Development and Application of Variable Strength Expression Vectors in *Shewanella oneidensis* MR-1

A Thesis
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Audrey J. Harris

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Dr. Jeffrey A. Gralnick

December, 2014

Acknowledgements

I am eternally indebted to my P.I., Jeffrey Gralnick for letting me join his lab. Thank you for creating a wonderful lab environment that encourages a love of bacteria and a passion for science in general. Thank you so much for your patience and kindness and for directing me through this process. You've shown me that you can be both a brilliant scientist and a great human being.

I would like to thank my committee members Daniel Bond and Brett Barney. I appreciate that you are both available to talk anytime; you have inspired me with your love of science and fantastic ideas. Most of all, I appreciate you not kicking me out of this program years ago!

Thanks to Ben Bonis for being my first reader. I am so sorry to have put you through that, but I appreciate you rewording and clarifying all the things I meant to say. I "literally" could not have written this without your help.

Thanks to Nick "Bestie" Kotloski for your bravery in pestering me to finish my thesis. You have been an avid supporter and somehow I can listen to you when you are helping me check in with reality. Also, your dance moves are truly inspirational.

Thanks to Aunica Kane and Brittany Bennett for all of your advice and support throughout the painful processes of life and thesis writing. Thanks to Caleb Levar and Eric Kees for brewing the beer that keeps us all chugging away.

Thanks to Megan McKenzie for telling me that I am smart and supporting my insane decision to move to the Midwest.

Thanks to all the former co-workers and friends in my life that told me graduate school was a good idea. Thanks to everyone in the Gralnick Lab, past and present, who made these last few years so much fun!

Dedication

I would like to dedicate this thesis to my family, who against all logic, continue to love and support me. Thanks to my parents for calling and visiting and checking in on me. Thanks for your interest in what I work on - it means so much to me. Thank you for believing in me and encouraging me to follow my own path, even if it takes me farther away from you guys than I ever wanted to go. Thanks to my brother for all the late night phone calls. Thanks to the rest of my family for loving and being proud of me.

Table of Contents

Table of Tables

Table of Figures

Chapter 1
Introduction

Dissimilatory metal reducing bacteria (DMRB) provide an example of the diversity of microbial survival strategies, powerfully impact environmental metal cycling, and are proving valuable in biotechnology applications (Hau & Gralnick, 2007). Many microorganisms consume sugars and respire oxygen, gaining considerable energy in the process while others utilize energetically less favorable fermentation or anaerobic respiration of soluble substrates. Organisms capable of dissimilatory metal reduction exploit a niche using anaerobic extracellular respiration to survive, coupling the oxidation of simple organic compounds to the reduction of insoluble metal oxides or other terminal electron acceptors. Metal oxides, though plentiful in the environment, prove difficult to access and utilize as terminal electron acceptors as this necessitates electron transfer from the cytosol, across membranes, and then further onto insoluble substrates (Richter et al., 2012).

The most well understood DMRB are members of *Shewanella* genera and in the last 50 years much has been discovered about the physiology, ecology, phylogeny and genetics of various species. *Shewanella* species can be found worldwide in water and sediment from the Amazon to the Arctic. *Shewanella oneidensis* MR-1 is a gamma-Proteobacteria with a completed genome available (Heidelberg et al., 2002) and the model organism of the genus. *S. oneidensis* MR-1 was isolated from Lake Oneida in NY, USA and demonstrates tremendous respiratory flexibility, being capable of utilizing an impressive array of substrates as terminal electron acceptors (Myers & Nealson, 1988). *S. oneidensis* MR-1 is a facultative anaerobe that is fast growing and genetically tractable. Recent studies have elucidated modes of metabolism, regulation, energy generation, and electron transport mechanisms that enable *S. oneidensis* MR-1 to survive on such a wide variety

of terminal electron acceptors (Coursolle & Gralnick, 2010; Gralnick & Newman, 2007; Pinchuk et al., 2011). Importantly, riboflavin derived compounds are exported and utilized as electron shuttling molecules which have a tremendous impact on the rate of reduction of insoluble substrates (Marsili et al., 2008; von Canstein et al., 2008; Brutinel & Gralnick, 2012).

DMRB dramatically impact the environment due to their involvement in globally occurring cycling of carbon and metals, affecting redox and, in turn, solubility. DMRB are responsible for seasonal metal fluctuations and nutrient release in sediment (Nealson et al., 2002). Use of DMRB for bioremediation has been explored; however a complete understanding of redox chemistry is important as substrates that become reduced can potentially result in release of toxic metals into soluble forms. In addition to remediating the environment, the ability to transfer electrons to extracellular acceptors enables DMRB to interface with electrodes in biosensors or microbial fuel cells. Redox active biosensors depend on bacterial promoters which respond to an environmental input signal, triggering a response by initiating expression of a gene pathway allowing transfer of current to create an output signal (Golitsch et al., 2013). Biosensors could be engineered to respond to a wide variety of specific input signals and communicate directly with a computer interface and have therefore attracted much interest. Microbial fuel cells are a rapidly expanding area of study, intriguing due to the possibility of harvesting electricity from a simple system and simultaneously treating waste streams. These systems consist of a chamber in which bacteria, often in consortia, oxidize organic molecules and pass electrons onto the anode. Both applications are attractive in remote locations as they are basically self-sustaining.

Regardless of the specific biotechnological applications, in order to effectively utilize these bacteria, it is critical to first have the molecular tools to control and modulate gene expression. Generally, plasmid selection, conjugation methods, and induction strategies successful in *Escherichia coli* and other gram negative bacteria are used successfully in *S. oneidensis* MR-1 including gene deletion, chromosomal integration, and

complementation. Previous studies have shown successful induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) and *β*-galactosidase assays to be effective in assessing protein production and reporting on promoter strength (Gao et al., 2010). This thesis describes the design and construction of a suite of expression vectors with modular promoter and ribosome binding site sequences resulting in a wide range of protein expression levels in *S. oneidensis* MR-1. To allow IPTG induction regardless of host background, a subsequent set of vectors was constructed containing *lacI*^{q}, the *E. coli* gene encoding *lac* operon repressor protein. The practical application of these inducible plasmids in *S. oneidensis* MR-1 was demonstrated by driving variable expression of genes involved in riboflavin biosynthesis and flavin adenosine dinucleotide transport across the inner membrane in order to increase extracellular flavin levels and thus extracellular respiration rates. This study demonstrates the benefit of being able to temporally control select gene expression and amplitude of expression by using a set of predesigned vectors. The modularity of this system will enable researchers to easily exchange alternative promoters or ribosome binding sites in order to modify protein expression for custom applications.

