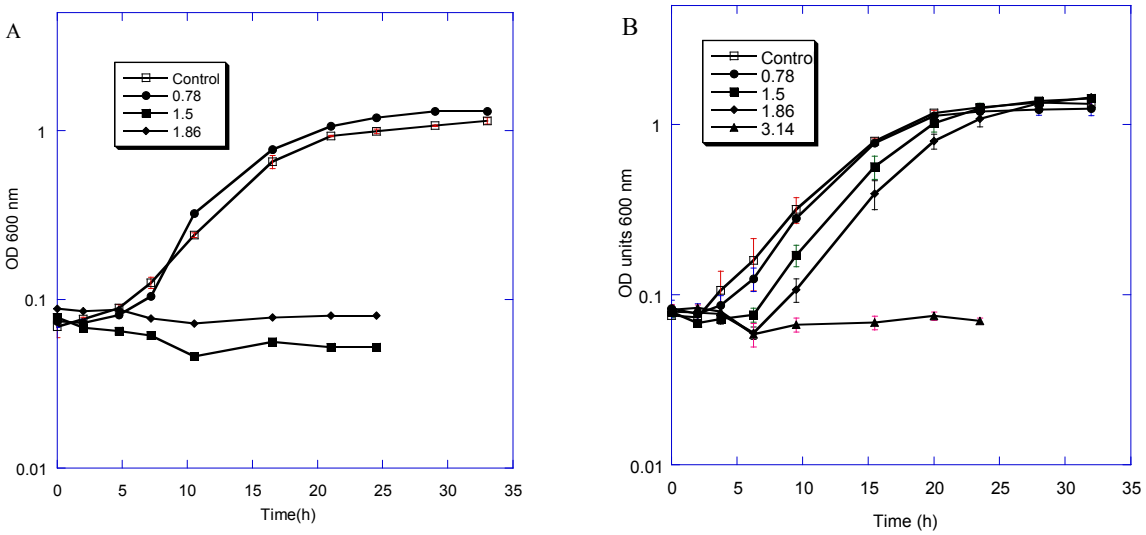


Figure 30: Growth of Δ*glg*A in the presence of various DO concentrations, i.e, 0.78, 1.5, 1.86 and 3.14mg/l DO with A) lactate and B) pyruvate as substrates

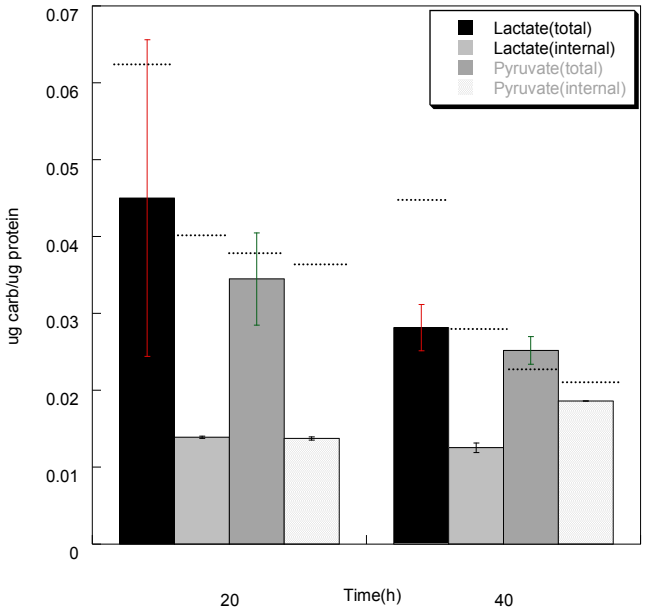


Figure 31: Total vs. internal carbohydrate levels for Δ*glg*A planktonic cells at 20 and 40h in the presence of lactate and pyruvate. Dotted lines on graph represent WT levels at the respective time point.

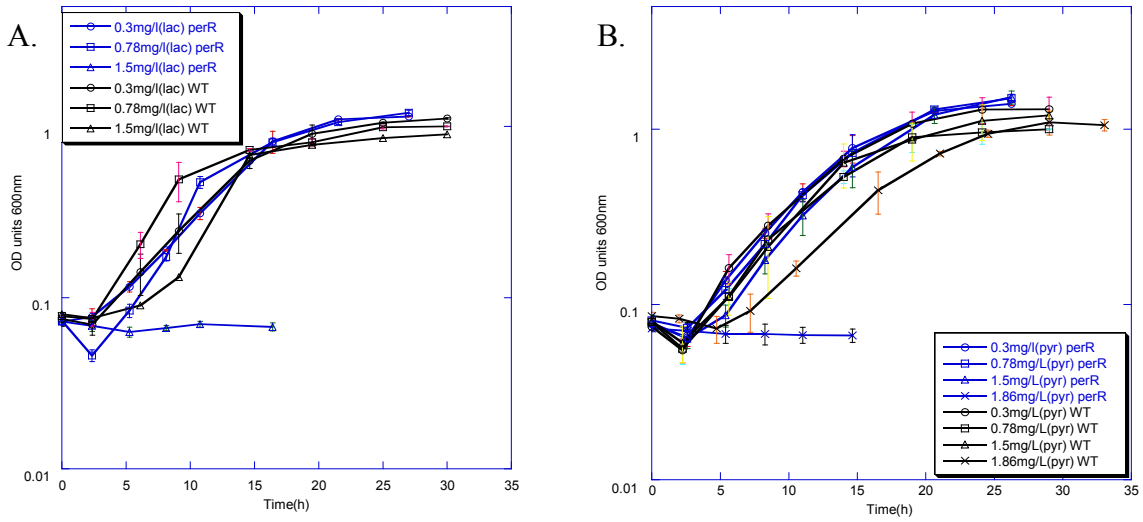


Figure 32: (A) Growth ofΔ *per*R and WT *D. vulgaris* planktonic cells in the presence of various concentrations of DO with lactate as substrate. (B) Growth of Δ*per*R and WT *D. vulgaris* planktonic cells in the presence of various concentrations of DO with lactate as substrate.

Effect of DO on Δ*per*R Planktonic, Lactate- and Pyruvate-Grown Cells

The Δ*per*R mutant displayed decreased tolerance to DO as compared to wild-type cells grown in either lactate or pyruvate. In the presence of lactate, Δ*per*R cells were inhibited at 1.5 mg/l DO while pyruvate-grown cells were only tolerant up to 1.5 mg/l DO. An increased growth rate was observed in the mutant in the presence of lactate similar to wild-type cells (Figure 32A and B). Δ*per*R complemented cells were grown in the presence of the different DO levels with lactate and pyruvate as substrates. For both substrates, the complemented strain had restored phenotype and similar DO tolerance as wild-type cells (Figure 33A and B). These results suggested that PerR was not essential for tolerance to lower DO levels, and that pyruvate-grown cells appeared to be more reliant upon a *per*R regulated system for oxygen tolerance.

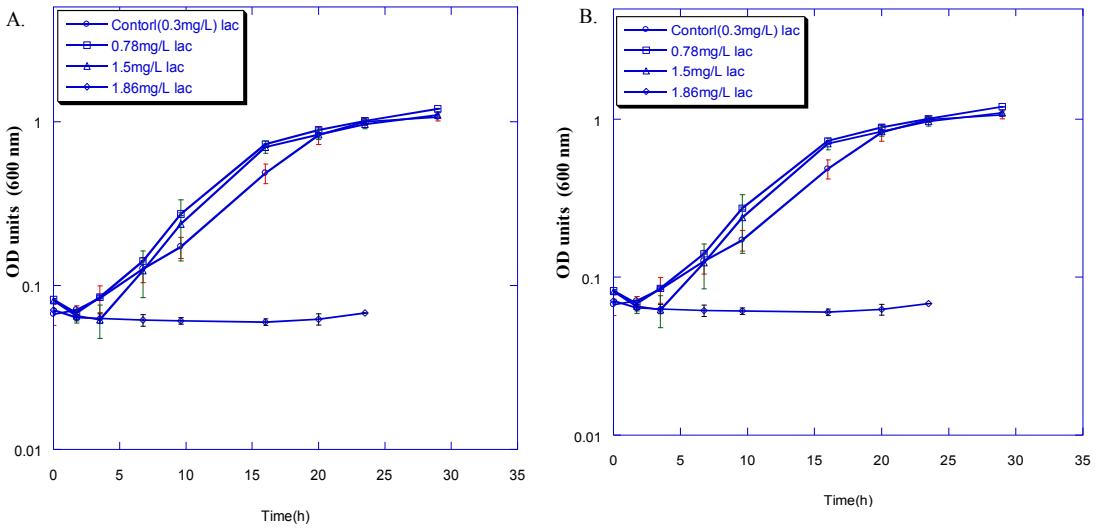


Figure 33: Growth of Δ*per*R complement in the presence of different DO concentrations with A) lactate and B) pyruvate as substrates.

Discussion

The described study demonstrated the substrate-dependent responses of *Desulfovibrio vulgaris* Hildenborough to dissolved oxygen. *D. vulgaris* was grown in the presence of lactate and pyruvate as substrates and exposed to various DO concentrations (0.78, 1.5, 1.86 and 3.1 mg/l DO). Although there have been a few reports of oxygen-dependent growth or aerobic respiration in SRB (Dilling and Cypoinka H, 1990; Johnson *et al*., 1997), none of have addressed a substrate based differential response. Moreover, the experimental conditions employed in these previous reports are distinctly different compared to data presented in this thesis, including the use of a defined medium.

Molecular oxygen can induce severe toxic and mutagenic effects on biological cells, and both aerobic and facultative organisms exercise stringent regulatory mechanisms to combat oxidative stress (Fournier *et al*., 2004). Catalase and super oxide dismutase were perceived to be absent in stringent anaerobes (including SRBs) and hence were thought to be incapable of survival in the presence of oxygen (Postgate, 1984). Over the past three decades; however, evidence has mounted to substantiate that these enzymes exist in anaerobes, including SRBs, and that different SRBs have a variety of mechanisms to deal with oxygen (Fournier *et al*., 2004).

Although categorized as strict anaerobes, various experiments conducted by many laboratories have suggested that *D. vulgaris* can exhibit aerotolerance (*i.e.*, capable of survival under microaerobic conditions (Abdollahi and Wimpenny, 1990; Hardy and Hamilton, 1981). In addition to exhibiting limited tolerance to oxygen, these organisms also show the presence of certain terminal oxygen reductases such as a cytochrome *c*

oxidase and cytochrome *bd* oxidases that are both found in facultative and aerobic organisms (Heidelberg *et al*., 2004). The presence of these genes suggests that *D. vulgaris* should be able to respire oxygen under microaerophilic or aerobic conditions (Santana, 2008); however, the experiments can be difficult due to confounding issues related to sulfur metabolism of the SRBs. Our data demonstrated oxygen consumption in *D. vulgaris* with ATP production and increased growth rates. However, this ability was substrate-dependent and linked to glycogen levels. Although pyruvate-grown cells displayed enhanced tolerance towards DO, lactate-grown grown cells exhibited a significant increase in growth rate at 0.8 and 1.5 mg/l DO.

Bacteria are capable of synthesizing polyglucose or poly-β- hydroxybutyrate as storage molecules, especially in nutrient-limiting conditions (Dawes and Senior, 1973). Although much is known in other bacteria, there have been few reports about storage polymers in the SRB (Stams *et al*., 1983), including *Desulfobulbus* and some strains of *Desulfovibrio* (Widdel and Pfenning, 1982; Laanbroek *et al*., 1982). It was in the nineties that the role of polyglucose in oxygen-dependent respiration was documented. vanNiel *et al*. (1996) proposed that the presence of polyglucose enabled the survival of *Desulfovibrio salixigens* under oxic conditions (*i.e.,* electrons for oxygen reduction came from glycogen). The study showed that strain Mast1 was able to respire glucose, pyruvate, or lactate in the presence of oxygen. *Desulfovibrio desulfuricans* and *D. gigas* have been shown to accumulate polyglucose as well, but the extent of accumulation is strain dependent (Krekeler and Cypionka, 1995; Dilling and Cypionka, 1990; Santos *et al*., 1993). Another report by DeBeers *et al.* (2003) summarized that certain compounds,

such as pyruvate, alanine and α-ketoglutarate supported aerobic respiration in SRBs such as *Desulfovibrio oxyclinae*, and oxygen-linked growth in terms of cell division has been reported in this organism (Jonkers *et al*., 2003; Sigalevich *et al*., 2000). Because there is not direct evidence to substantiate active polyglucose respiration in *D. vulgaris* in the presence of oxygen, experiments were conducted to evaluate the role of polyglucose respiration in substrate-dependent, oxygen responses of *D. vulgaris.* Glycogen measurements suggested that there were comparable levels of endogenous carbohydrate for both lactate- and pyruvate-grown cells. These results indicated that differential oxygen tolerance/consumption cannot solely be attributed to polyglucose accumulation and respiration.

At least two groups have conducted experiments to study transcriptional responses of *D. vulgaris* to oxygen/air (Pereira *et al*., 2008; Mukhopadhyay *et al*., 2007). Transcriptomic data revealed that within the first 20 min upon exposure to air, several genes belonging to the carbohydrate metabolism COG were up-expressed in lactate-grown cells (Table 10; pyruvate-cells were not tested).

Pgm	phosphoglucomutase, alpha-D-glucose phosphate-specific	DVU1428
DVU1658	transaldolase, putative	DVU1658
fbp	fructose-1,6-bisphosphatase	DVU1841
gmhA	phosphoheptose isomerase	DVU1889
Fba	fructose-1,6-bisphosphate aldolase, class II	DVU2143
*glg*B	1,4-alpha-glucan branching enzyme	DVU2243
***glg*A**	**glycogen synthase**	**DVU2244**
	NAD-dependent epimerase/dehydratase family protein	DVU2455
Pyk	pyruvate kinase	DVU2514
Gpm	phosphoglycerate mutase	DVU2935
DVU3356	NAD-dependent epimerase/dehydratase family protein	DVU3356
Eno	Enolase	DVU0322
	HPCH/HPAI aldolase family protein	DVU0343
gap-1	glyceraldehyde 3-phosphate dehydrogenase	DVU0565

Table 10: List of genes up-expressed in *D. vulgaris* planktonic cells grown in the presence of lactate within the first 20 min of exposure to air.

GlgA, a putative protein that encodes 489 amino acids is annotated as a glycogen synthase in *D. vulgaris*. Both lactate- and pyruvate-grown ΔglgA cells exhibited increased sensitivity towards DO and lactate-grown cells did not increase the growth rate in response to intermediate DO levels. Data revealed there were decreased internal carbohydrate levels for Δ*glg*A compared to WT *D. vulgaris*. Between lactate and pyruvate cells, there were no significant differences observed which suggests that internal polyglucose levels are not solely an indication of oxygen reduction. When ATP levels were measured in lacate grown cells (data not shown), the levels were lower than WT cells grown with the same substrate which indicated that perhaps GlgA is involved in oxygen utilization in lactate-grown cells and not pyruvate-grown cells.

A previous study involving chromium as a stressor revealed that pyruvate-grown grown cells had increased tolerance to Cr(VI) compared to lactate-grown cells, and this result corresponds to pyruvate-grown cells being less sensitive to oxygen. The increased tolerance was attributed to the fact that pyruvate-grown cells can yield more energy and efficient growth when compared to lactate (Klonowska *et al*., 2008; Traore *et al*., 1981).

In a transcriptomic comparison between lactate- and pyruvate-grown cells, subsets of genes that belonged to oxidative stress/defense were up-expressed in lactate-grown cells compared to pyruvate-grown cells (unpublished data). These included *ahpC*, an alkyl hydroperoxide reductase, *fld*, a flavodoxin, *rdl*, a rubredoxin and *perR*, a peroxide responsive regulator. On the other hand, two ferridoxins and *sodB*, a super oxide dismutase was down-expressed in lactate-grown cells compared to pyruvate-grown cells. These data suggest that there is inherently different metabolisms and defense systems that are expressed in a substrate-dependent manner for *D. vulgaris*.