Chapter 2

Design and Construction of Expression Vectors with Modular Promoters and Ribosome Binding Sites

Introduction:

Controlling protein production levels has long been the primary goal of bacterial metabolic engineering. Typically, the objective is to tune gene expression to maximize protein production while minimizing input cost. However, in many circumstances, less than maximal protein production is desirable. Perhaps the product is detrimental to the growth of the cell, toxic, or cannot be effectively folded if the genes encoding it are too highly expressed. Or perhaps microbial products are desired that require tuning complex cellular pathways by controlling the expression of key enzymes to supply precursors or redirect flux through the system. For microbial physiology studies it may be beneficial to express a gene at biologically relevant levels rather than the extremely high levels often achieved by commonly used expression vectors. The field of molecular biology has developed rapidly to provide the knowledge for genetic manipulation in many microbes, resulting in the ability to control protein production at any level.

There are many established ways to alter the DNA surrounding genes to control gene expression and therefore protein production (Brewster et al., 2012). Specific steps in the process, from DNA transcription into RNA and then RNA translation into protein, can be tuned to result in the desired outcome. On the transcriptional level, the gene copy number, as well as the affinity of the promoter region for transcription factors and RNA polymerase may be manipulated. On the translational level, gene expression is affected by the affinity of ribosome binding and the transcript mRNA decay rate. Native genomic DNA can be altered but it is often advantageous to introduce foreign fragments of DNA into the cell to produce the desired affect without impacting the background genetic

material of the host cell. Conveniently, many bacteria are able to carry extra-chromosomal DNA in the form of plasmids stably within the cytosol.

Plasmids occur naturally in bacterial populations and are often responsible for carrying genetic pathways involved in toxin production, antibiotic resistance, or other specialized functions (Eberhard, 1989). These self-replicating circles of DNA are often passed between bacteria via conjugation to other members in a population who can benefit from such pathways. The ability of many bacteria to transfer and receive plasmids has been exploited and improved by molecular biologists to introduce desired functions to bacterial cells expressing plasmid-borne genes. Plasmids that carry all necessary genetic information to mobilize and replicate themselves can be maintained in a wide variety of bacterial species and are therefore referred to as broad host range plasmids. The DNA sequence of plasmids can be manipulated to include selection markers (typically antibiotic resistance), genes or pathways of interest, reporter genes (often encoding florescence), regulatory proteins, or any other desired pieces.

Currently, laboratories studying *Shewanella* species generally rely on genetic techniques established for *E. coli* and other gram negative bacteria. Early work involved mutagenesis and isolation of phenotypes deficient in various aspects of anaerobic respiration. Genetic work has been facilitated by the complete sequencing of *S. oneidensis* MR-1 (Heidelberg et al., 2002) and studies investigating the coding sequence, regulation, and transcriptome have been conducted (Hau & Gralnick, 2007). Many genetic tools familiar to microbiologists have been successfully employed in *S. oneidensis*, including transposons (Koch et al., 2001), commercially available pUC and pET vectors (Marx & Lidstrom, 2001), suicide vectors for gene deletion (Saltikov & Newman, 2003) or insertion (Teal et al., 2006), and a variety of other broad range plasmids used to compliment deletions or for heterologous expression. More unique applications have been designed, for example a biosensor in which the input of arabinose activates a promoter driving expression of electron conduit genes in the Mtr pathway, resulting in an output of signal in the form of current production (Golitsch et al., 2013). A practical application of a *S. oneidensis*

biosensor providing a current based output signal is found in the creation of a strain capable of arsenic detection for monitoring water pollution (Webster et al., 2014). Gao, et al. (2010) designed a *lacZ* based reporter system to evaluate the strength of native *S. oneidensis* promoters in response to global regulators ArcA, CRP, and EtrA. This thesis work seeks to enable improved control in manipulation of *S. oneidensis* MR-1 by examining the effects of promoter and ribosome binding site (RBS) choice on protein production in relevant laboratory conditions.

In this project, a suite of expression vectors was created with modular, non-native promoters and ribosome binding sites resulting in a range of protein production levels for use in *S. oneidensis* MR-1 and other, similar bacteria. The study of dissimilatory metal reduction will be aided if researchers can more accurately estimate the effect of gene expression in these unique organisms. The vectors resulting in the highest protein expression could be used to purify protein for further characterization as individual proteins are implicated in electron transport throughout the cell. The plasmids resulting in low protein expression could potentially be used to study gene expression by complementing deletions at more physiologically relevant levels. Most importantly, understanding the impact of promoter and ribosome binding site choice in *S. oneidensis* MR-1 will enable appropriate module selection to control expression for future applications. Computational predictions for gene expression based on promoter binding and RBS affinity can be made, but experimental validation of these estimates under different growth conditions is necessary. The rest of this chapter will explain the construction and resulting protein production levels of the created expression vectors.

Plasmid Backbone and Construction Plan:

The expression plasmids used in this study were designed using pBBR1-MCS2 as a backbone. pBBR1 is a cryptic, broad range, medium copy number plasmid originally isolated from *Bordetella bronchiseptica* by Antoine and Locht and modified to include a chloramphenicol resistance cassette to allow for selection, creating pBBR1CM (Antoine & Locht, 1992). This plasmid was further modified by Kovach to add a Stratagene

polylinker that includes the *lacZα* fragment interrupted by 16 restriction enzyme recognition sites, resulting in pBBR1MCS (Kovach et al., 1994). In order to make this broad range plasmid more useful in a wide range of gram negative bacteria (some with native chloramphenicol resistance), four new plasmids were created from pBBR1MCS, each displaying a different antibiotic resistance. pBBR1MCS-2 contains an aminoglycoside 3'-phosphotransferase coding sequence conferring kanamycin resistance as described by Kovach in 1995 (Kovach et al., 1995). This is an ideal starting vector for this work as it is small, transferable to both *Shewanella* and *Geobacte*r species, and has many restriction enzyme sites distributed usefully throughout the plasmid to allow further modification.

For this study, the promoter region, ribosome binding site (RBS), and the first 56 base pairs coding for *lacZα* fragment were removed from pBBR1-MCS2 by restriction digest while maintaining most of the multiple cloning site. The two promoter and three RBS segments (explained in the following sections) were ligated together in all six possible combinations and then inserted into the cut vector to create pAH01 through pAH06 (see Figure 2.1). In order to show that the observed expression levels are driven by the promoter and ribosome binding sites and not by read-through from other plasmid borne genes, a vector lacking those two elements was created (pAH07).