Studies in *Clostridium acetobutylicum*, a stringent anaerobe revealed that PerR is a multi-functional protein that plays a crucial role in oxidative stress (Hillman *et al*., 2008). The study demonstrated that by fine-tuning the PerR regulatory mechanisms, prolonged aerotolerance, limited growth, and oxygen consumption could be achieved in this anaerobic bacterium (Hillman *et al*., 2008). This organism possibly uses the oxidation sensor PerR as an oxygen-dependent switch that triggers a temporary response to immediate exposure to oxygen.

The Δ*per*R experiments conducted in *D. vulgaris* to elucidate if another system existed in lactate-grown cells which facilitate oxygen consumption similar to *Clostridium*

cells. Experiments revealed that there was decreased tolerance to DO both in lactate- and pyruvate-grown cells that was restored in the complemented strains. However, significant differences were not observed in growth rates at lower DO levels in lactate-grown cells.

Past studies have suggested that oxygen reduction by SRBs is a protective mechanism rather than an energy-coupled conservation mechanism that allows growth; however, a few reports have suggested that ATP formation could be coupled to oxygen reduction along with sulfate/sulfite (Dilling and Cypionka, 1990). Oxygen reduction in *Desulfovibrio desulfuricans* CSN with hydrogen and other electron acceptors yielded ATP formation (Dilling and Cypionka, 1990). Similarly it has been shown that *Desulfovibrio desulfuricans* and *Desulfobulbus propionicus* are capable of sulfite oxidation with oxygen as electron acceptor concomitant with ATP formation (Dannerberg *et al*., 1992). In 1993, Marschall *et al.* (1993) documented weak aerobic growth of *Desulfovibrio desulfuricans* Essex and *Desulfobacterium autotrophicum* in homogeneously aerated cultures.

D. vulgaris could utilize oxygen coupled to energy production and growth, but only with lactate and not pyruvate. Glycogen was required for the increased growth rate, but again, only in lactate-grown cells. Pyruvate-grown cells had similar glycogen levels, but did not increase ATP synthesis or increased growth. However, pyruvate-grown cells could tolerate higher DO levels and a PerR regulated system was more important to oxygen tolerance when cells were metabolizing pyruvate versus lactate. Future work is needed to delineate the basis of substrate-dependent systems for oxygen tolerance in

SRBs, but the results underscore the diversity of phenotypic responses linked to different metabolisms and electron flow even in a single microorganism. The presented work has implications for both bioremediation and biocorrosion in the context of SRB responses to oxygen.

References

Abdollahlahi, H. , and J. W. T. Wimpenny.1990. Effects of oxygen on the growth of *desulfovibrio-desulfuricans*. J. Gen. Microbiol. 136: 1025-1030.

Bender, K. S., H. C. B. Yen, C. L. Hemme, Z. Yang, Z. He, Q. He, J. Zhou, K. H. Huang, E. J. Alm, T. C. Hazen, A. P. Arkin, and J. D. Wall.2007. Analysis of a ferric uptake regulator (Fur) mutant of *Desulfovibrio vulagris* Hildenborough. Appl. Environ. Microbiol. 73: 5389-5400.

Brandis, A., and R. K. Thauer.1981.Growth of *Desulfovibrio* species on hydrogen and sulfate as sole energy sourse. J. Gen. Microbiol. 126: 249-252.

Chaplin, M. F.1986. Monosaccharides, p. 1-2. In M.F. Chaplin and J. F. Kennedy (ed.), Carbohydrate analysis. IRL Press, Oxford.

Clark, M. E., Z. He, K. H. Huang, E. J. Alm, X. F. Wan, T. C .Hazen, A. P. Arkin, J. D. Wall, J. Z. Zhou, and M. W. Fields. 2006. Temporal transcriptomic analysis as *Desulfovibrio vulgaris* hildenborough transitions into stationary phase during electron donor depletion. Appl Environ Microbiol. 72: 5578-5588

Cypionka, H., F. Widdel, and N. Pfennig. 1985.Survival of sulfate-reducing bacteria after oxygen stress, and growth in sulfate free oxygen-sulfide gradients. FEMS. Microbiol.Ecol. 31:39–45.

Dannenberg, S., M. Kroder, W. Dilling, and H. Cypionka.1992. Oxidation of H2, organic compounds and inorganic sulfur compounds coupled to reduction of O2 or nitrate by sulfate-reducing bacteria. Arch. Microbiol. 158: 93-99.

Dawes, E. A., and P. J. Senior.1973. The role and regulation of energy reserve polymers in micro-organisms. Adv. Micro. Phys. 10: 135-266.

Dilling, W., and H. Cypionka.1990. Aerobic respiration in sulfate reducing bacteria. FEMS. Microbiol. Ecol. 73:123-128.

Dos Santos, W. G., I. Pacheco, M. Y. Liu, M. Texeira, A.V. Xavier, and J. DeGall.2000. Purification and characterization of an iron superoxide dismutanse and a catalase from the sulfate reducing bacterium *Desulfovibrio gigas*. J. Bacteriol. 182:796-804.

Eschemann, A., M. Kuhl, and H. Cypionka.1999. Aerotaxis in Desulfovibrio. Environ. Microbiol. 6:489-494.

Fareleira, P., J. LeGall, A. V. Xavier and H. Santos.1997. Pathways for utilization of carbon reserves in *Desulfovibrio gigas* under fermentative and respiratory conditions. J. Bacteriol. 179:3972-3980.

Fournier, M., Y. Zhang, J. D. Wildschut, A. Dolla, J. K. Voordouw, D. C. Schriemer and G. Voordouw. 2003. Function of oxygen resistance proteins in the anaerobic sulfate reducing bacterium *Desulfovibrio vulgaris* Hildenborough. J. Bacteriol. 185:71-79.

Fournier, M., Z. Dermoun, M.C. Durand, and A. Dolla. 2004. A new function of the *Desulfovibrio vulgaris* Hildenborough (Fe) hydrogenase in the protection against oxidative stress. J. Biol. Chem. 279:1787-1793.

Hardy, J. A., and W. A. Hamilton. 1981. The oxygen tolerance of sulphate-reducing bacteria isolated from north-sea waters. Curr. Microbiol. 5: 259-262.

Hatchikian, E., and Y. A. Henry.1977.An iron-containing superoxide dismutase from the strict anaerobe *Desulfovibrio desulfuricans* (Norway 4). Biochemie. 59: 153-161.

Heidelberg, J. F., R. Seshadri, S. A. Haveman, C. L. Hemme, I. T. Paulsen, J. F. Kolonay, J. A Eisen, N. Ward, B. Methe, L. M. Brinkac, S. C. Daugherty, R. T. Deboy, R. J. Dodson, A. S. Durkin, R. Madupu, W. C. Nelson, S. A. Sullivan, D. Fouts, D. H. Haft, J. Selengut, J. D. Peterson, T. M. Davidsen, N. Zafar, L. W. Zhou, D. Radune, G. Dimitrov, M. Hance, K. Tran, H. Khouri, J. Gill, T. R. Utterback, T. V. Feldblyum, J. D. Wall, G. Voordouw, and C. M. Fraser.2004. The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. Nat. Biotechnol. 22:554-559.

Hillmann, F., R. J. Fischer, F. Saint-Prix, L. Girbal, and H. Bahl.2008. PerR acts as a switch for oxygen tolerance in the strict anaerobe *Clostridium acetobutylicum*. Mol. Microbiol. 68: 848-860.

Ishimoto, M., J. Koyama, and Y. Nagai.1954. Biochemical studies on sulfate-reducing bacteria. IV. The cytochrome system of sulfate-reducing bacteria. J. Biochem. 41:763-70.

Johnson M. S, I. B. Zhulin, M. E. R. Gapuzan, and B. L. Taylor.1997. Oxygen-dependent growth of the obligate anaerobe *Desulfovibrio vulgaris* Hildenborough. J. Bacteriol. 179: 5598-5601.

Jonkers, H. M., I. O. Koh, P. Behrend, G. Muyzer, and D. de Beer.2003. Aerobic organic carbon mineralization by sulfate-reducing bacteria in the oxygen-saturated photic zone of a hypersaline microbial mat. Micro. Ecol. 49: 291-300.

Klonowska, A., M. E. Clark, S. B. Thieman, B. J. Giles, J. D. Wall and M. W. Fields. 2008. Hexavalent chromium reduction in Desulfovibrio vulgaris causes transitory

inhibition of sulfate reduction and cell growth. Appl. Microbiol. Biotechnol. 78: 1007-1016.

Krekeler, D., and H. Cypionka.1995. The preferred electron-acceptor for *Desulfovibrio dedulfuricans* CSN. FEMS. Microbiol.17: 271-277.

Krekler, D., A. Teske, and H. Cypionka.1998. Strategies of sulfate-reducing bacteria to escape oxygen stress in a cyanobacterial mat. FEMS. Microbiol. Ecol. 25: 98-96.

Laanbroek, H. J., T. Abee, and I. L. Voogd.1982. Alcohol Conversions by *Desulfobulbus-propionicus* lindhorst in the presence and absence of sulfate and hydrogen. Arch. Microbiol. 133: 178-184.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-75.

Marshall, C., P. Frenzel, and H. Cypionka.1993. Influence of oxygen on sulfate reduction and growth of sulfate reduction and growth of sulfate-reduction bacteria. Arch. Microbiol. 159: 168-173.

Moura, I., P. Tavares, and N. Ravi. 1994. Characterization of 3 proteins containing multiple iron sites—rubrerythrin, desulfoferrodoxin, and a protein containing a six-iron cluster. Methods. Enzymol. 243:216–240.

Mukhopadhyay, A., Z. L. He, E. J. Alm, A. P. Arkin, E. E. Baidoo, S. C. Borglin, W. Q. Chen, T. C. Hazen, Q. He, H. Y. Holman, K. Huang, R. Huang, D. C. Joyner, N. Katz, M. Keller, P. Oeller, A. Redding, J. Sun, J. Wall, J. Wei, Z. M. Yang, H. C. Yen, J. Z. Zhou, and J. D. Keasling.2006. Salt stress in *Desulfovibrio vulgaris* Hildenborough: An integrated genomics approach. J. Bacteriol. 188: 4068-4078.

Mukhopadhyay, A., A. M. Redding, M. P. Joachimiak, A. P. Arkin, S. E. Borglin, P. S. Dehal, R. Chakraborty, J. T. Geller, T. C. Hazen, Q. He, D. C. Joyner, V. J. J. Martin, J. D. Wall, Z. K. Yang, J. Zhou, and J. D. Keasling.2007. Cell-wide responses to low-oxygen exposure in *Desulfovibrio vulgaris* Hildenborough. J. Bacteriol. 189: 5996-6010.

Postgage, J. R.1984.The sulfate-reducing bacteria, 2nd Edn, p.208 Cambridge University Press, Cambridge.

Rabus, R., A. Ruepp, T. Frickey, T. Rattei, B. Fartmann, M. Stark, M. Bauer, A. Zibat, T. Lombardot, I. Becker, J. Amann, K. Gellner, H. Teeling, W. D. Leuschner, F. O. Glockner, A. N. Lupas, R. Amann, and H. P. Klenk.2004. The genome of *Desulfotalea psychrophila*, a sulfate-reducing bacterium from permanently cold Arctic sediments. Environ. Microbiol. 6: 887-902.

Ruwisch, R. C.1985.A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. J. Microbiol. Methods. 4:33-36.

Santana, M.2008. Presence and expression of terminal oxygen reductases in strictly anaerobic sulfate-reducing bacteria isolated from salt-marsh sediments. Anaerobe. 14: 145-156.

Santos. H., P. Fareleira, A. V. Xavier, L. Chen, M. Y. Liu, and J. LeGall.1993. Aerobic metabolism of carbon reserves by the 'obligate anaerobe' *Desulfovibrio gigas*. Biochem. Biophys. Res. Comm. 195:551-557.

Sigalevich, P., E. Meshorer, Y. Helman, and Y. Cohen.2000. Transition from anaerobic to aerobic growth conditions for the sulfate-reducing bacterium Desulfovibrio oxyclinae results in flocculation. Appl. Environ. Microbiol. 66: 5005–5012.

Stams, F. J. M., M. Veenhuis, G. H. Weenk, and T. A. Hansen,.1983. Occurrence of polyglucose as a storage polymer in desulfovibrio species and desulfobulbus-propionicus. Arch. Microbiol. 136: 54-59.

Traore, A. S., C. E. Hatchikian, J. P. Belaich, and J. LeGall.1981.Microcalorimetric studies of the growth of sulfate-reducing bacteria: energetic of *Desulfovibrio vulgaris* growth. J. Bacteriol. 145: 191-199.

van Niel, E. W. J. , and J. C. Gottschal.1998. Oxygen consumption by *Desulfovibrio* strains with and without polyglucose. App.l Environ. Microbiol. 64: 1034-1039.

vanNiel, E. W. J., T. M. P. Gomes, A. Willems, M. D. Collins, R. A. Prins, and J. C. Gottschal.1996.The role of polyglucose in oxygen-dependent respiration by a new strain of *Desulfovibrio salexigens*. FEMS. Microbiol.Ecol. 4:243-253.

Widdel, F., and N. Pfenning.1981. Studies on dissimilatory sulfate reducing bacteria that decompose fatty acids. I. Isolation of new sulfate reducing bacteria enriched with acetate from saline environments: description of *Desulfobacter postgatei.* gen. nov., sp. nov. Arch. Microbiol. 129:395-400.

Widdel, F., and N. Pfenning.1982.Studies on dissimilatory sulfate reducing bacteria that decompose fatty acids. II. Incomplete oxidation of propionate by *Desulfobulbus propionicus*. gen. nov., sp. nov. Arch. Microbiol. 131:360-365.

Zhang, X., P. Bishop, and B. K. Kinkle.1999.Comparison of extraction methods for quantifying extracellular polymers in biofilms. Wat. Sci. Tech. 39: 211-218.

CHAPTER 5

RESPONSE OF *DESULFOVIBRIO VULGARIS* HILDENBOROUGH BIOFILM CELLS TO DISSOLVED OXYGEN

Abstract

Desulfovibrio vulgaris Hildenborough (*D. vulgaris*) is a Gram-negative sulfate-reducing bacterium that belongs to the δ-*proteobacterium*. Through direct and indirect mechanisms, SRBs can promote heavy metal bioremediation via reduction, but also can contribute to metal and concrete corrosion via the production of sulfides. In natural environments, microbes are often found as multi-species biofilms rather than free-living cells and reports suggest that biofilm structures are more resilient to stressors compared to planktonic counterparts. Although there are several studies on the effect of oxygen on *Desulfovibrio* planktonic cells, very few studies report effects of oxygen on biofilm cells. *D. vulgaris* biofilms were cultivated using CDC reactors and exposed to varying concentrations of dissolved oxygen in the presence of two different substrates, lactate and pyruvate. Growth experiments revealed, that biofilm-grown cells were inhibited at 1.5 mg/l DO both in the presence of lactate and pyruvate as carbon/energy sources while planktonic cells survived 1.5 mg/l DO when grown with lactate and 1.9 mg/l DO when grown with pyruvate (Chapter 3). In addition, regardless of DO, biofilm cells had a prolonged lag phase when transferred to the planktonic state, and this result suggested that biofilm cells required a transition period between biofilm and planktonic growth modes.