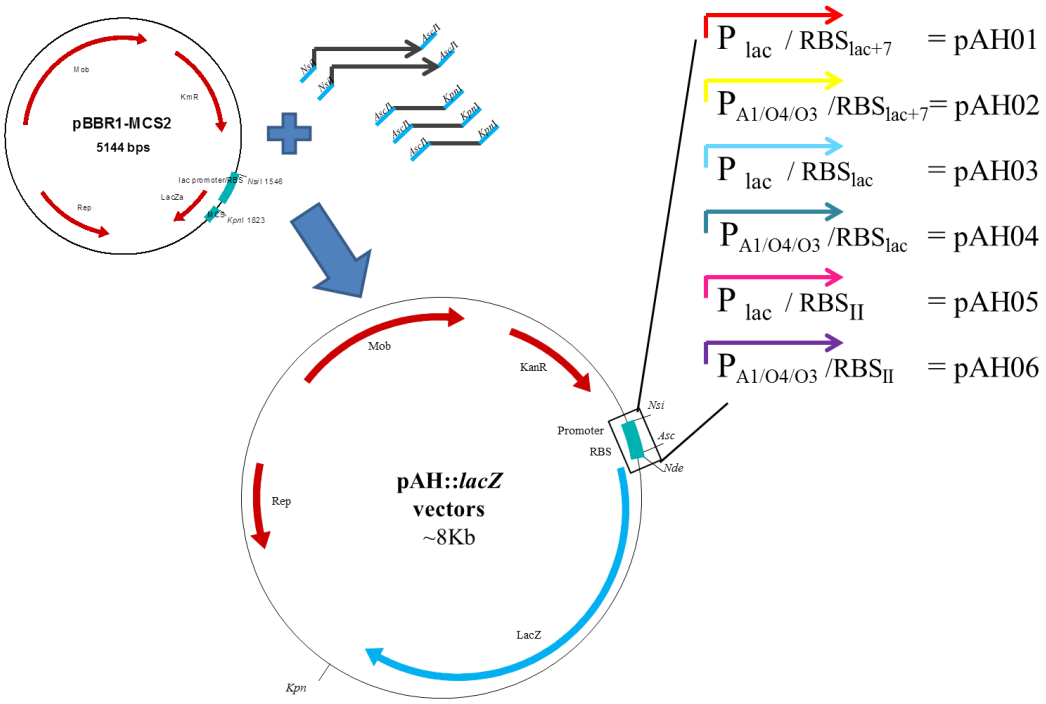

Figure 2.1 – Expression vector flowchart
The promoter, RBS, and start of *lacZα* are replaced in pBBR1-MCS2 with modular promoter and RBS segments to create six expression vectors of varying strength driving expression of full length *lacZ*.

Promoters:

Much of the early bacterial genetic work was done in *E. coli* and excellent experimental evidence enables accuracy in predicting expression levels in that organism. Many factors impact the efficacy of a stretch of DNA as a promoter. Generally, promoters are areas upstream of a gene or operon and rich in A's and T's to facilitate strand separation as the RNA polymerase complex begins to unwind and transcribe the downstream region. The sequence of promoters also determines which transcription factors are able to interact with that region. Sigma factors are part of the complete assemblage of the RNA polymerase holoenzyme and allow promoter binding and initiation of transcription. Cells produce different levels of various sigma factors throughout their growth cycles in response to stress or environmental conditions, controlling which genes are transcribed at a given time. Transcription factors interact with the promoters at two specific sites

referred to as the conserved hexamers. These two, six base pair long regions are typically located 10 and 35 base pairs upstream of the transcription start site and strongly impact binding with the RNA polymerase holoenzyme. Because of the very specific interaction between the DNA in the promoter region and the RNA polymerase protein, the spacing between the -10 and -35 regions is generally tightly conserved at 17+/-1 base pairs – any variation in the number of base pairs negatively impacts RNA polymerase binding (Chen et al., 1994). Also, the nucleotide sequence of this spacer region can strengthen or weaken the interaction and affect transcription although the impact of mutations is not as drastic as variations in the -10 and -35 regions.

Two well-studied promoters known to drive expression at different levels in *E. coli* were selected. The first is the *lac* promoter (P_{lac}) which comes from perhaps the most thoroughly investigated genetic regulatory system in microbiology. P_{lac} has been used since the earliest days of microbial genetics to drive fairly robust transcription as characterized in 1986 (Deuschlel et al., 1986). The region contains conserved -35 and -10 hexamers, two LacI binding sites (LacI operators), and two experimentally validated CRP binding sites (see Figure 2.2). The other promoter used in this study is $P_{A1/O4/O3}$ and is derived from T7 phage promoter A1. The A1 promoter is known to drive much stronger expression than other commonly used promoters, including those from other phages (T5 and λ) and other *E. coli* operons (P_{bla} and P_{tac})(Deuschlel et al., 1986). $P_{A1/O4/O3}$ used in this project was constructed based on segments originally created by Lanzer and Bujard. They examined the efficacy of operon repression by modifying the repressor protein binding position within the promoter region (Lanzer & Bujard, 1988). A suite of promoters was investigated after inserting the core LacI operator sequence into multiple sites including one site in the same position as the native *E. coli lac* operon (O3) and one approximately 30 base pairs upstream between the conserved hexamers (O4)(see Figure 2.2). Andersen et al. (1998) created $P_{A1/O4/O3}$ and used it (in combination with a synthetic RBS from a Qiagen expression vector pQE70) to drive strong expression of mutated green fluorescent protein. The region contains conserved -35 and -10 hexamers as well as the two LacI operator sites and two potential CRP binding sites. Both promoters have

been used in the Gralnick lab previously to drive gene expression in MR-1. Expression from multi-copy plasmids with the *lac* promoter and from chromosomal integrations with the A1/O4/O3 promoter has been successful but never quantified.

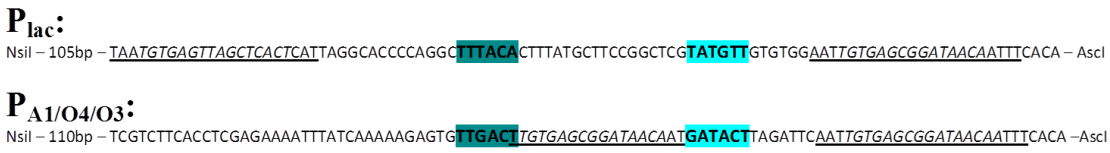

Figure 2.2 – Promoter comparison
Both promoter regions are of similar size and flanked by the same restriction enzymes. The conserved hexamers are shaded in blue (-35 dark blue, -10 light blue), LacI repressor binding sites are underlined and potential CRP binding sites are italicized.

Ribosome Binding Sites (RBS):

The ribosome binding site encompasses an area of mRNA immediately upstream and downstream of the translation initiation codon and includes a standby site for ribosome loading as well as some variation on the Shine-Dalgarno sequence (Salis, 2011). The Shine-Dalgarno (SD) sequence is complimentary to a short segment of the 16S rRNA portion of the ribosomal 30S subunit. The number of base pairs (as well as their sequence and secondary structure) between the ribosome binding site and the gene starting codon affect the rate of translation. Generally, five base pairs separating the 3' end of the SD from the initiator codon is optimal in *E. coli* which allows the assembled ribosome complex to interact with both sites (Chen et al., 1994). The three different ribosome binding sites used in this study vary in SD to start codon spacing. From shortest to longest, RBS_{lac} has a 5 base pair spacing, RBS_{II} has a seven base pair spacing and $RBS_{lac+7bp}$ has a SD to start codon spacing of 12 base pairs (see Figure 2.3). The RBS from the *lac* operon in *E. coli* and the synthetic RBS_{II} from the Qiagen vector were used based on previous reports of medium and high expression, respectively. In order to create vectors with lower expression levels, the spacing between the ribosome binding site found in front of the *lac* operon and the start codon for *lacZ* was increased by inserting seven extra base pairs. The expression vectors were designed to have an NdeI

(CA^T**ATG**) site just downstream of the SD. Cloning with NdeI is convenient as genes that start with **ATG** can easily be inserted without disrupting the spacing between the RBS and start codon and therefore preserving expression levels established with reporter genes.

RBS_{lac}: AscI – CACAC**AGGAAA**CA-CAT*ATG*(NdeI) – KpnI

RBS_{II}: AscI – CACACATCTAGAATTAAAG**AGGAGA**AATTA-CAT*ATG*(NdeI) – KpnI

$RBS_{lac+7bp}$: AscI – CACAC**AGGAAA**CACATTCAA-CAT*ATG*(NdeI) – KpnI

Figure 2.3 – Ribosome binding site comparison
All RBS are flanked by the same restriction enzymes. The Shine-Dalgarno sequence is bolded and shaded in red. Reporter genes are inserted at the NdeI ATG (underlined and italicized). The spacing between the 3' end of the shaded region and the start codon of the reporter gene varies from 5bp in RBS_{lac}, 8bp in RBS_{II} and 12bp in $RBS_{lac+7bp}$.

β-galactosidase (LacZ) and the *lac* Operon:

The full length sequence of *lacZ* was used as a reporter gene for all plasmid expression studies. *lacZ* encodes β-galactosidase, a homotetramer which in its native setting cleaves lactose into glucose and galalactose. β-galactosidase has been used extensively in molecular biology and is one of the earliest discovered and best studied cases of bacterial catabolism regulation. Jacob and Monod studied *E. coli* in the 1960s and observed that growth initially stalled but then resumed in a medium where glucose was absent and lactose was present. They deduced that *E. coli* was turning on expression of different genes giving the cells the ability to utilize lactose but only when the preferred carbon source (glucose) was unavailable. The ability to turn on specific pathways when necessary, and then to turn them off again, allows cells to maximize efficiency by avoiding wasteful enzyme production in the absence of usable substrate. Maintaining diverse pathways on the genome (even if rarely expressed) gives cells the ability to rapidly respond to changes in their environment. The genes enabling lactose catabolism are *lacZ*, *lacY* and *lacA*, collectively termed the *lac* operon, and microbiologists began studying how bacteria were able to control this pathway. LacY is a lactose permease that increases entry of the substrate into the cell, LacZ cleaves the new sugar while the

function of LacA (a transacetylase) is not well understood. An astonishing amount of what we know today about multi-tiered genetic regulation stems from insight gathered studying the *lac* operon.

Jacob and Monod characterized and mapped many mutants until they were able to deduce that full expression of the genes in the *lac* operon depended on a positive signal as well as repression relief. The positive regulation is a result of elevated cyclic AMP levels caused by glucose starvation. This molecule can bind to catabolite activator protein (CAP) (also called cAMP receptor protein (CRP)) which causes a conformational shift that facilitates binding of CRP-cAMP compound to the promoter region - this enhances RNA polymerase binding and therefore elevates transcription of the *lac* operon. In the same region of the *E. coli* chromosome, a gene encoding the DNA-binding repressor protein LacI is constitutively expressed regardless of carbon source utilization. LacI binds to the operator sequence of the promoter region upstream of the *lac* operon and prevents the binding of RNA polymerase, thereby effectively preventing expression of the operon. When lactose is present, it is imported into the cell where it binds to the LacI protein, causing a conformational shift that leads to LacI disassociating with the promoter DNA. The unbound promoter region allows DNA polymerase to bind and transcribe the *lac* operon. If LacI is not produced in the system (as is the case with the expression vectors in *S. oneidensis* MR-1 and *E. coli* UQ950 described in this chapter), the genes downstream of the *lac* promoter are constitutively expressed.

The *lac* operon/repressor system is very effective in nature and therefore has been used extensively by molecular biologists. Many variations on these genes and regulation strategy are commercially available. Perhaps most familiar is blue/white screening in which a successful insertion of a cloned gene of interest into a predesigned expression vector will disrupt the coding sequence for the alpha fragment of *lacZ* (the other *lacZ* subunits are found in the genome of the host cell, usually *E. coli*). When plated on a medium containing X-gal, cells bearing undisrupted β-galactosidase will cleave X-gal resulting in blue colonies where successful clones will be unable to cleave X-gal and

therefore will result in typical white colonies. There are many commercially available substrates which enable colorimetric, fluorescent or chemiluminescent measurement of β-galactosidase production levels. Substrate choice depends on sensitivity and range desired. For our purposes, β-galactosidase assays were performed using ortho-nitrophenylβ-galactoside (ONPG) as a substrate. β-galactosidase cleaves colorless ONPG (a lactose analog) into galactose and ortho-nitrophenyl, a yellow compound that can be measured on a spectrophotometer colorimetrically. This assay allows quick and sensitive differentiation of *lacZ* expression levels.

The sequence of *lacZ* already contains an NdeI site approximately 99bp upstream of the stop codon which is problematic for directed cloning into the RBS NdeI site. To circumvent this, the *lacZ* insert was generated by heterostagger PCR. Heterostagger PCR is a technique that generates single stranded sticky ends without using restriction enzymes. The sequence of the forward primers was designed to build in the TA overhang provided by digestion with NdeI without the undesirable cutting of the *lacZ* coding sequence as shown in Figure 2.4.