Introduction

Biofilms have numerous definitions but can be simply defined as a self-assembled complex that is a spatially and metabolically structured microbial community attached to a surface. The biofilm is typically encased in an extracellular polymer matrix (combination of polysaccharide, protein and/or nucleic acid). Biofilms exhibit a great degree of structural, chemical and genetic heterogeneity, and mature biofilms that exhibit structural complexity display concentration gradients of metabolic substrates and products (Rani *et al*., 2007; deBeer *et al*., 1993; Xu *et al*., 1998 and Stewart and Franklin, 2008). Studies on *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* biofilms suggest that within these complex structures exist heterogenous patterns with respect to DNA synthesis, protein synthesis and oxygen concentration (Rani *et al*., 2007; Xu *et al*., 1998). Using microelectodes, it has been demonstrated that oxygen concentration gradients can exist within biofilms (deBeer *et al*., 1993). Biofilms are the more preferred mode of existence for bacteria as they are considered to be more resilient to stressful conditions, such as changes in chemical environments, presence of detergents, anti-microbial agents, and biofilms have an increased ability to persist and survive under adverse conditions (Szomolay *et al*., 2005; Teitzel and Parsek, 2003; Mah and O'Toole, 2001; Stoodley *et al*., 2002; Hansen *et al*., 2007)

Physiological variation can occur along biofilm depth as a function of oxygen availability/distribution, and anaerobic pockets can be present within the lower biofilm layers (Stewart and Franklin, 2008). However, most biofilm work has been performed with aerobic or facultative organisms, and few studies have addressed the potential

heterogeneity issues that arise in anaerobic biofilms. Bactericidal effects of reactive oxygen species (*e.g.*, peroxide, superoxide ion, hypochlorous acid and hydroxyl radical) have been characterized in planktonic cells (Demple, 1991; Farr and Kogoma, 1991), but fewer studies have characterized the effects directly with biofilms. Studies on oxidative stress response in *Pseudomonas aeruginosa* biofilm cells revelaed that biofilm cells responded in a similar fashion as compared to planktonic cells, although induction of *kat*B in biofilms required substantially more H_2O_2 (Hasset *et al*., 1999; Elkins *et al*., 1999).

Sulfate-reducing bacteria (SRBs), implicated in biocorossion of steel as well as important for heavy metal bioremediation, have been studied extensively as biofilms (Lopes *et al*., 1997; Feugeas *et al*., 1997; Viveros *et al*., 2006; Cantero *et al*., 2003). Moreover, biofilm formation in *Desulfovibrio vulgaris* Hildenborough has been studied by several groups in the last decade (Clark *et al*., 2007; Beyenal and Lewandowski, 2004; Beyenal *et al*., 2004; Marsili *et al*., 2005; Zhang *et al*., 2007). In the described study, biofilms grown in a stirred reactor were used to compare responses to dissolved oxygen (DO) between biofilm and planktonic cells. The results indicated that biofilm cells were more susceptible to DO exposure than planktonic cells irrespective of the substrate tested (lactate vs. pyruvate).

Materials and Methods

Growth Conditions

Growth of *Desulfovibrio vulgaris* Hildenborough ATCC 29579 was done in LS4D (Clark et al., 2006) minimal medium with 60 mM lactate and in PS4D minimal medium with 60 mM pyruvate as the carbon/electron donor. The S4D based medium contained 50 mM sulfate as the electron acceptor as well as ammonium chloride (20 mM), magnesium chloride (8 mM), potassium phosphate (2.2 mM), PIPES (30 mM), calcium chloride (0.6 mM), resazurin, 80X mineral solution, 100 X vitamin solutions as previously described (Clark et al., 2006). Growth experiments were performed in 10 ml anaerobic tubes with anoxic nitrogen gas as the head-space and sealed with butyl rubber stoppers and aluminum crimp seals.

Biofilm Growth under Nutrient Limiting Conditions in CDC Reactor

D. vulgaris biofilm cells were cultivated using a modified CDC (Concurrent Downflow Contactor, Biosurface Technologies, MT). The dilution rate was maintained at 0.09 h^{-1} with a flow rate of 0.56 ml/min. The inoculua for the reactors were grown to exponential phase in a 60 ml serum bottle uner nitrogen head-space, and for some experiments the cells were cultivated with either with modified electron donor and acceptor concentrations. Exponential cells at 10% inoculua (0.8 OD_{600}) were inoculated into the CDC reactor that contained 400 ml modified LS4D or PS4D. Growth was allowed for 24 h and then the reactor was run in continuous mode. Stable biofilms were allowed to form for 72 h, and thereafter removed at 12 h time-points.

Biofilm Growth in an Annular Reactor with Varying rpm

An annular reactor (Model 1320, Biosurface Technologies, MT) was used to grow *D. vulgaris* biofilms at varying speeds in order to compare potential affects form different fluid shears. An inner rotating cylinder provides controlled shear to sampling surfaces and a high surface-to-volume ratio provides copious biofilm production. For the experiments, the reactor was run at 150, 200 and 400 rpms in order to determine if altered speeds affected *D. vulgaris* biofilm. *D. vulgaris* planktonic cells were grown to exponential phase and 10% inoculum was used introduced into the annular reactor. Dilution rate of the reactor was 0.09 h^{-1} and the flow rate was maintained at 0.56 ml/ min. The reactor contained 1000 ml LS4D with 60 mM lactate and 50 mM sulfate. The biofilms were allowed to stabilize for 70 to 72 hrs and biofilm samples removed every 24 h. Protein and carbohydrate measurements were made on the collected samples. In addition, biofilm thickness (microtome slices prepared with a Leica CM1850 cryostat) was measured using a cryo-microtome and imaged on Nikon Eclipse E800.

Protein and Carbohydrate Measurements

Protein and carbohydrate levels were measured for *D. vulgaris* biofilm cells grown under nutrient limiting conditions (CDC reactor) and at varying speeds (annular reactor). Under nutrient limiting conditions, the electron donor (lactate and pyruvate) concentration was maintained at 7.5mM and electron acceptor concentration was 6.25mM. Annular reactors had 60mM lactate and 50mM sulfate and on biofilm formation, cells were scraped aseptically into 1.5ml microfuge tubes for protein and carbohydrate analysis. Protein concentration was routinely obtained by Lowry's method. Reagents include copper reagent: Lowry 1(20g/1000ml Na_2CO_3 in 0.1M NaOH), 2%$CuSO_4$, 4% w/v Na tartarate and phenol reagent (2N Folin Ciocalteau solution). Bovine serum albumin was used as the standard. Samples were boiled with 0.2N NaOH for 15 min and copper reagent was added, mixed immediately and incubated for 45 min at 39°C. On cooling, 1ml phenol reagent was added and incubated at room temperature for 30 min. Reaction was measured at 660nm., a standard curve of R= 0.998 and 0.999 was obtained and protein concentrations of unknown was extrapolated from the standard curve (Lowry, 1951).

Carbohydrates were quantified by the cysteine-sulfuric acid method. Samples were heated to 100°C for 3 min with 700mg/l L-cysteine hydrochloride prepared in 86% ice-cold sulfuric acid. The mixture was rapidly cooled to room temperature and absorbance was measured at 415nm. A Standard curve with R=0.988 was obtained and unknown carbohydrate concentrations were extrapolated from the standard curve
(Chaplin, 1986).

Optimization of Dissolved Oxygen (DO) Levels in 10ml Anaerobic Tubes

LS4D and PS4D media were prepared typically under anoxic conditions in contact with a N_2 headspace. Different volumes of air (2 to 10ml) were introduced post-autoclaving into 10-ml balsch tubes (Bellco Glass, NJ, USA) aseptically with media (LS4D and PS4D). An equal volume of N_2 headspace was recovered from the tubes with a sterile syringe before introduction of air. Tubes were allowed to stand for a few minutes to equilibrate before DO concentration in the medium was measured (10 replicates) using a DO probe (Mettler Toledo). For the tested experimental conditions, 2 ml air corresponded to 0.8± 0.1 mg/L DO, 5 ml air corresponded to 1.5± 0.2 mg/l DO, 8 ml air corresponded to 1.9 ± 0.1mg/l DO and 10 ml air corresponded to 3.2±0.1mg/L DO. The untreated medium tubes (*i.e.*, tubes without added air) had 0.3± 0.1 mg/l DO.

Biofilm Cultivation for Re- inoculation into 10ml Balsch Tubes

D. vulgaris cells were cultivated in CDC reactors either in LS4D or PS4D with typical electron donor and acceptor concentrations. Stable biofilms were allowed to form for 72 hrs on glass coupons. The reactor was then transferred to an anaerobic glove bag (H_2, CO_2 gas mix) and eight glass coupons were carefully recovered from the reactor, rinsed to remove planktonic cells and aseptically scraped into 10 ml anaerobic balsch tubes. Biofilm cells were re-suspended in S4D minimal mineral medium. Recovered *D. vulgaris* biofim cells (grown with LS4D or PS4D) were directly inoculated into 10 ml of respective medium tubes with pre-determined DO concentrations to a final OD_{600} of 0.07 and growth was monitored over time. For another set of growth experiments, recovered *D. vulgaris* biofilm cells (either with LS4D or PS4D) were first vortexed vigorously.

Vortexed cells were then inoculated into 10ml LS4D or PS4D tubes (with pre-determined DO concentration) to a final inoculation OD_{600} of 0.07 and growth response was tracked over time

SEM for Biofilms Exposed to DO

Biofilm cells were grown in CDC reactors on circular glass coupons. They were allowed to stablilize for 72 hrs and carefully transferred anoxically into LS4D without carbon source to wash off excess planktonic cells. The glass coupon was subsequently transferred into a 10ml LS4D medium without any added DO and into another tube with 3.14mg/L DO to establish if DO had any effect on cell morphology. Coupons were placed in fixative that contained 2.5% glutaraldehyde (w/v), 2.0% paraformaldehyde (w/v) and 0.05 mM sodium cocadylate buffer (pH 7.0) for scanning-electron microscopy. The biofilms were fixed overnight and were then washed four times (20 min each) with ddH2O. Slides were dehydrated by incubation in increasing concentrations of ethanol and then dried at the critical point with a CO_2 critical point drier (Tousimis) as previously described (Clark et al., 2007). Samples were sputter coated with gold (20 nm) and viewed at the Imaging Chemical Analysis Laboratory facility (Montana State University) on Zeiss Supra 35 FEG-VP or Supra 55VP PGT/HKL field emission scanning electron microscopes.

Results and Discussion

Growth of Biofilms in Annular Reactors

D. vulgaris biofilm was grown using an annular reactor designed by Bio Surface technologies. This reactor facilitates biofilm growth at varying rpms which in turn helps control biofilm thickness. Biofilms were grown in the presence of 60 mM lactate and 50 mM sulfate at 150, 200 and 400 rpm. Glass coupons were sampled every 24 hrs for protein and carbohydrate levels. The protein content varied from 0.5 to 2.5 ug/mm^2 with the highest amount at 200 rpm. Moreover, at 200 rpm, *D. vulgaris* produced significantly higher levels of carbohydrates compared to other speeds (at least 10-fold more). The carbohydrate content was almost similar to the protein level at this speed, i.e., 0.8-1.6ug/mm^2 (Figure 34). In addition, carbohydrate to protein ratio was approximately 20 to 30-fold higher at 200 rpm compared to 150 and 400 rpm (Figure 34 B). This suggests that at 200rpm *D.vulgaris* produced significantly more EPS compared to the 150 and 400rpm even though a significant difference in biofilm thickness could not be observed.

Biofilm thickness was measured via cryo-microtome technique and subsequently imaged using a light microscope. At relatively lower speeds, i.e, 150 and 200 rpm, biofilm thickness averaged at approximately 50 microns (data not shown) and at a comparatively high speed, i.e., 400 rpm, biofilm thickness averaged at approximately 78 microns. A less complex biofilm (few microns in thickness) could not be cultivated using an annular reactor by altering the speed of the motor.

Previous studies conducted with mixed-species biofilms have shown that at high flow velocities, biofilm thickness increases at a higher rate than at lower flow velocities (Garny *et al*., 2008). It was demonstrated that higher flow velocity facilitated greater substrate transport rates toward and into the biofilm that in turn enhanced biomass production rates (Wa¨sche et al., 2002). The speeds employed in our experiments did not establish a controlled biofilm thickness. Therefore, biofilm growth was characterized in response to substrate limitation.

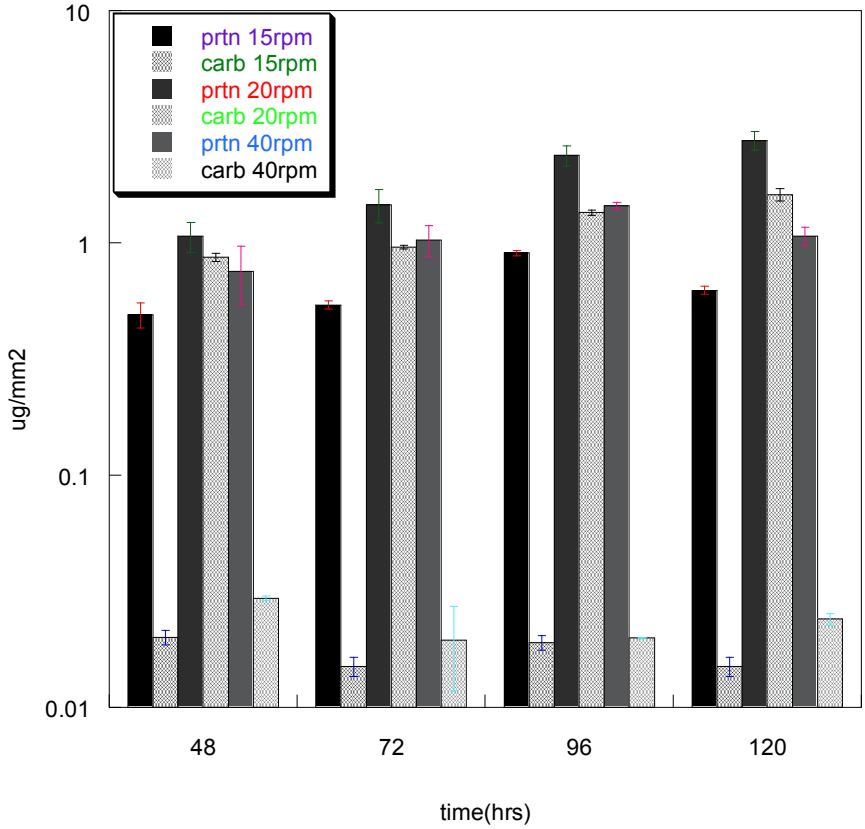


Figure 34A: Protein and carbohydrate concentrations of scraped biofilm cells from coupons recovered from Annular Reactors run at 150, 200 and 400 rpm. Black solid and hashed bars represent protein and carbohydrate concentrations at 15 rpm respectively. Dark grey soild and hashed bars represent protein and carbohydrate concentrations at 20rpm respectively and light grey solid and hashed bars represent protein and carbohydrate concentrations at 40rpm respectively. Biofilm cells were cultivated in LS4D using 60mM lactate and 50mM sulfate. n=2.

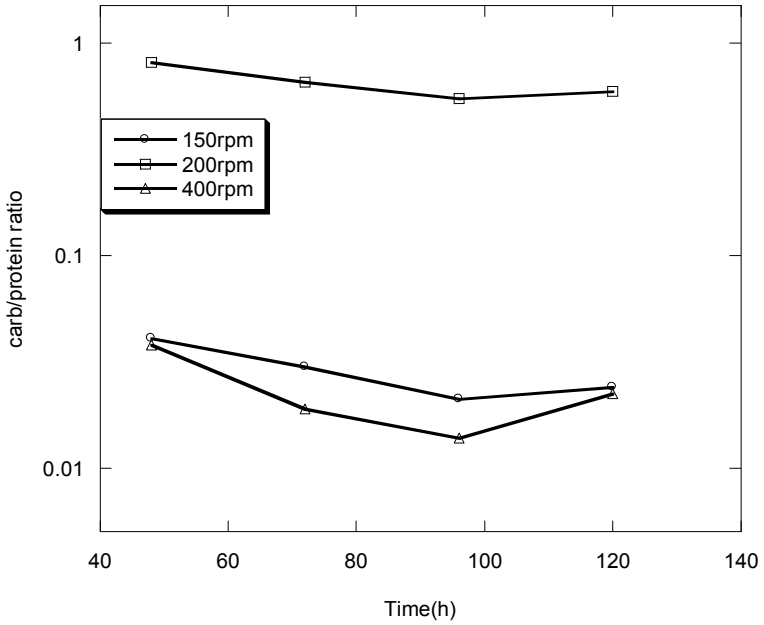


Figure 34 B: Carbohydrate to protein ratio of biofilm cells at 48, 72, 96 and 120h. Biofilms were grown in annular reactors with 60mM lactate and 50mM sulfate as carbon and energy source and electron acceptor respectively. Reactor was run at 150, 200 and 400rpm. n=3.

CDC Reactor Biofilm Cultivation

In the previous chapter (Chapter 4), *D. vulgaris* planktonic cells were subjected to various concentrations of DO and their growth response was evaluated with lactate and pyruvate as substrates. To establish if biofilm cells were more resilient to this treatment, *D. vulgaris* were first grown on glass coupons in a CDC reactor under substrate limiting conditions. Substrate concentrations were 7.5mM lactate/pyruvate and electron acceptor, sulfate, was maintained at 6.25 mM. At these low concentrations it was hypothesized that *D. vulgaris* would form a less heterogeneous biofilm with limited thickness.

Biofilms were grown for 72 hours and allowed to stabilize. Samples were collected every 12 h for protein and carbohydrate measurements. In the presence of lactate as substrate, at 72 hrs, protein concentrations were approximately 200 ug/cm^2 and increased to approximately 600 ug/cm^2 at 156 h. Thickness measurements were made via cryo-microtome that was subsequently imaged using a light microscope. The biofilm thickness ranged from 60 to 100 microns. In the presence of pyruvate, protein levels were more uniform compared to lactate cells, (between 70 to100 ug/cm^2) once stable biofilms were formed at 72 h. Carbohydrate levels were lower than levels measured in the presence of lactate (approximately 1 to 2.9 ug/ul carbohydrate), and biofilm thickness ranged from 40 to 60 μm (Figure 35 A and B). Carbohydrate to protein ratios were similar for lactate and pyruvate biofilm cells as shown in Figure 35 C (*i.e.,* approximately between 0.015 to 0.03).

Apart from hydrodynamic conditions, an important factor that governs biofilm growth is nutrient concentration or nutrient availability (Stoodley *et al*., 2001; Telemann *et al*., 2004; Wijeyekoon *et al*., 2004; Garny *et al*., 2008). It has been demonstrated for different *Pseudomonas* species that at higher nutrient loads, biofilm formation is promoted (Peyton *et al*., 1996). Starvation on the other hand, leads to detachment of biofilms (Hunt *et al*., 2004). In addition, Huang *et al.* (1994) demonstrated that when nutrient supply is increased, polysaccharide/protein ratio is also increased based upon studies with *E.coli*. Our goal was to establish a less complex biofilm structure by lowering nutrient loads in a tractable system. However, because biofilm thickness was similar between the different reactors, a CDC reactor was chosen

due to ease of use and the presence of previous data (chapter 4). Therefore, an experimental design was used so that biofilm cells could be directly compared to the data with planktonic cells presented in Chapter 4.

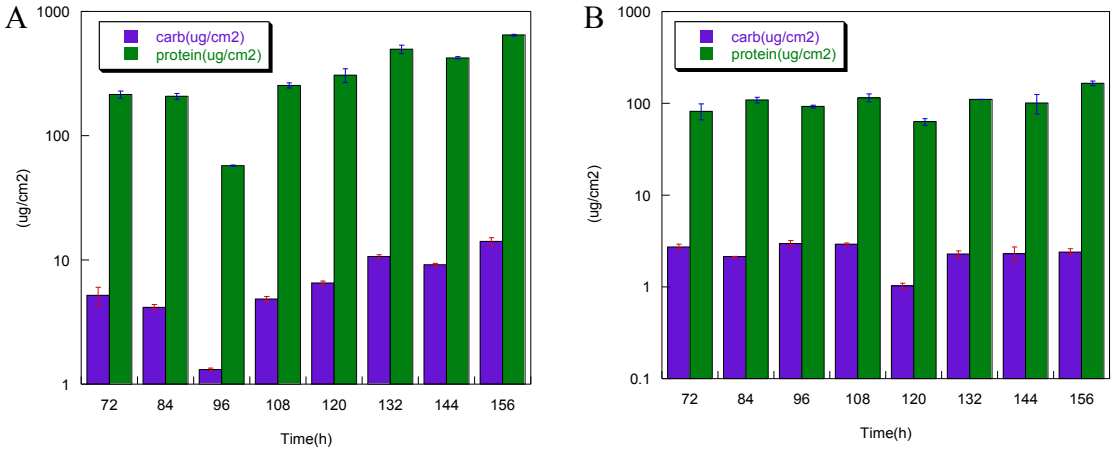


Figure 35: Graphs representing protein and carbodydrate concentrations of *D. vulgaris* biofilm cells grown in CDC reactors under nutrient limiting conditions. Measurements were made over a period of 156 hrs by scraping biomass from glass slides. (A) Green bars represent protein concentrations and purple bars represent carbohydrate concentrations of *D. vulgaris* biofilm grown on 7.5mM lactate and 6.25mM sulfate. (B) Green bars represent protein concentrations and purple bars represent carbohydrate concentrations of *D. vulgaris* biofilm grown on 7.5mM pyruvate and 6.25mM sulfate.

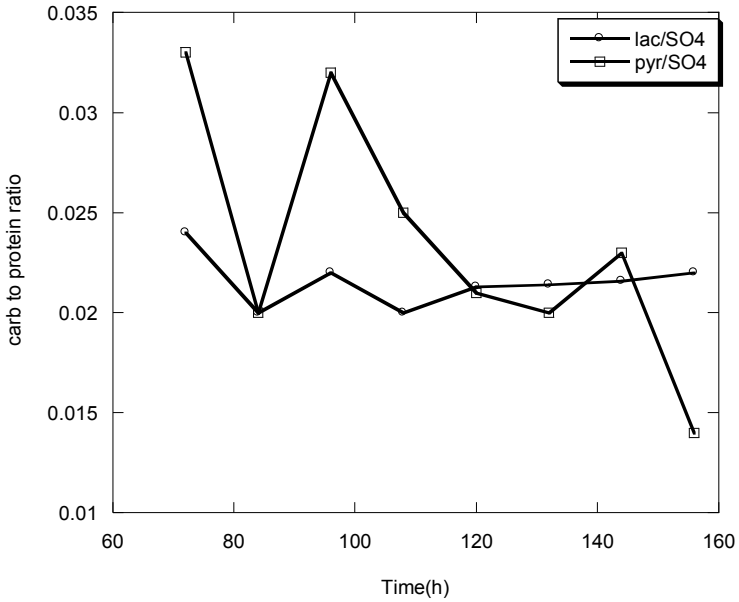


Figure 35 C: Carbohydrate to protein ratio of *D. vulgaris* biofilm cells grown in CDC reactor with lactate and pyruvate as substrates.

Biofilms were grown on glass slides in a CDC reactor with 60 mM lactate or pyruvate and 50 mM sulfate. The cells were scraped and inoculated into fresh LS4D/PS4D tubes with various concentrations of DO (0.78, 1.5, 1.86 mg/l) to an initial OD_{600} of 0.07. Biofilm cells were rinsed with S4D medium anoxically to lower the sulfide levels upon re-inoculation in the presence of DO, and sulfide levels normalized to protein were similar between pyruvate and lactate-grown biofilm cells. This is in contrast to planktonic cells, in which lactate-grown cells had elevated sulfide/protein ratio compared to pyruvate-grown cells (Chapter 4). In addition, planktonic cell and biofilm cell inoculua had similar sulfide/protein values.

Initial experiments were conducted by simply inoculating biofilm cells to a final OD of approximately 0.07 and testing growth responses to different DO concentrations. Lactate-grown biofilm cells were tolerant up to 1.5 mg/l DO, measured by MPN, similar to planktonic cells when the samples were not vigorously vortexted. In addition, the resuspended biofilm cells exhibited increased growth rates at 0.8 and 1.5 mg/l DO similar similar to planktonic cells (Figure 36A and B and 37A). The lactate-grown biofilms were inhibited at 1.9 mg/l DO similar to planktonic cells (Figure S5). When cells were vortexed during sample preparation, the cell tolerance decreased. The vortexed biofilm cells grew with 0.8 mg/l DO and had an increased growth rate compared to lactate control cells but were inhibited at 1.5 mg/l DO (Figure 36C and 37; (Table 11). These results indicated that biofilm cells did not have increased tolerance to DO compared to planktonic cells and were even more sensitive when the biofilm cells vortexed. The data suggested that *D. vulgaris* biofilm structure may contribute to biofilm tolerance to DO, but that biofilm cells in general were more sensitive than planktonic cells.

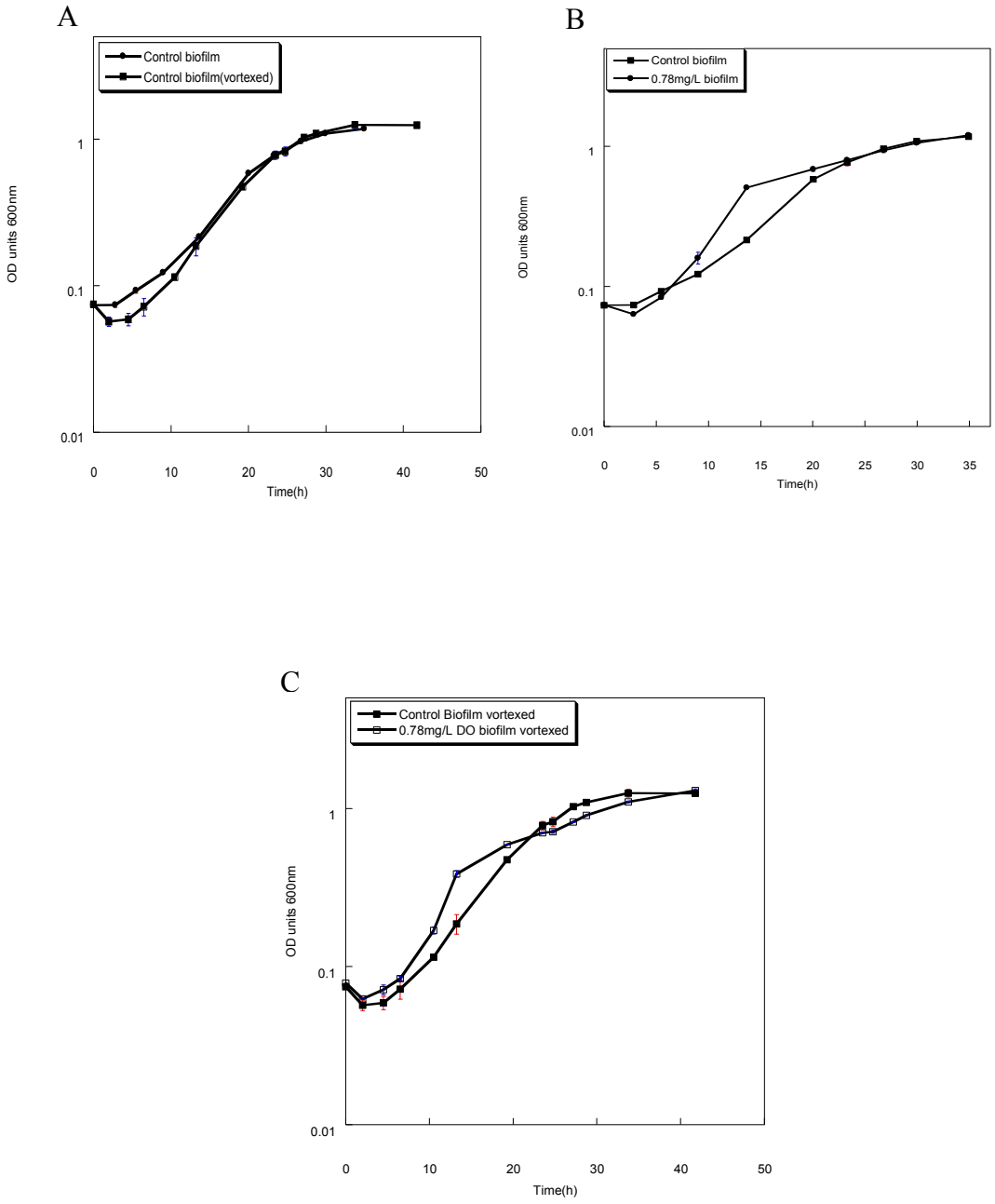
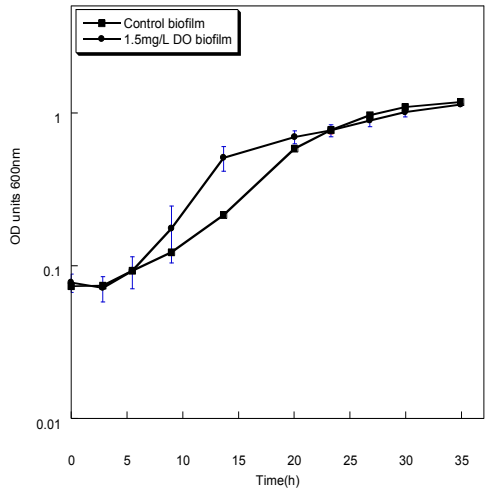


Figure 36 A, B, C: Growth of *D. vulgaris* cells on 60mM lactate and 50mM sulfate. A) *D. vulgaris* biofilm cells transferred to planktonic mode grown without added oxygen (0.3mg/L), unvortexed (●) and vortexed (■). B) *D. vulgaris* biofilm cells (unvortexed) in planktonic mode at 0.3mg/L and 0.78mg/L DO. C) Vortexed *D. vulgaris* biofilm cells in planktonic mode at 0.3mg/L and 0.78mg/L DO.

A.



B.

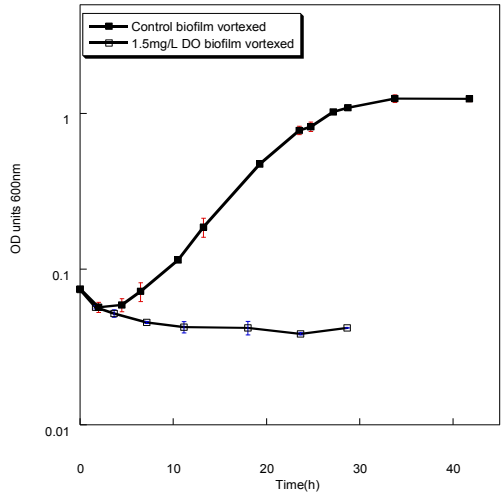


Figure 37 A, B: Growth of *D. vulgaris* cells on 60mM lactate and 50mM sulfate. A) *D. vulgaris* biofilm cells (unvortexed) in planktonic mode at 0.3mg/l and 1.5mg/l DO. B) Vortexed *D. vulgaris* biofilm cells in planktonic mode at 0.3mg/l and 1.5mg/l DO.