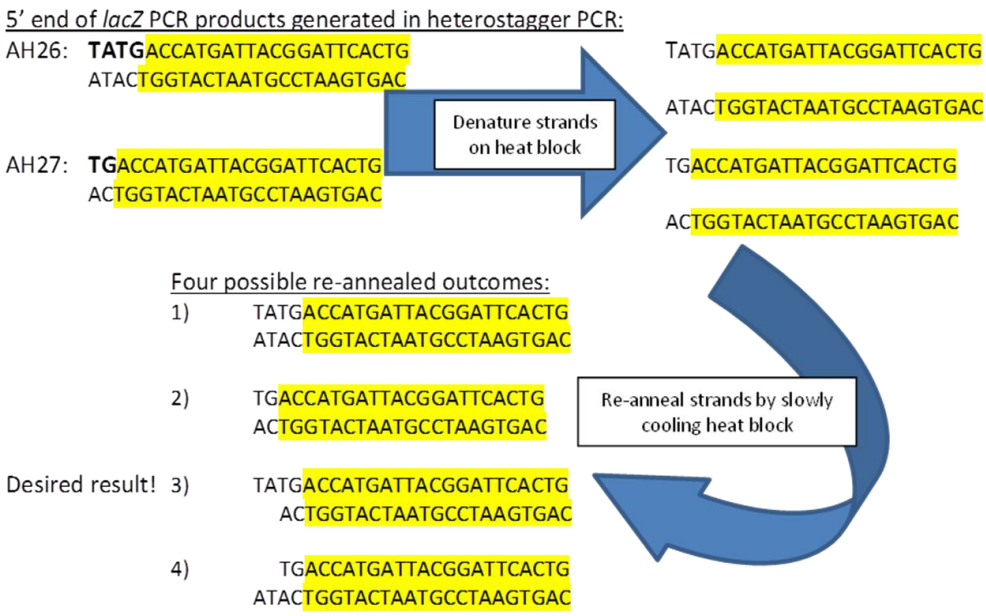

Figure 2.4 – Heterostagger PCR to create TA overhang on *lacZ*
Two PCR products were generated using AH28R as a reverse primer and either AH26F or AH27F as forward primers. The difference in the 5' region is shown in bold. Only one (#3) out of the four possible re-annealed products has the proper 5' TA overhang necessary for ligation into the vector cut with NdeI.

Heterostagger PCR is especially convenient for modifying the expression vectors described in this chapter as it allows sticky end addition on any piece of DNA to make it compatible with built in vector restriction enzyme recognition sites. Therefore any promoter, RBS or reporter gene can be swapped into the system regardless of insert sequence and without the need for site directed mutagenesis to alter codon usage. The modular nature of the parts of these expression vectors will therefore make future alterations easy. Protein production from these expression vectors will be assessed in the rest of the chapter. Once reporter protein levels are established, researchers will have a suite of modifiable plasmids resulting in different expression levels to best suit the desired application.

Materials and Methods:

Culture Conditions:

Strains and plasmids used in this study are listed in Table 2.1. *E. coli* strains were streaked from 15% glycerol freezer vial stocks onto Luria-Bertani (LB) plates and single colonies picked for inoculation into 2 mL LB supplemented with 50 µg/mL kanamycin (Km) for plasmid bearing strains. Cultures were grown overnight (~14 hours) at 37^{o} C with constant shaking at 225 rpm. *S. oneidensis* strains were grown in the same way at 30^{o} C. For *S. oneidensis* growth in minimal media, Shewanella Basal Media (SBM) was used as described previously with 0.05% casamino acids, 20 mM sodium lactate, and 40 mM sodium fumarate added (Hau et al., 2008).

Table 2.1 – Strains and plasmids used in this study

***E. coli* strains:**	**Features:**	**Source/Reference:**
UQ950	DH5α λ(*pir*); F-Δ(*argF-lac*)169 Φ80d*lacZ*58(ΔM15) *glnV* 44(AS) *rfbD* 1 *gyrA* 96(NalR) *recA* 1 *endA* 1 *spoT*1 *thi*-1 *hsdR* 17 *deoR* λpir+; Used for cloning, plasmid preps and expression assays	D. Lies, Caltech/ Saltikov & Newman, 2003
WM3064	*thrB* 1004 *pro thi rpsL hsdS lacZ* ΔM15 RP4–1360 Δ(*araBAD*)567 Δ*dapA* 1341::[*erm pir*(wt)]; Donor strain for conjugation	W. Metcalf, Univ of Illinois, Urbana
***Shewanella oneidensis* strain:**		
MR-1	Wild type strain; Used as host strain for expression assays	J. Gralnick, U of MN/ Nealson, et al., 1999
Plasmids:		
pBBR1-MCS2	5.1-kb broad range host plasmid, Km^{r}, *lacZ*α; Backbone of pAH vectors	Kovach, 1999
pucBB:*lacZ*	Source of full length *lacZ* from *E. coli* MG1655	D. Bond, U of MN
pSMV3::$p_{A1/O4/O3}$ -*gfp* mut3*	Source of $p_{A1/O4/O3}$ and RBS_{II}	J. Gralnick, U of MN/ Teal et al., 2006
pAH01	5.1-kb broad-range expression vector, Km^{r}, pro_{lac} + $RBS_{lac+7bp}$	This study
pAH02	5.1-kb broad-range expression vector, Km^{r}, $pro_{A1/O4/O3}$ + $RBS_{lac+7bp}$	This study
pAH03	5.1-kb broad-range expression vector, Km^{r}, pro_{lac} + RBS_{lac}	This study
pAH04	5.1-kb broad-range expression vector, Km^{r}, $p_{A1/O4/O3}$ + RBS_{lac}	This study
pAH05	5.1-kb broad-range expression vector, Km^{r}, pro_{lac} + RBS_{II}	This study
pAH06	5.1-kb broad-range expression vector, Km^{r}, $pro_{A1/O4/O3}$ + RBS_{II}	This study
pAH07	5.1-kb broad-range expression vector, Km^{r}, no promoter or RBS	This study
pAH08	5.1-kb broad-range expression vector, Km^{r}, no promoter + $RBS_{lac+7bp}$	This study
pAH09	5.1-kb broad-range expression vector, Kmr, no promoter + RBS_{lac}	This study
pAH10	5.1-kb broad-range expression vector, Km^{r}, no promoter + RBS_{II}	This study

Reagents:

All restriction enzymes, T4 ligase, and Antarctic phosphatase were purchased from New England Biolabs. GoTaq Green Mix was supplied by Promega and *pfu* ultra high fidelity polymerase was procured from Agilent. The protocols provided by the suppliers were followed. Plasmid isolation, gel purifications, and PCR clean up were performed using

Invitrogen's PureLink HiPure Plasmid Miniprep Kit, PureLink Gel Extraction Kit, and PureLink PCR Purification Kit. Cut vectors were concentrated using a Zymo Clean and Concentrate Kit. All oligonucleotides and primers were ordered from University of Minnesota Genomics Center (UMGC) and all promoters and RBS were sequenced by the same facility. Primers used in this study are listed in Table 2.2.