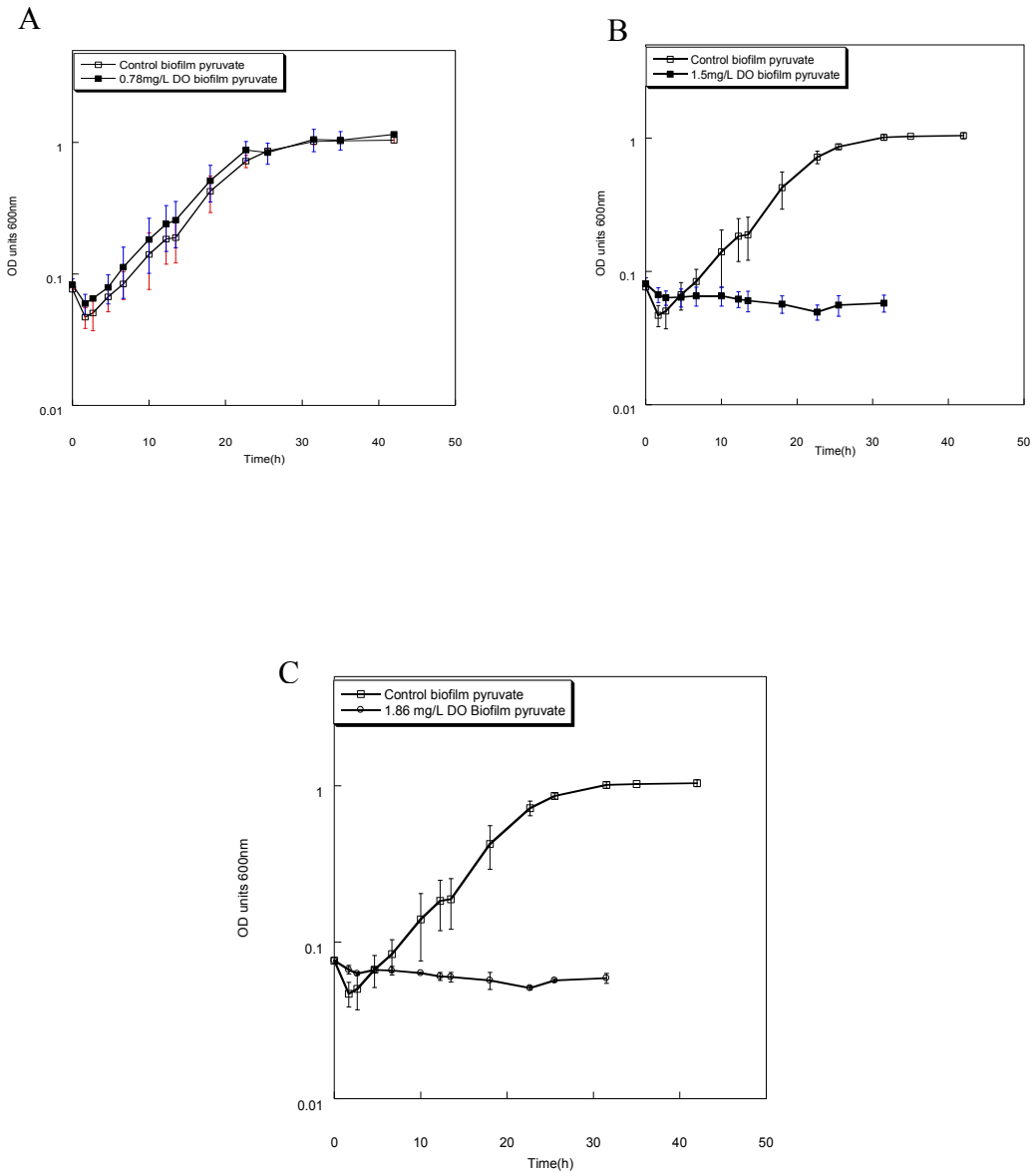


Figure 38 A, B, C: Growth of *D. vulgaris* cells on 60mM pyruvate and 50mM sulfate. A) Vortexed *D. vulgaris* biofilm cells grown in the presence of 0.3mg/l (control) and 0.78mg/l DO. B) Vortexed *D. vulgaris* biofilm cells grown in the presence of 0.3mg/l (control) and 1.5mg/l DO. C) Vortexed *D. vulgaris* biofilm cells grown in the presence of 0.3mg/l (control) and 1.86mg/l DO.

When pyruvate was the electron donor for biofilm cells, response to DO was similar to lactate-grown biofilm cells. Cell preparations that were not vortexed had similar DO tolerance as planktonic cells. The vortexed (inoculum) cells were inhibited at 1.5 mg/l DO (Figure 38 A, B, C). In addition, there was no increased growth rate in pyruvate-grown biofilm cells, similar to planktonic cells (Table 11). However, contrary to planktonic cells, no differential response was observed with respect to tolerance between lactate and pyruvate cells (*i.e.,* lactate- and pyruvate-grown biofilm cells had similar levels of DO tolerance).

Vortexed biofilm (lactate and pyruvate) cells appeared to be more sensitive to DO compared to planktonic cells contrary to the popular theory that biofilm cells (aggregated cells) are more resilient to stressors compared to planktonic cells. The physiological switch from biofilm to planktonic mode perhaps induces a modified response in *D. vulgaris* cells. It is plausible that intact biofilm cells would respond differently had the biofilm cells been tested directly with DO as stressor. To test this hypothesis, intact biofilms were processed in an anaerobic glove bag and exposed to 1.9 mg/l DO in balsch tubes similar to planktonic cells. The planktonic cells and intact biofilms were tolerant to 1.9 mg/l DO for a 2.5 h time period as assessed via viability (MPN). Viability for planktonic cells and intact biofilms both decreased to the same extent and the populations retained 25% viability. These results further indicate that biofilm cells did not have increased tolerance to DO exposure under the tested conditions.

DO(mg/l)	Biofilm cells (Lactate)	Biofilm cells (Pyruvate)
Control (0.36)	$0.11\pm0.02h^{-1}$	$0.13\pm0.01h^{-1}$
0.78	$0.17\pm0.04h^{-1}$	$0.13\pm0h^{-1}$
1.5	-	-

Table 11: Growth rates of *D. vulgaris* cells grown with 60mM lactate or pyruvate as carbon/energy sources and 50mM sulfate as the electron acceptor. Source of inoculum was vortexed scraped biofilm cells grown with the respective electron donors. No growth was observed at 1.5mg/l DO.

Scanning Electron Microscopy to Test
Morphology of Biofilm in the presence of DO

Previous reports have indicated that DO can not only alter the physiology of cells, but can have an effect on morphology as well (Sass *et al*., 1998). To determine if high DO concentrations induced a morphological change to *D. vulgaris* cells, cells were examined under SEM.

Biofilm cells were grown in CDC reactors (lactate, sulfate) on small circular glass coupons. Two glass coupons were introduced into fresh LS4D tubes with 3.14mg/l DO. One coupon was sampled at 2.5h and the other at 24h. Control coupons, not exposed to any DO were examined at similar time points.

Data indicated that the morphology of *D. vulgaris* cells was not altered significantly in the presence of high DO levels. Cells appeared normal (similar to control) at 2.5h post exposure to DO. At 24 h, significant detachment seemed to have occurred as there were fewer cells in the microscopic field. (Figure 39 A, B, C, D)

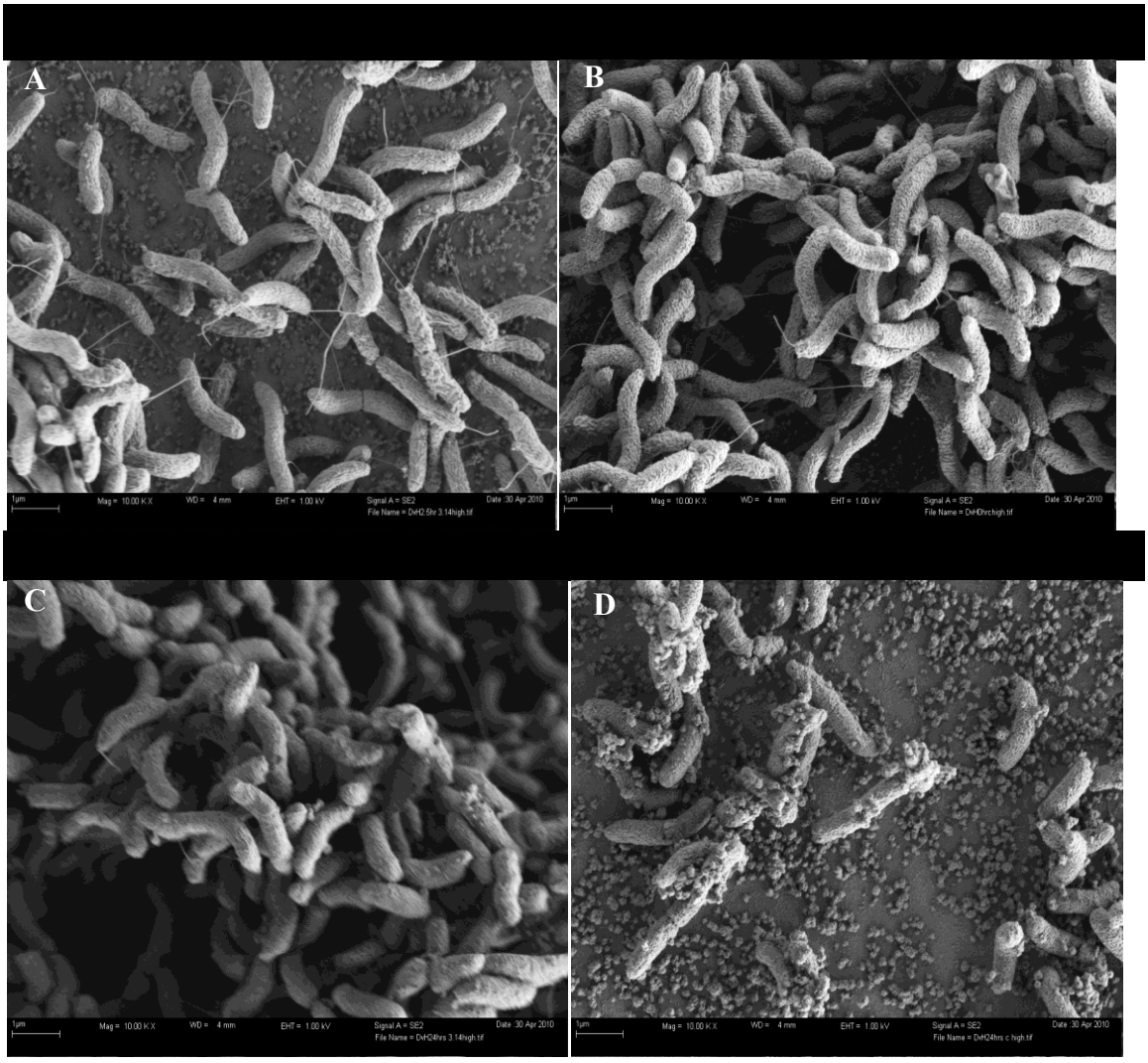


Figure 39: Electron micrographs of *Desulfovibrio vulgaris* Hildenborough biofilm cells grown on glass coupons in a CDC reactor with 60mM lactate as the electron donor and 50mM sulfate as the electron acceptor. Mag=10,000X (A) EM of *D. vulgaris* cells, unexposed to dissolved oxygen at 0h. (B) EM of *D. vulgaris* cells, unexposed to DO at 24h (C) EM of *D. vulgaris* cells exposed to 3.14mg/l DO at 2.5h and (D) EM of *D. vulgaris* cells exposed to 3.14mg/l DO at 24h.

This study investigates effects of DO on *D. vulgaris* biofilm cells. In order to circumvent the difficulties in studying biofilms exposed to oxygen, efforts were made to cultivate biofilm structures with lesser complexity. This was acheieved by growing

biofilm cells in an annular reactor with different speeds and by dramatically lowering nutrient loads in a CDC reactor. Results from both experiments did not however help controlled biofilm formation. Subsequent experiments conducted in this chapter then elucidate effects of biofilm grown cells to DO in the presence of lactate and pyruvate as substrates, in line with planktonic experiments performed in Chapter 4. Contrary to planktonic cells, however, no differences were observed with respect to tolerance towards DO in biofilm cells with lactate and pyruvate. This was further corroborated with viability experiments which suggest biofilm cells did not have increased tolerance to DO exposure under the tested conditions. In nature, bacterial populations occur as biofilms rather than individual cells. A major caveat in studying biofilm responses is most reports merely provide a more average response of cells rather than targetting individual cells within the biofilm structure. Future work will incorporate biofilm cultivation techniques with speed control as well as nutrient depleting conditions.

References

Beyenal, H. and Z. Lewandowski .2004. Dynamics of lead immobilization in sulfate reducing biofilms.Water Res 38: 2726-36.

Beyenal, H., R. K. Sani, et al. 2004. Uranium immobilization by sulfate-reducing biofilms. Environ Sci Technol 38: 2067-74.

Clark, M.E., R. E. Edelmann, M. L. Duley, J. D. Wall, and M. W. Fields. 2007. Biofilm formation in *Desulfovibrio vulgaris* Hildenborough is dependent upon protein filaments. Environ Microbiol. 9:2844-2854.

Clark, M. E., Z. He, K. H. Huang, E. J. Alm, X. F. Wan, T. C .Hazen, A. P. Arkin, J. D. Wall, J. Z. Zhou, and M. W. Fields. 2006. Temporal transcriptomic analysis as *Desulfovibrio vulgaris* Hildenborough transitions into stationary phase during electron donor depletion. Appl Environ Microbiol. 72: 5578-5588

deBeer, D., P. Stoodley, F. Roe, and Z. Lewandowski. 1993. Effects of Biofim stuuctures on oxygen distribution and mass transport. Biotechnol. Bioeng. 43:1131-1138.

Demple, B. 1991. Regulation of bacterial oxidative stress genes. Annu. Rev. Genet. 25:315–337.

Cantero, E.V., J. J. Cabriales and E. M. Romero. 2003. The corrosion effects of sulfate- and ferric-reducing bacterial consortia on Steel. Geomicrobiol. J. 20: 2, 157-169.

Elkins, J. G., D. J. Hassett, P. S. Stewart, H. P. Schweizer, and T. R. McDermott. 1999. Protective role of catalase in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide. Appl Environ. Microbiol. 65: 4594-4600.

Farr, S. B., and T. Kogoma. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. 55:561–585.

Feugeas, F., J. P. Magnin, A. Cornet, and J. J. Rameau. 1997. Microbiologically influenced corrosion: Biofilm influence on steel corrosion, recent techniques and results. J Phys III. 7: 631-663.

Garny, K., H. Horn, and T. Neu. 2008. Interaction between biofilm development, structure and detachment in rotating annular reactors. Bioprocess Biosyst Eng. 31:619–629.Genet. 25:315–337.

Hansen, S. K., P. B. Rainey, J. A. Haagensen, and S. Molin.2007. Evolution of species interactions in a biofilm community. Nature. 445:533–536.

Hassett, D.J., J. G. Elkins, J. F. Ma, and T. R. McDermott. 1999. *Pseudomonas aeruginosa* biofilm sensitivity to biocides: Use of hydrogen peroxide as model antimicrobial agent for examining resistance mechanisms. Methods Enzymol. 310: 599-608.

Huang, C. T., S. W. Peretti, and J. D. Bryers. 1994. Effects of medium carbon-to-nitrogen ratio on biofilm formation and plasmid stability. Biotechnol Bioeng. 44:329–336.

Hunt, S. M., E. M. Werner, B. Huang, M. A. Hamilton, and P. S. Stewart. 2004. Hypothesis for the role of nutrient starvation in biofilm detachment. Appl Environ Microbiol. 70:7418–7425.

Lopes, F. A., P. Morin, R. Oliveira, and L. F. Melo. 2006. Interaction of *Desulfovibrio desulfuricans* biofilms with stainless steel surface and its impact on bacterial metabolism. J. Appl. Microbiol. 101: 1087-1095.

Mah, T. C., and G. A. O'Toole. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9: 34-39.

Marsili, E., H. Beyenal, et al.2005. Uranium removal by sulfate reducing biofilms in the presence of carbonates. Water Science Technol 52(7): 49-55.

Padilla-Viveros, A., E. Garcia-Ochoa, and D. Alazard. 2006. Comparative electrochemical noise study of the corrosion process of carbon steel by the sulfate-reducing bacterium *Desulfovibrio alaskensis* under nutritionally rich and oligotrophic culture conditions. Electrochimichia Acta. 51: 3841-3847.

Peyton, B. M. 1996. Effects of shear stress and substrate loading rate on Pseudomonas aeruginosa biofilm thickness and density. Water Res. 30:29–36.

Rani, S. A., B. Pitts, H. Beyenal, R. A. Veluchamy, Z. Lewandowski, W. M. Davidson, K. B. Meyer, and P. S. Stewart. 2007. Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. J.Bacteriol. 189:4223-4233.

Sass, H., M. Berchtold, J. Branke, H. Konig, H. Cypionka, and H. D. Babenzien.1998. Psychrotolerant sulfate-reducing bacteria from an oxic freshwater sediment, description of *Desulfovibrio cuneatus* sp. nov. and *Desulfovibrio litoralis* sp. nov. Syst. Appl. Microbiol. 21: 212-219.

Stewart, P. S., and M. J. Franklin. 2008. Physiological heterogeneity in biofilms. Nature Rev. 6:199-210.

Stoodley, P., K. Sauer, D. G. Davies, and J. W. Costerton.2002. Biofilms as complex differentiated communities. Annu Rev Microbiol. 56:187–209.
Stoodley, P., S. Wilson, L. Hall-Stoodley, J. D. Boyle, H. M. Lappin-Scott, and J. W. Costerton. 2001. Growth and detachment of cell clusters from mature mixed-species biofilms. Appl Environ Microbiol. 67:5608–5613.

Szomolay, B., I. Klapper, J. Dockery, and P. S. Stewart. 2005. Adaptive responses to antimicrobial agents in biofilms. Environ Microbio. 7: 1186–1191.

Teitzel, G. M., and M. R. Parsek.2003. Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa.* Appl Environ Microbiol. 69: 2313–2320.

Telgmann, U., H. Horn, and E. Morgenroth. 2004. Influence of growth history on sloughing and erosion from biofilms. Water Res. 38:3671–3684.
Wäsche, S., H. Horn, and D. C. Hempel.2002.) Influence of growth conditions on biofilm development mass transfer at the bulk/biofilm interface. Water Res. 36:4775–4784.

Wijeyekoon, S., T. Mino, H. Satoh, and T. Matsuo. 2004. Effects of substrate loading rate on biofilm structure. Water Res. 38:2479–2488.

Xu, K. D., P. S. Stewart, F. Xia, C. T. Huang, and G. A. McFeters. 1998. Spatial Heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. Appl Environ Microbiol. 64:4035-4039.

Zhang, W. W., D. E. Culley, et al. 2007. Comparative transcriptome analysis of *Desulfovibrio vulgaris* grown in planktonic culture and mature biofilm on a steel surface. Appl Microbiol Biotechnol 76(2): 447-457.

CHAPTER 6

EPILOGUE

As oxygen evolved and subsequently the concentrations increased in the atmosphere, microorganisms started developing strategies to utilize molecular oxygen. While utilization mechanisms on the one hand could be a detoxification strategy, on the other hand, more energy can be produced by aerobic respiration that provides organisms a metabolic advantage. Organisms started to develop more elaborate electron transport chains and included terminal oxidases (such as, cytochrome *aa3*) capable of transferring electrons to oxygen. While reduction of oxygen is metabolically advantageous to microbes, this process leads to the formation of several reactive intermediate products. Toxic radicals (hydroxyl, superoxide, and hydrogen peroxide) damage DNA, proteins and lipids. Enzymes such as superoxide dismutase and catalase convert these radicals to water and oxygen to alleviate oxidative stress. Anti-oxidative enzymes seemingly have ancient origins and appear to be widely distributed in obligately anaerobic organisms that have existed for at least several billion years (White, 2007; Konhauser 2009; Hewitt and Morris, 1975).

Chapters 2 and 3 of this dissertation demonstrate how a facultative γ-*Proteobacterium*, *Shewanella oneidensis* MR-1 utilizes sensor/signaling domains, usually part of larger peptides, to sense oxygen for optimal regulation of metabolism. Facultative organisms shift from aerobic to anaerobic metabolism, depending on oxygen availability, and in doing so undergo extensive changes in metabolism. Many regulatory systems

govern gene expression accompanying the shift to anaerobiosis. Several two component and one-component signal transduction pathways have been implicated in gene regulation in such facultative organisms. Many open reading frames in bacteria and archaea contain domains like PAS, PAC, GGDEF, EAL (discussed in Chapter 1) that have been reported to receive/ transmit signals and consequently elicit a response (White, 2007; Hoch and Silhavy, 1995, Stock *et al*., 2000; Hoch, 2000; Taylor and Zhulin, 1999; Galperin *et al*., 2001; Anantharaman *et al*., 2001; Galperin *et al*., 1999).

In this study, a deletion mutant for ORF SO3389 was characterized. SO3389 encodes for a sensory box protein which is comprised of a unique domain architecture including PAS, PAC, GGDEF and EAL. Closest hits to this protein were found in three other *Shewanella* genomes with 84-85% sequence identity and also found in *Pseudomonas spp., Vibrio spp.* and *Mangetococcus spp*. The closest non-*Shewanella* relative, in terms of sequence identity was *Vibrio*. Physiological functions have not been attributed yet to these proteins (sensory box proteins or conserved hypothetical proteins). Since at least 40% of MR-1 is comprised of hypothetical proteins, tremendous amount of time will be consumed in verifying the functions of these proteins (Heidelberg *et al*., 2002; Yost *et al*., 2003). One approach is to use computational methods to make functional predictions. Another approach, more tedious, and used in this study, is to employ mutagenesis to characterize such proteins. Although more tedious, it is a more direct and accurate method to assign functions to proteins (Brenner, 2001; Fetrow *et al*., 2001).

PAS proteins are capable of binding to cofactors such as FAD, FMN, heme, and Fe-S clusters. PAS proteins exhibit structural plasticity, and upon sensing an environmental change can undergo a change in quaternary structure (Slavny *et al*., 2010). Phenotypic analysis of SO3389 suggested that during trasition from aerobic to anoxia, irrespective of the electron acceptor tested, mutant cells could not grow. The mutant was impaired in sensing the absence of oxygen. Heme-gels for cytochrome analysis revealed that the deletion mutant was deficient in at least three kinds of cytochromes during this transition. Increased fumarate reductase activity in WT cells (qualitative and quantitative) further corroborated the idea that the mutant was deficient in sensing and responding to anoxic conditions. Furthermore, gene expression data suggested that SO3389 possibly regulated certain cytochromes, reductases and other regulators directly and indirectly during transition to anoxia. In addition to phenotypic characterization, structure prediction was carried out for SO3389-PAS to obtain insight on (i) structural similarity with other PAS domains (with solved structures), (ii) to predict if SO3389-PAS binds to oxygen via cofactors and (iii) if it exhibits protein-protein interactions or interactions with other ligands. NifL, studied in *Azotobacter vinelandii* contains two PAS domains. Although the PAS does not directly bind oxygen, it senses the redox state of the cell by binding to an FAD moiety (Hill *et al*., 1996). Another example of an FAD-binding PAS is MmoS, methane monooxygenase, studied in *Methylococcus capsulatus*. This PAS sensor also aids in sensing changes in cellular redox levels (Ukaegbu and Rosenzweig, 2009). YtvA, a photosensor in *Bacillus subtilis*, contains PAS sensors that bind to FMN cofactors. Via FMN, these photoreceptors absorb and react to blue light

(Moglich and Moffat, 2007). In this study structure prediction using DALI Protein Structure Database and Phyre server was carried out to predict the possible structure of SO3389- PAS. The sequence and structure comparisons suggested that SO3389 could be a FAD-based O_2/redox sensor, (closest match with MmoS) and future work includes the characterization of ligand interactions for SO3389.

SO3389 also contains putative GGDEF and EAL domains, and chapter 2 describes other phenotypes associated with SO3389. Apart from being deficient in transitions to anoxia (PAS domains, discussed above), phenotypic analysis revealed that ΔSO3389 was impaired in biofilm formation and motility. Impediment in cell differentiation was one of the first phenotypes described for a GGDEF domain in *Caulobacter cresentus* (Hecht and Newton, 1995). Cellulose production was demonstrated to be activated by the GGDEF domain. Extrapolysaccharide production in turn affects morphology and increases biofilm formation in many gram negative bacteria (Amikam and Galperin, 2006; Lee *et al*., 2007; Sudarsan *et al*., 2008; Weinhouse *et al*., 1997). Decrease in c-di-GMP concentration on the other hand (overproduction of EAL) leads to suppressed biofilm formation but activates formation of structural components that assist in various types of motility (Kirillina *et al*., 2004; Simm *et al*., 2004). Chapter 2 of this thesis demonstrates how SO3389, containing GGDEF and EAL domains are possibly responsible for biofilm formation and motility in MR-1. Evidence has emerged for c-di-GMP as a secondary messenger molecule in bacteria that is involved in the regulation of planktonic, motile, and sedentary lifestyles. Since c-di-GMP molecules appear to be involved in a plethora of regulatory processes integrating varied signals, it

has become extremely important to assign molecular and physiological function to these domains and identify the effector and target molecules that are responsible for eliciting a response. There is a possibility that these molecules are involved in protein-protein interactions, that have perhaps led to the evolution of systems with degenerate GGDEF and EAL domains that do not depend on c-di-GMP metabolism to function. Despite the theories, these domains remain captured in their physiological context: regulating motility and/or biofilm related functions (Hengge, 2009).

Thus, understanding the physiological responses of an iron-reducer such as MR-1 to oxygen is very important in assessing the potential impact of environmental perturbations on metal-reducing activity. MR-1 responds to and/ or senses oxygen via sensory domain proteins that contain PAS sensors. Although oxygen is considered to be the preferred electron acceptor, MR-1 will, in the absence of oxygen, activate machinery to utilize alternate electron acceptors. In turn, this alternative machinery allows the organism to produce energy. *Desulfovibrio vulgaris* Hildenborough is another example of a metal-reducing bacterium implicated in bioremediation. *D. vulgaris* is a 'strict' anaerobe, commonly studied with respect to heavy metal and radionuclide immobilization. Responses to oxygen in the environment also impact how this stringent anaerobe performs metal-reduction. Chapters 4 and 5 of this dissertation demonstrate examples of oxygen responses in *Desulfovibrio vulgaris* Hildenborough.

With respect to anaerobic microorganisms, evolution of molecular oxygen was considered to be baneful. It was believed that anaerobes are perhaps not equipped to deal with the deleterious effects of oxygen. To deal with the mutagenic effects of oxygen and

eradicate toxic oxidative metabolites, aerobes and facultatives posses a set of regulated enzymes like catalase and superoxide dismutase. Over the past three decades, there is ample evidence to substantiate the presence of anti-oxidative measures in 'strict' anaerobes. This includes behavioral response to oxygen, aerotolerance and survival in the presence of oxygen (by reducing molecular oxygen) (Hatchikian, 1977; Dos Santos *et al*., 2000; Krekler *et al*., 1998).

The results described herein explain substrate based differential response of a sulfate-reducing bacterium, *Desulfovibrio vulgaris* Hildenborough to dissolved oxygen. There are no reports thus far on oxygen tolerance based on carbon/energy source used. The electron donors used for this study were lactate and pyruvate since most of the studies have used lactate and pyruvate as carbon/energy sources. Early studies also suggest *Desulfovibrio vulgaris* demonstrates maximum growth yields in the presence of pyruvate, followed by lactate during dissimilatory sulfate reduction (Barton *et al*., 1978). Our experiments, however, suggest that *D. vulgaris* responds with similar growth rates in the presence of lactate and pyruvate as electron donors (Chapter 4). However, sulfide production varies significantly between the two electron donors, *i.e*., lactate grown cells produce higher levels of sulfide compared to pyruvate grown cells (this study, Chapter 4). In order to address the proposed hypothesis, physiology based experiments were systematically carried out which led to our conclusion that even though pyruvate grown cells seemed to be more tolerant towards DO, lactate cells perhaps had the capability of utilizing oxygen, coupling it to growth as demonstrated by an increase in ATP levels.

Occurrence of glycogen (polyglucose) has been investigated in sulfate-reducing bacteria and the role of polyglucose in oxygen-dependent respiration has been reported in *Desulfovibrio salexigens* (Stams *et al*., 1983 and vanNiel *et al*., 1996). To ascertain a possible role in *D. vulgaris* substrate-based differences with respect to oxygen tolerance, polyglucose (carbohydrate) was measured in lactate and pyruvate cells. In addition, GlgA, a putative glycogen synthase was up-expressed within 20 min of exposure to air in lactate-grown cells (microbesonline.org). Polyglucose measurements and growth response of Δ*glg*A in the presence of lactate and pyruvate revealed that there were decreased internal carbohydrate levels for Δ*glg*A compared to WT *D. vulgaris*. Carbohydrate levels tested at 0.8mg/l DO at 10h in Δ*glg*A cells revealed that lactate-grown cells had more total carbohydrate compared to pyruvate-grown cells (0.022 ug carb/ug protein for pyruvate- grown cells vs. 0.035 ugcarb/ug protein for lactate-grown cells).

When ATP levels were measured in lactate-grown Δ*glg*A cells at 0.8mg/L DO (0.38 ± 0.13 nmol ATP/ng protein), the levels were lower than WT cells (5.03 ± 0.79 nmol ATP/ng protein) grown with the same substrate. These results indicated that the inability to synthesize glycogen in Δ*glg*A cells diminished ATP production in the presence of intermediate DO concentrations, but only in lactate-grown and not pyruvate-grown cells. Results from polyglucose levels and growth response in the presence of the two substrates along with ATP measurements indicate that GlgA plays a role in the observed oxygen response in lactate-grown cells.

In an attempt to exploit the existence of another system in lactate cells that might affect the ability of *D. vulgairs* to consume oxygen, experiments were carried out with Δ*per*R. Studies in *Clostridium acetobutylicum*, a stringent anaerobe revealed that PerR is a multi-functional protein that plays a crucial role in coping with oxidative stress (Hillman *et al*., 2008). Authors demonstrate that by fine-tuning the regulatory mechanisms of the defense system involving PerR, prolonged aerotolerance, limited growth and oxygen consumption can be achieved in this organism. Growth results on Δ*per*R with lactate and pyruvate suggest that there are no significant differences in growth rates at lower DO levels in lactate-grown cells and the cells were still able to increase the growth rate at 0.8 mg/l DO. The decreased tolerance with both substrates at higher DO levels could perhaps simply be attributed to the absence of PerR and the ability to up-express detoxification systems for oxidative stress. The results also indicate that PerR is not required for the increased growth rates when grown with lactate and intermediate DO concentrations.

Lactate-grown cells exhibited increased growth rate (concomitant with increased ATP levels) in the presence of intermediate levels of dissolved oxygen despite the lower tolerance. On the other hand, pyruvate-grown cells exhibited increased tolerance without increase in ATP production at intermediate DO levels. The Δ*glg*A results suggest the involvement of polyglucose for lactate-grown cells and work is needed to definitively dissect the electron route of oxygen in the presence of these two substrates. In addition, future work is needed to understand why despite similar levels of glycogen in lactate- and

pyruvate-grown cells, only lactate-grown cells can increase ATP production upon oxygen exposure.

Even though *D. vulgaris* is capable of growth with lactate, pyruvate, or hydrogen as electron donors and sulfate as the terminal electron acceptor, the mechanisms involved in shuttling the electrons (generated by substrate oxidation) from cytoplasm to periplasm via various hydrogenases and subsequently re-routing these electrons back into the cytoplasm for sulfate-reduction are not well understood. Figure 40 illustrates possible electron flow in the presence of different substrates in *Desulfovibrio spp.* Lactate and pyruvate are both three-carbon energy sources with electron equivalents of 12 and 10 e^- /mole, respectively. It is intriguing to specultate how a difference of two electrons affects the decision to use glycogen differently for a *D.vulgaris* cell which inturn confers differential tolerance toards DO. In addition, future studies should be directed towards deciphering electron shuttling by substrate oxidation (lactate and pyruvate) in the presence of glycogen (endogenous substrate) and oxygen and studying the possible involvement of other electron transport complexes while utilizing oxygen as a terminal electron acceptor. These studies will have significant implications in addressing substrate dependent variations towards oxygen susceptibility in the subsurface which in turn could help improve bioremediation strategies.