Transformation of Strains:

S. oneidensis MR-1 isolates and *E. coli* UQ950 isolates were transformed with plasmids pAH01-pAH06 via conjugation with diaminopimelic acid (DAP) auxotroph *E. coli* WM3064. LB plates with added DAP were cross-streaked with donor strain (WM3064) and recipient strain (*E. coli* UQ950 or *S. oneidensis* MR-1) and incubated overnight. Overnight growth was streaked out onto new LB+ Km plates. Only recipient cells successfully transformed with plasmids conferring resistance to Km can grow as there is no DAP on the plate to support growth of the DAP auxotroph donor strain.

Table 2.2 – Primers/ oligos used in this study

Name:	5' - 3' sequence:	Target:
AH7F	TCAGTCAGGGCGCGCCGTCACGACGTTGTAAAACG	*lac* promoter from pBBR1-MCS2
AH8R	TCAGTCAGGGCGCGCCATATTATACGCAAGGCGACAAG	*lac* promoter from pBBR1-MCS2
AH9F	TCAGTCAGGGCGCGCCTCGCGCATTACCTCACTAAG	A1/O4/O3 promoter from JG1592
AH11R	CAGTCAGGGCGCGCCTGTGAAATTGTTATCCGCTCAC	separate promoters from RBS
AH16F	CAGTCAGGGCGCGCCCACACATCTAG	RBS_{II}
AH17R	CGCGGTGCCGGTACCCATATGTAATTTCTCC	RBS_{II}
AH19F	CAGTCAGGGCGCGCCCACACAGGAAACACATATGGGTACCACGTCCG	RBS_{lac}
AH20R	CGGACGTGGTACCCATATGTGTTTCCTGTGTGGGCGCGCCCTGACTG	RBS_{lac}
AH21F	CAGTCAGGGCGCGCCCACACAGGAAACACATTCAACATATGGGTACCACGTCCG	$RBS_{lac+7bp}$
AH22R	CGGACGTGGTACCCATATGTTGAATGTGTTTCCTGTGTGGGCGCGCCCTGACTG	$RBS_{lac+7bp}$
AH26F1	TATGACCATGATTACGGATTCACTG	Heterostagger PCR for *lacZ*
AH27F2	TGACCATGATTACGGATTCACTG	Heterostagger PCR for *lacZ*
AH28R	GATACTGAGGTACCTTATTTTTGACACCAGAC	Heterostagger PCR for *lacZ*
AH73F	TTCAGGCATGCATGACTTCACGTCGCTAGCGGCGCGCCCATATGTCGTCCG	linker for vectors without promoters or RBS
AH74R	CGGACGACATATGGGCGCGCCGCTAGCGACGTGAAGTCATGCATGCCTGAA	linker for vectors without promoters or RBS

Construction of Promoters, Ribosome Binding Sites and Linkers:

P_{lac}-

The *lac* promoter region was amplified with primers AH8F and AH11R from *E. coli* K12 MG1655 starting at position 365369 producing a 206bp fragment flanked with an NsiI

site upstream and an AscI site downstream of the promoter region.

$P_{A1/O4/O3}$-

The A1/O4/O3 promoter region was amplified from strain JG1592 with primers AH18F and AH11R to produce a 211bp fragment flanked by NsiI upstream and AscI downstream.

RBS_{lac}-

The ribosome binding site found in front of the *lac* operon in *E. coli* K12 MG1655 was synthesized by ordering two single stranded, complimentary oligonucleotides (AH 19 and AH20) and annealing them to form a double stranded product in 10 mM Tris buffer. The oligos were designed to contain an AscI site upstream of the RBS as well as NdeI and KpnI sites downstream of the RBS.

RBS_{II}-

A synthetic ribosome binding site (RBS_{II}) from Qiagen cloning vector pQE70 was amplified from strain JG1592 using primers AH16 and AH17 designed to contain an AscI site upstream as well as NdeI and KpnI sites downstream.

$RBS_{lac+7bp}$-

$RBS_{lac+7bp}$ was synthesized by ordering two single stranded, complimentary oligonucleotides (AH 21 and AH22) and annealing them to form a double stranded product in 10 mM Tris buffer. The oligos were designed to create a 37 base pair fragment containing an AscI site upstream of the RBS as well as NdeI and KpnI sites downstream of the RBS.

28 base pair linker-

A linker with multiple restriction enzyme recognition sites was created by ordering two single stranded, complimentary oligonucleotides (AH73F and AH74R) and annealing them to form a 37 base pair, double stranded product in 10 mM Tris buffer. The restriction sites present are NsiI, AhdI, NheI, AscI, and NdeI.

Expression Vector Construction:

pAH01-

P_{lac} and $RBS_{lac+7bp}$ were digested with AscI, ligated together, and PCR amplified with

primers AH8F and AH22R to produce a 243 base pair product. These products were digested with NsiI and KpnI and ligated into pBBR1-MCS2 cut with NsiI and KpnI to create pAH01.

pAH02-

$P_{A1/O4/O3}$ and $RBS_{lac+7bp}$ were digested with AscI, ligated together, and PCR amplified with primers AH18F and AH22R to produce a 248 base pair product. These products were digested with NsiI and KpnI and ligated into pBBR1-MCS2 cut with NsiI and KpnI to create pAH02.

pAH03-

P_{lac} and RBS_{lac} were digested with AscI, ligated together, and PCR amplified with primers AH8F and AH20R to produce a 236 base pair product. These products were digested with NsiI and KpnI and ligated into pBBR1-MCS2 cut with NsiI and KpnI to create pAH03.

pAH04-

$P_{A1/O4/O3}$ and RBS_{lac} were digested with AscI, ligated together, and PCR amplified with primers AH18Fand AH20R to produce a 241 base pair product. These products were digested with NsiI and KpnI and ligated into pBBR1-MCS2 cut with NsiI and KpnI to create pAH04.