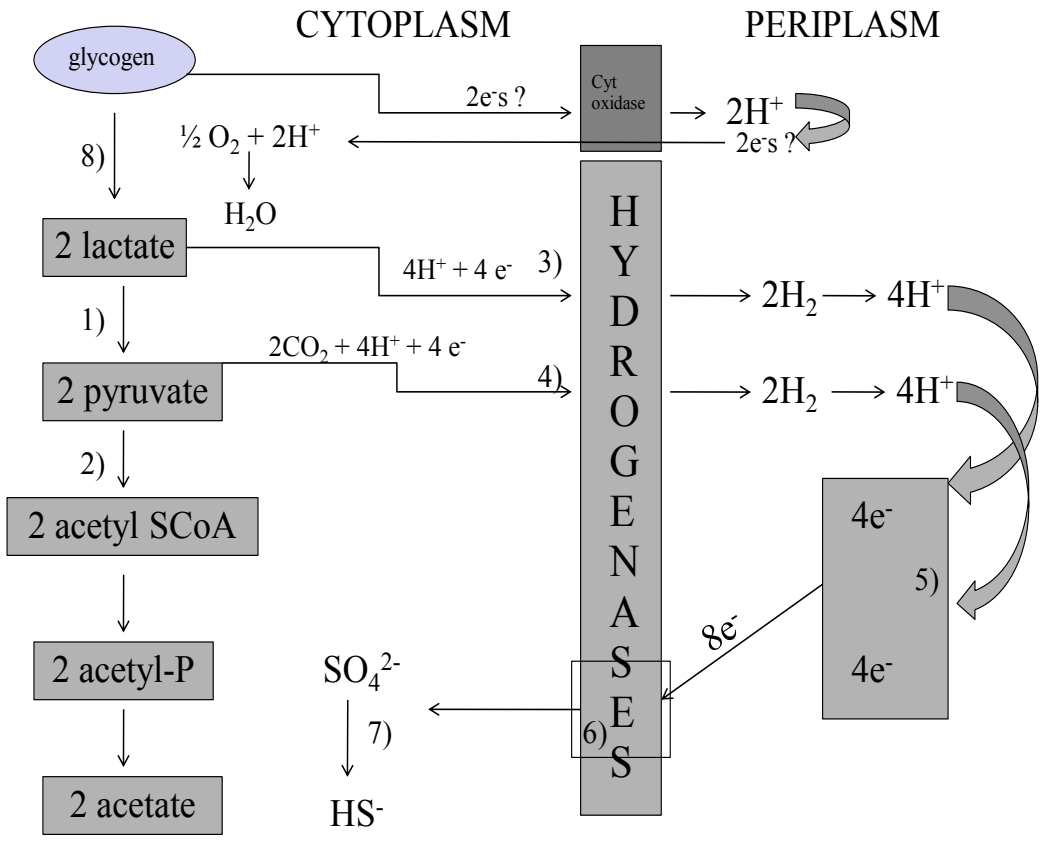


Figure 40: Model for possible electron flow with lactate or pyruvate as carbon sources. 1) lactate dehydrogenase. 2) pyruvate-ferridoxin oxidoreductase or pyruvate formate lyase. 3) cytoplasmic, membrane bound hydrogenase. 4) CO-dependent hydrogenase. 5) cytochrome c_3 network. 6) transmembrane electron transport complex. 7) enzymes that reduce sulfate to sulfide. 8) enzymes for polyglucose utilization.

In addition to the aforementioned efforts, cytochrome profile analysis (qualitative methods by heme-stained gels and RT-PCR), specifically of cytochrome *c* oxidase, cytochrome *bd* oxidase, other *c*-type cytochromes could provide insight into the involvement of these cytochromes in oxygen reduction when grown in the presence of lactate but not pyruvate. In addition, gene expression can be measured by RT-PCR, for

genes involved in oxidative stress, which will establish what genes specifically confer tolerance when grown in the presence of lactate and what genes are involved when grown with pyruvate. Experiments that directly and non-invasively measure oxygen consumption (in the presence of dissolved oxygen) when grown with the two substrates will validate if the increase in ATP levels in lactate cells is a result of oxygen depletion (coupled to growth). In order to non-invasively measure oxygen consumption, a fiber optic DO probe was purchased from Ocean Optics with autoclavable sensor patches. The fiber optic oxygen sensor uses the fluorescence of a chemical complex in a sol-gel to measure partial pressure of oxygen. The sensor formulation in the sol-gel could not be autoclaved successfully. The gel-matrix disintegrated at 121°C and consequently the experiment could not be performed. PreSens, a German-based company made similar optic probes butcould not be calibrated successfully in the growth medium used for the reported experiments. If oxygen measurements could be made successfully, results obtained will provide tremendous insight on aerobic growth of 'stringent' anaerobes. The above endeavors would help establish if depletion occurs at different rates during consumption as opposed to detoxification in anaerobes such as sulfate-reducing bacteria.

Chapter 4 addresses the effects of DO on planktonic *D. vulgaris* cells. As an extension to our original hypothesis to study effects of DO on *D.vulgaris* planktonic cells, we performed experiments to evaluate effects of oxygen on *D. vulgaris* biofilm cells as well (Chapter 5). However, to evaluate effects of a parameter like oxygen on biofilm cells, it is critical to recognize the inherent heterogeneity that exists in a biofilm. Biofilm responses in terms of concentrations of metabolic substrates and products is one

of the characteristics of the main parameters that dictates biofilm thickness and morphology. Stratified distribution of oxygen has been demonstrated in aerobic biofilms (deBeer *et al*., 1994), and oxygen profiling has been shown in *Staphylococcal* colony biofilms (deBeer *et al*., 1994). Experiments conducted by Rani *et al*., (2007), demonstrate as the depth of biofilm increases, oxygen concentration decreases. Similar results were observed with *Pseudomonas* biofilms (Rani *et al*., 2007; Xu *et al*., 1998). Keeping the nature of biofilms in mind, efforts were made to grow biofilms with less structural complexity to simplify the heterogeneity within these structures. Annular reactors and CDC reactors were used at varying speeds and CDC reactors were used under nutrient limiting conditions. This was undertaken to test if any one of the applied conditions yielded a less complex biofilm structure based upon biofilm thickness. This, however, could not be achieved. When cultivated using annular reactors at different speeds, depth of *D. vulgaris* biofilms ranged between approximately 50-80 microns. In addition, there was high variability between the samples analysed from the same run. This variability persisted on switiching to a CDC reactor with limited nutrient load. Thickness measurements were made via cryo-microtome that was subsequently imaged using a light microscope. The biofilm thickness ranged from 60 to 100 microns in the case of CDC reactors and this variability was not substrate-dependent. In addition to gradients in oxygen concentrations, SRB biofilms are faced with the complication of sulfide gradients which inevitably adds another level of physiological complexity and impacts the ability to accurately measure analytes due to the reducing nature of sulfides.

Biofilms are typically considered to be more resilient compared to the planktonic counterparts in terms of tolerance to chemical insults. In order to determine if a physiological switch from biofilm to planktonic mode alters tolerance of biofilms to dissolved oxygen, growth experiments were carried out with lactate and pyruvate as substrates. Results indicate similar tolerance of lactate- and pyruvate-grown biofilm cells towards the stressor and both were more sensitive to the treatment compared to planktonic cells. Therefore, contrary to the original prediction, biofilm cells were not more tolerant to oxygen. Viability experiments with intact biofilms further corroborated the idea that even intact biofilms were not more tolerant to oxygen exposure compared to the planktonic counterparts. Viability for planktonic cells and intact biofilms both decreased to the same extent and the populations retained 25% viability.

This dissertation acknowledges molecular oxygen as a molecule that could potentially be a repellant or an attractant to a microorganism, and surprisingly, could be a potential attractant for a strict anaerobe. Facultatives, equipped to survive with or without oxygen, have evolved several strategies to intelligently sense and utilize this molecule to optimize their metabolism, and SO3389 in *S. oneidensis* was identified as a unique sensory protein that was involved in cell responses to increasing and decreasing oxygen concentrations. A prior function for this type of protein had not been reported, and thus, the reported work will provide insight across biology.

It is traditionally assumed the anaerobes, on the other hand, have built-in protection mechanisms to eradicate deleterious effects of oxidative stress without any

comitant growth. Although several protective features have been demonstrated in sulfate-reducing bacteria towards oxygen, to our knowledge, this is the first report that illustrates substrate based differential response of *D.vulgaris* towards dissolved oxygen. It also shows that oxygen consumption in this organism is likely coupled to growth. While the proposed experiments provide better insight and understanding of response of anaerobes towards oxygen, results presented in this dissertation compel us to re-define 'strict' anaerobes like *Desulfovibrio vulgaris* Hildenborough as microaerophiles or aero-opportunists, capable of oxygen utilization at discrete oxygen concentrations. Discrete attributes such as this may play a significant role in niche differentiation.

Shewanella oneidensis MR-1 and *Desulfovibrio vulgaris* Hildenborough represent different physiological groups, yet both can be associated with dissimilatory reduction of radionuclides and heavy metals (Wu *et al*., 2007; Ganesh *et al*., 1997; Lovley *et al*., 2009; Mohapatra *et al*., 2010; Lloyd, 2003). Microorganisms (two discussed in this dissertation) play a significant role in confining and limiting the spread of radioactivity in the environment through their metabolic processes. *Shewanella spp.* is implicated in Cr(VI), U(VI), Fe(III), Mn(IV), Tc(VII) reduction processes. *Desulfovibrio spp.* is capable of reducing all of the above compounds efficiently as well (Mohapatra *et al*., 2010). Even though both phylogenetic groups reduce heavy metals and radionuclides, due to their metabolic diversity, the process proceeds differently. For instance, Ganesh *et al*., (1997) reported differential capabilities with respect to U(VI) reduction using a *Shewanella* and *Desulfovibrio spp*. While *Desulfovibrio* used a periplasmic cytochrome c_3 to carry out the reduction, *Shewanella* used a multicomponent enzyme system to do the

same. The reduction process progressed slower in *Desulfovibrio* compared to *Shewanella* and it was affected by various organic compounds when either bacterium was used (Ganesh *et al*., 1997). The nature of the organisms found in the subsurface environment largely depends on oxygen availability. If the subsurface is anaerobic, sulfate-reducing bacteria will predominate once nitrate and iron have been depleted. On the other hand, if conditions are more aerobic, growth of facultatives like *Shewanella* will be favored.

There are many environmental conditions, including oxygen and electron donor availability that can govern metabolic status of microbial populations (biofilm and planktonic) during bioremediation (Roling and VanVersveld, 2002; Edwards et al., 2007). Exposure of strict anaerobic bacteria to oxidative stress is a common phenomenon in subsurface environments, and could greatly impact bioremediation strategies. Efforts were made to address this phenomenon in the presence of different electron donors and these types of cellular responses could impact the rate of bioremediation. By assessing the physiological stress responses of model bacteria such as *Shewanella oneidensis* MR-1 and *Desulfovibrio vulgaris* Hildenborough in regards to contaminated waste-sites, it will be possible to implement improved bioremediation strategies. For example, dependent upon the nature of oxygen exposure, lactate or pyruvate could be used as the carbon and energy source. If low levels of DO are expected, lactate may be a more desirable substrate as opposed to pyruvate for higher DO levels. Future experiments could also include community level responses to the different physiological behavior of SRBs during oxygen exposure.

References

Amikam, D., and M. Y. Galperin.2006. PilZ domain is part of the bacterial c-di-GMP binding protein. Bioinformatics. 22:3-6.

Anantharaman, V., E. V. Koonin, and L. Aravind.2001. Regulatory potential, phyletic distribution and evolution of ancient, intracellular small-molecule-binding domains. J. Mol. Biol. 307:1271-1292.

Brenner, S.A.2001.Tour of structural genomics. Nat Rev Genet. 2:801-809.

deBeer. D., P. Stoodley, and Z. Lewandowski.1994.Effects of biofilm structures on oxygen distribution and mass transport. Biotechnol. Bioeng. 43: 1131-1138.

Dos Santos, W. G., I. Pacheco, M. Y. Liu, M. Texeira, A.V. Xavier, and J. DeGall.2000. Purification and characterization of an iron superoxide dismutanse and a catalase from the sulfate reducing bacterium *Desulfovibrio gigas*. J. Bacteriol. 182:796-804.

Edwards. L., K. Kusel, H. Drake, and J. E. Kostka. 2007. Electron flow in acidic subsurface sediments co-contaminated with nitrate and uranium. Geochim. Cosmochim. Acta. 71:643-654.

Fetrow, J. S., N. Siew, and M. M. Di Gennaro.2001. Genomic-scale comparison of sequence and structure based methods of function prediction: does structure provide additional insight. Protein Sci. 10: 1005-1014.

Galperin, M. Y., A. N. Nikolskaya, and E. V. Koonin.2001.Novel domains of the prokaryotic two-component signal transduction systems. FEMS. Microbiol. Lett. 203:11-21.

Galperin, M. Y., D. A. Natale, L. Aravind, and E. V. Koonin.1999.A specialized version of the HD hydrolase domain implicated in signal transduction. J. Mol. Microbiol Biotechnol 1:303-305.

Ganesh. R., K. G. Robinson, G. D. Reed, and G. S. Sayler. 1997. Reduction of hexavalent uranium from organic complexes by sulfate- and iron-reducing bacteria. Appl. Environ. Microbiol. 63:4385-4391

Hecht, G. B., and A. Newton.1995.Identification of a novel response regulator required for the swarmer-to-stalked-cell transition in *Caulobacter cresentus*. J. Bacteriol. 177:6223-6229.

Heidelberg, J. F., I.T. Paulsen, K. E. Nelson, E. J. Gaidos, and W. C. Nelson. 2002. Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. Nat. Biotech. 20:1118-1123.

Hengee, R.2009.Principles of c-di-GMP signaling in bacteria. Nature Rev. 7:263-273.

Hewitt, J., and J. G. Morris.1975. Superoxide dismutase in some obligately anaerobic bacteria. FEBS. lettrs. 50:315-318.

Hill, S., S. Austin, T. Eydmann, T. Jones, and R. Dixon. 1996. *Azotobacter vinelandii* NIFL is a flavoprotein that modulates transcriptional activation of nitrogen-fixation genes via a redox-sensitive switch. Proc. Nat. Acad. Sci. USA. 93: 2143-2148.

Hillmann, F., R. J. Fischer, F. Saint-Prix, L. Girbal, and H. Bahl.2008. PerR acts as a switch for oxygen tolerance in the strict anaerobe Clostridium acetobutylicum. Mol. Microbiol. 68: 848-860.

Hoch, J. A., and T. J. Silhavy. eds1995. Two-component signal transduction, ASM Press.

Hoch, J. A.2000.Two-component and phosphorelay signal transduction. Curr. Opin. Microbiol. 3:165-170.

Kirillina, O., J. D. Fetherston, A. G. Bobrov, J. Abney, and R. Perry. 2004. HmsP, a putative phosphodiesterase, and HmsT, a putative diguanylate cyclase, control Hms-dependent biofilm formation in *Yersinia pestis*. Mol. Microbiol.54:75-88.

Konhauser, K.2009.Introduction to geomicrobiology. Blackwell publishing.

Lee, V. T., J. M. Matewish, J. L. Kessler, M. Hyodo, Y. Hayakawa, and S. Lory.2007. C cyclic-di-GMP receptor required for bacterial exopolysaccharide production. Mol. Microbiol. 65:1474-1484.

Lloyd. J. R. 2003. Microbial reduction of metals and radionuclides. FEMS. Microbiol. Rev. 27: 411-425.

Magee, E. L., B. D. Ensley, and L. L. Barton.1978. An Assessment of growth yields and energy coupling in *Desulfovibrio*. Arch. Microbiol. 117:21-26.

Moglich, A., R. A. Ayers, and K. Moffat. 2009. Structure and signaling mechanism of Per-ARNT-Sim domains. Cell. Struct. 17:1282-1294.

Mohapatra. B. R., O. Dinardo, W. D. Gould, and D. W. Koren. 2010. Biochemical and genomic facets on the dissimilatory reduction of radionuclides by microorgansisms-a review. Min. Engg. 23:591-59

Mouser. P. J., D. E. Holmes, L. A. Perpetua, R. DiDonato, B. Postier, A. Liu, and D. R. Lovley. 2009. Quantifying expression of *Geobacter spp*. Oxidative stress genes in pure culture and during in situ uranium bioremediation. ISME. J. 3:454-465.

Rani, S. A., B. Pitts, H. Beyenal, R. A. Veluchamy, Z. Lewandowski, W. M. Davidson, K. B. Meyer, and P. S. Stewart. 2007. Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. J. Bacteriol. 189:4223-4233.

Roling. W. F. M., and H. W. vanVersveld. 2002. Natural attenuation: what does the subsurface have in store? Biodegradation. 13:53-64

Simm, R., M. Morr, A. Kader, M. Nimtz, and U. Romling.2004.GGDEF and EAL domains inversely regulate c-di-GMP levels and transitions from sessility to motility. Mol. Microbiol. 53:1123-1134.

Slavny, P., L. Richard, P. Salinas, T. A. Clarke, and R. Dixon. 2009. Quaternary structure changes in a second Per-Arnt-Sim domain mediate intramolecular redox signal relay in the NifL regulatory protein. Mol. Microbiol. 75: 61 – 75.

Stams, F. J. M., M. Veenhuis, G. H. Weenk, and T. A. Hansen,.1983. Occurrence of polyglucose as a storage polymer in *Desulfovibrio* species and *Desulfobulbus propionicus*. Arch. Microbiol. 136: 54-59.

Stock, A. M., V. L. Robinson, P. N. Goudreau.2000.Two component signal transduction. Annu. Rev. Biochem. 69:183-215.

Sudarsan, N., E. R. Lee, Z. Weinberg, R. H. Moy, J. N. Kim, K. H. Link, and R. R Breaker.2008. Riboswitches in eubacteriasense the second messenger cyclic-di-GMP. Science. 321:411-413.

Taylor, B. L., and I. B. Zhulin.1999. PAS domains: Internal sensors of oxygen, redox potential and light. Microbiol. Mol. Bio. Rev. 63:479-506.

Ukaegbu, U.E., and A. C. Rosenzweig. 2009. Structure of the redox sensor domain of *Methylococcus capsulatus* (Bath) MmoS. Biochemistry. 48:2207– 2215.

vanNiel, E. W. J., T. M. P. Gomes, A. Willems, M. D. Collins, R. A. Prins, and J. C. Gottschal.1996.The role of polyglucose in oxygen-dependent respiration by a new strain of *Desulfovibrio salexigens*. FEMS. Microbiol.Ecol. 4:243-253.

Voordouw, G. 2002. 2006. Carbon Monoxide Cycling by *Desulfovibrio vulgaris* Hildenborough. J. Bacteriol. 184: 5903–5911.

Weinhouse, H., S. Sapir, D. Amikam, Y. Shilo, G. Volman, P. Ohana, and M. Benziman.1997. c-di-GMP binding protein, a new factor regulating cellulose synthesis in *Acetobacter xylinum*. FEBS. Lettr. 416:207-211.

White, D.2007.The physiology and biochemistry of prokaryotes, III Edition, Oxford University Press.

Wu, W.M,. J. Carley, J. Luo, M. A. Ginder-Vogel, E. Cardenas, M. B. Leigh, C. Hwang, S. D. Kell, C. M. Ruan, L. Y Wu, J. Van Nostrand, T. Gentr, K. Lowe, T. Mehlhorn, S. Carroll, W. S. Luo, M. W. Fields, B. H. Gu, D. Watson, K. M. Kemner, T. Marsh, J. M. Tiedje, J. Z. Zhou, S. Fendorf, P. K. Kitanidis, P. M. Jardine, and C. S. Criddle. 2007. In situ bioreduction of uranium (VI) to submicromolar levels and reoxidation by dissolved oxygen. Environ Sci Technol. 41: 5716-5723

Xu, K. D., P. S. Stewart, F. Xia, C. T. Huang, and G. A. McFeters. 1998. Spatial heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. Appl. Environ. Microbiol. 64:4035-4039.

Yost, C., L. Hauser,F. Larimer, D. K. Thompson, A. S. Beliaev, J. Zhou, Y. Xu, and D. Xu. 2003. A computational study of *Shewanella oneidensis* MR-1: Structural prediction and functional inference of hypothetical proteins. OMICS. 7:177-191.

APPENDIX A

Supplemental Figures

Supplemantal Figure S1

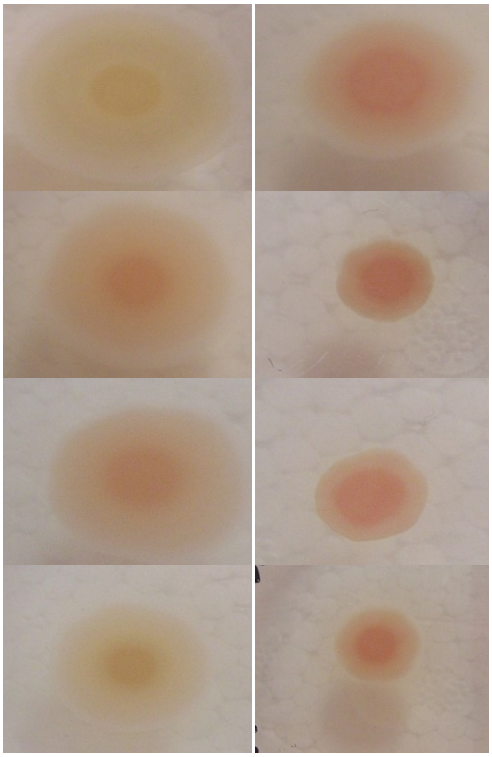


Figure S1: WT and SO3389 were spot inoculated on 0.3% motility agar inoculated from exponential phase aerobic cultures. 1, 2, 3, and 4 represent the four transfers of cultures. At every transfer, motility assay was carried out.

Supplemental Figure S2

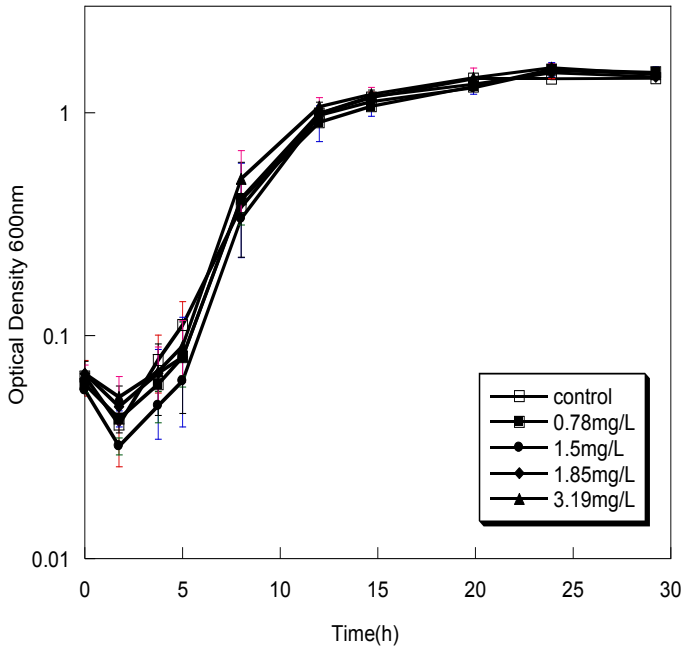


Figure S2: Growth of *D. vulgaris* planktonic cells at various DO concentrations with lactate as substrate in the presence of yeast extract**.**

Supplemental Figure S3

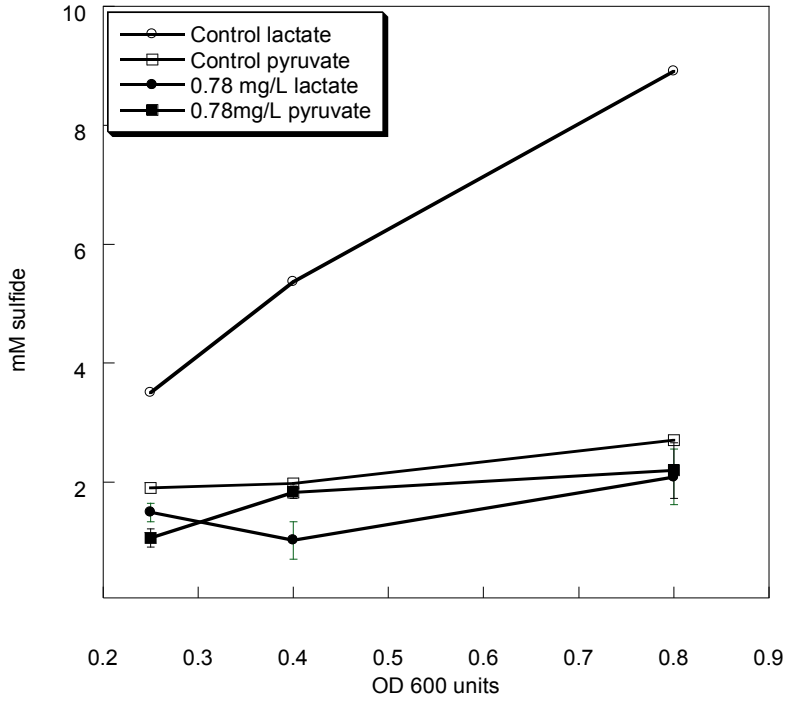


Figure S3: Sulfide levels of washed *D. vulgaris* planktonic cells grown with lactate and pyruvate as carbon sources at 60mM concentration at 0.3 and 0.78mg/l DO concentrations. Open circle and square represents WT *D. vulgaris* washed cells at 0.3 mg/l DO grown with lactate and pyruvate respectively. Closed circle and square represents WT *D. vulgaris* washed cells at 0.78 mg/l DO grown with lactate and pyruvate respectively.

Supplemental Figure S4

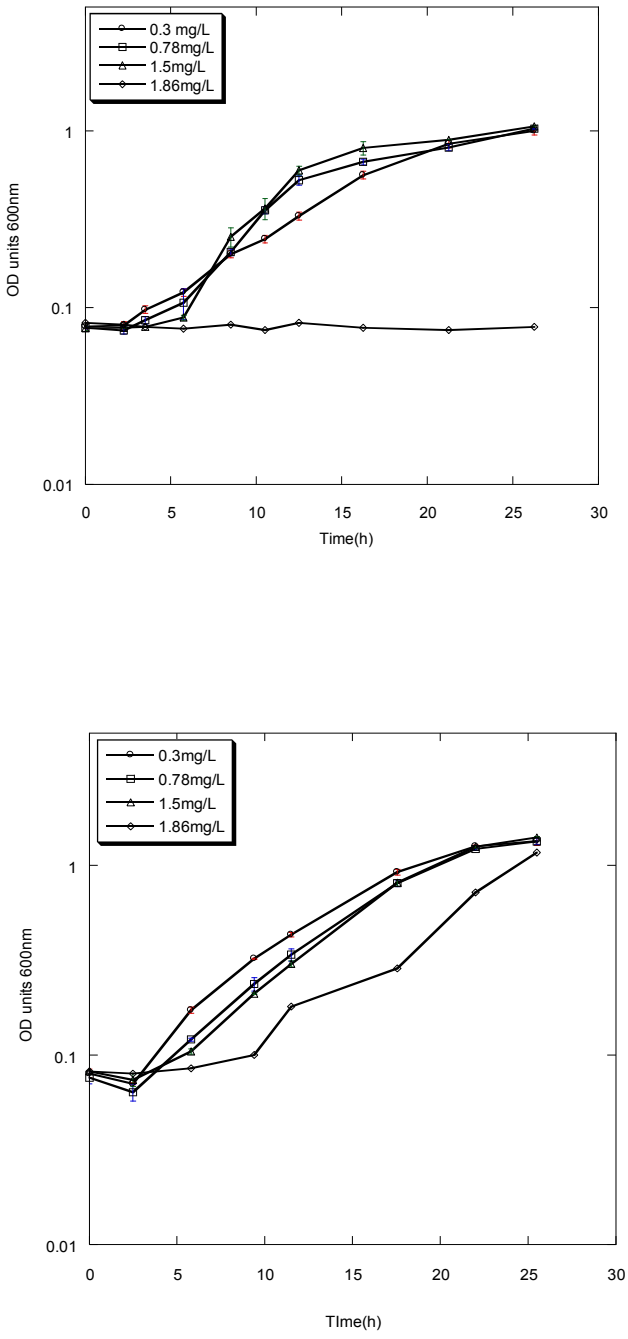


Figure S4: GlgA complemented strain grown with (A) lactate and (B) pyruvate as carbon and energy source. GlgA cells were exposed to 0.78, 1.5 and 1.86mg/l DO.

Supplemental Figure S5

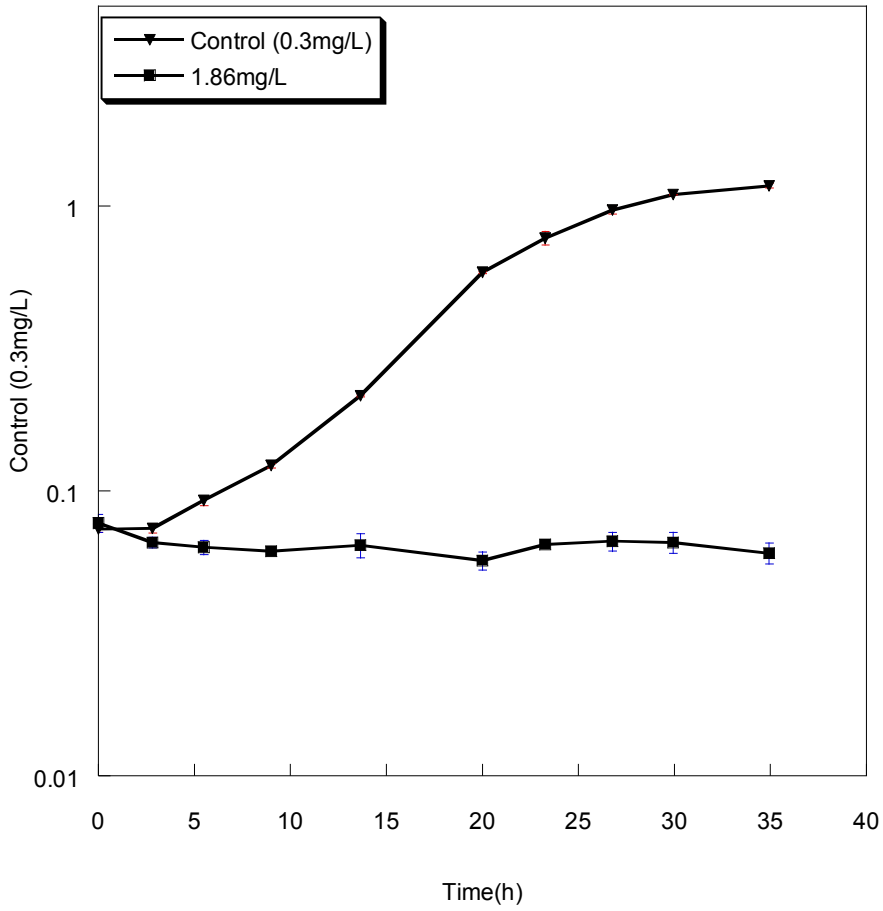


Figure S5: *D. vulgaris* biofilm cells (non-vortexed) grown in LS4D with 60mM lactate and 50mM sulfate in the presence of 1.86mg/L DO.