pAH05-

P_{lac} and RBS_{II} were digested with AscI, ligated together, and PCR amplified with primers AH8F and AH17R to produce a 253 base pair product. These products were digested with NsiI and KpnI and ligated into pBBR1-MCS2 cut with NsiI and KpnI to create pAH05.

pAH06-

$P_{A1/O4/O3}$ and RBS_{II} were digested with AscI, ligated together, and PCR amplified with primers AH18F and AH17R to produce a 258 base pair product. These products were digested with NsiI and KpnI and ligated into pBBR1-MCS2 cut with NsiI and KpnI to create pAH06.

pAH07-

The 28 base pair linker was digested with NsiI and NdeI and ligated into pAH01 digested

with NsiI and NdeI to create pAH07. This vector contains no promoter and no RBS.

pAH08-

The 28 base pair linker was digested with NsiI and AscI and ligated into pAH01 digested with NsiI and AscI to create pAH08. This vector contains no promoter but retains $RBS_{lac+7bp}$.

pAH09-

The 28 base pair linker was digested with NsiI and AscI and ligated into pAH03 digested with NsiI and AscI to create pAH09. This vector contains no promoter but retains RBS_{lac}.

pAH10-

The 28 base pair linker was digested with NsiI and AscI and ligated into pAH05 digested with NsiI and AscI to create pAH10. This vector contains no promoter but retains RBS_{II}.

Growth Curves:

Overnight LB cultures from wild type and transformed strains (*E. coli* UQ950 and *S. oneidensis* MR-1) bearing plasmids pAH01-pAH05 and an empty vector control were back diluted into 2 mL SBM and allowed to grow overnight again. These overnight cultures were pelleted for 2 minutes at 8,000 rpm, washed once with SBM and resuspended in SBM. The suspension was back diluted into sterile 48-well plates filled with either 500 µL LB or SBM to achieve an OD600 of ~0.005. Plates were shaken at 30^{o}C and absorbance at 600 nM read on a SpectraMax5.1. For anaerobic *S. oneidensis* investigations, this resuspension was back diluted into anaerobic Balch tubes containing 10 mL SBM. Tubes were incubated at 30^{o}C and OD_{600} read on a Thermo Electron Corp Spectronic 20Dx spectrophotometer. Media was supplemented with 50 µg/mL Km for plasmid bearing strains. All growth curves were performed with biological triplicates.

Miller Assays:

Overnight cultures from wild type and transformed strains (*E. coli* and *S. oneidensis*) bearing plasmids pAH01-pAH05 and pAH07 were spun down and the cell pellets washed twice in 1 mL LB twice. Pellets were resuspended in 1 mL LB. For aerobic investigations in *E. coli* and *S. oneidensis*, the washed and resuspended culture was back diluted into 2

mL of either LB or SBM to achieve an OD_{600} of ~0.005-0.01. For anaerobic investigations in *S. oneidensis*, this resuspension was back diluted into anaerobic Balch tubes containing 10mL SBM. Media was supplemented with 50 µg/mL Km for plasmid bearing strains. All β-galactosidase assays were performed with cultures from biological triplicates and performed two or three separate times. OD_{600} absorbance was recorded from a Spectronic 20Dx spectrophotometer for aerobic cultures and a Genesys 10uv spectrophotometer for anaerobic tubes, both made by Thermo Electron Corporation.

β-galactosidase assays were performed according to Miller's 1992 Cold Spring Harbor Protocol (Miller, 1992) with some modifications. 100 µL of *S. oneidensis* and *E. coli* cultures was harvested in mid log phase (OD_{600} between 0.300-0.400, precise readings recorded) and added to centrifuge tubes containing 3.5 µL β-mercaptoethanol, 10 µL chloroform, 10 µL sodium dodecyl sulfate (SDS), and 900 µL Z-buffer (60 mM $Na_2HPO_4{\cdot}7H_20$, 40 mM $NaH_2PO_4{\cdot}H_20$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_20$) and then vortexed. 200 µL of colorless 4 mg/mL ortho-nitrophenylβ-galactoside (ONPG) is added to begin the reaction. The reaction is stopped when tubes become visibly yellow by adding 250 µL 1M $NaCO_3$ and vortexing. The time taken to achieve the color change is recorded. Permeabilized cells are then pelleted for 4 minutes at 8,000 rpm and 300 µl of the supernatant transferred to a clear 96 well plate. The absorbance of the supernatant at 420 nm was determined by a SpectraMax 5.1 plate reader. A cell free blank (using 100 µL of the media in which cells were tested – either LB or SBM/lactate/fumarate) was run through the same procedure and the absorbance reading of the blank subtracted from the sample readings.

The equation used to determine Miller Units is as follows:

$$MU = 1000 * \left(\frac{Abs420}{t * v * Abs600}\right)$$

Where *Abs420* measures the color generated by cleavage of ONPG, *t* is time in minutes, *v* is volume of cell culture in milliliters, and *Abs600* is the optical density of the culture.

Heterostagger PCR of *lacZ*:

lacZ was amplified via heterostagger PCR from *E. coli* MG1655. Two ~3,075 base pair products were generated with AH26F/28R and AH27F/28R, each with a KpnI restriction site just after the stop codon. The products were column cleaned, mixed together in equal proportions in 10 mM Tris buffer and denatured on a heat block set to 93^{o}C for 5 minutes. The heat block was removed and allowed to cool on the bench top to room temperature. After reannealing, PCR products were digested with KpnI, separated by gel electrophoresis and those bands excised and cleaned. This fragment was cloned into each of the six expression vectors digested with NdeI and KpnI. All constructs containing *lacZ* were Sanger sequenced by the University of Minnesota Genomics Center to confirm that no mutations occurred in the sequence of the gene, promoter, or RBS.

Results and Discussion:

Vector Impact on Growth:

E. coli and *S. oneidensis* MR-1 cultures bearing empty plasmids pAH01-pAH06 showed no growth deficiencies compared to wild type strains as demonstrated by the empty vector control in Figures 2.5-2.8. In *E. coli*, all but one of the strains expressing the reporter gene grew slightly more slowly than wild type and the empty vector control. The strain containing pAH01::*lacZ* grew at a rate similar to wild type. In *S. oneidensis* MR-1, strains carrying plasmids expressing low levels of the reporter gene (pAH01::*lacZ*, pAH02::*lacZ*, and pAH03::*lacZ)* also grew at rates similar to wild-type and empty vector control strains. Strains bearing plasmids resulting in high levels of *lacZ* expression (pAH04::*lacZ* and pAH05::*lacZ*) grew more slowly in all conditions but achieved final optical densities similar to wild type strains. The burden caused by overexpression of *lacZ* was more pronounced in minimal media and anaerobically (Figures 2.7 and 2.8). Note the absence of pAH06::*lacZ* strains in growth curves. The extremely high level of activity from this promoter/RBS combination resulted in mutations in the promoter regions in sequenced plasmids and was therefore unstable for use in expression assays (data not shown).

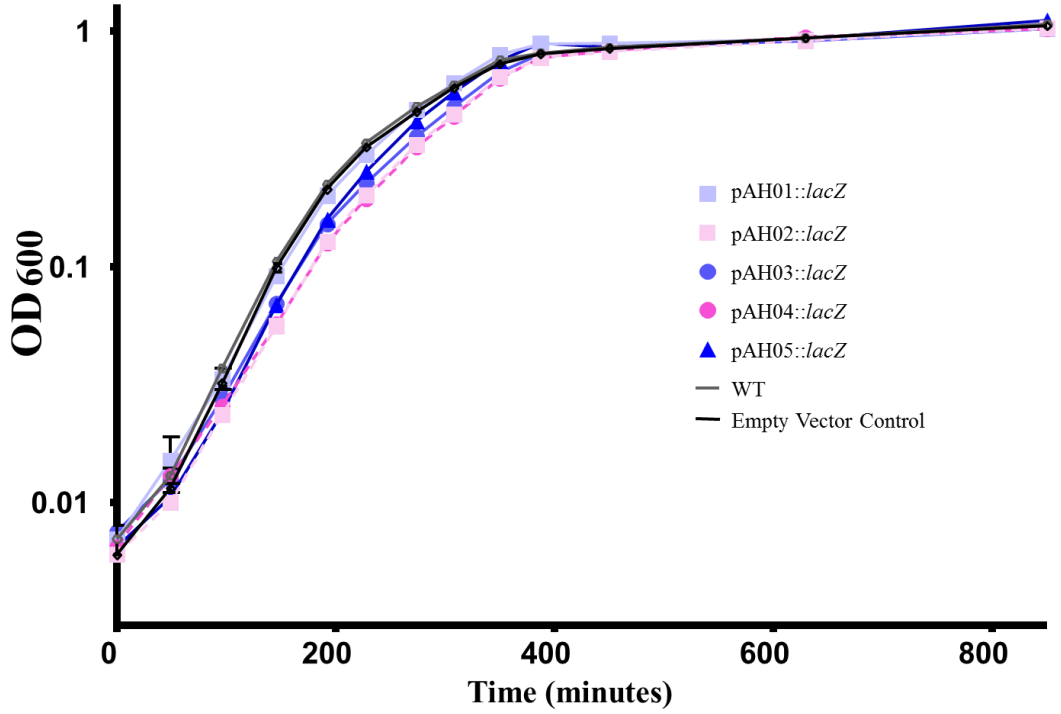

Figure 2.5 – *Escherichia coli* UQ950 growth curve in LB aerobically
E. coli UQ950 (WT) is shown by the solid black line; Empty vector control is shown by the solid grey line. Transformed UQ950 strains carrying vectors pAH01-pAH05 are shown in color - plasmids with the P_{lac} promoter are shades of blue (solid lines) and plasmids with the $P_{A1/O4/O3}$ promoter are shown in shades of red (dashed lines). Symbol shape represents the RBS on the plasmids – square = $RBS_{lac+7bp}$; circle = RBS_{lac}; triangle = RBS_{II}.

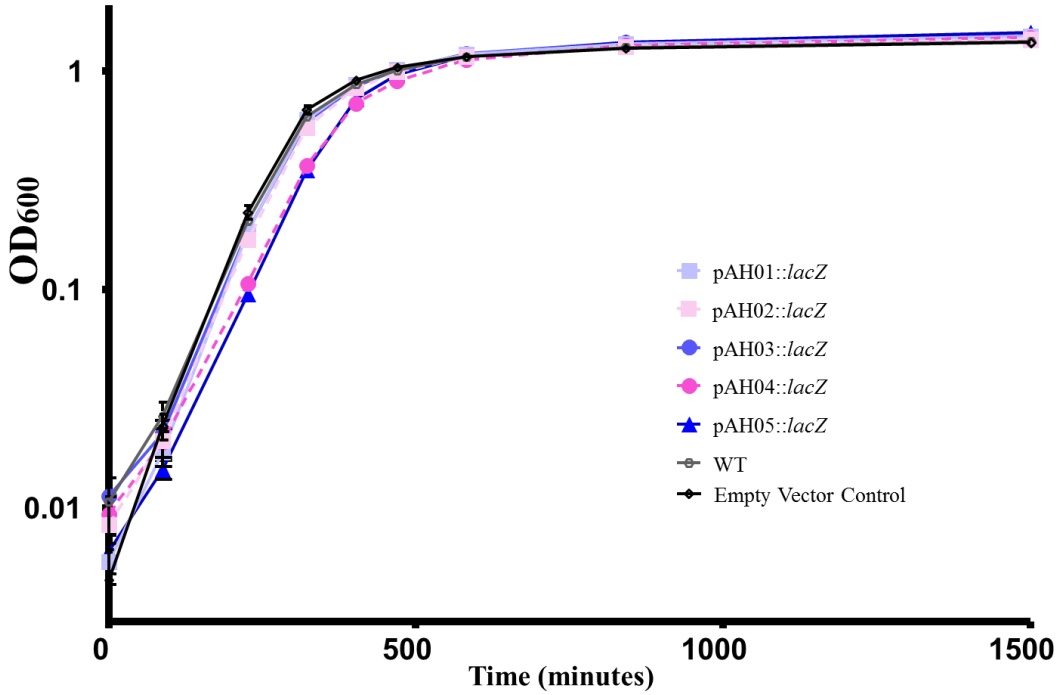

Figure 2.6 – *Shewanella oneidensis* MR-1 growth curve in LB aerobically

S. oneidensis MR-1 (WT) is shown by the solid black line; Empty vector control is shown by the solid grey line. Transformed MR-1 strains carrying vectors pAH01-pAH05 are shown in color - plasmids with the P_{lac} promoter are shades of blue (solid lines) and plasmids with the $P_{A1/O4/O3}$ promoter are shown in shades of red (dashed lines). Symbol shape represents the RBS on the plasmids – square = $RBS_{lac+7bp}$; circle = RBS_{lac}; triangle = RBS_{II}.

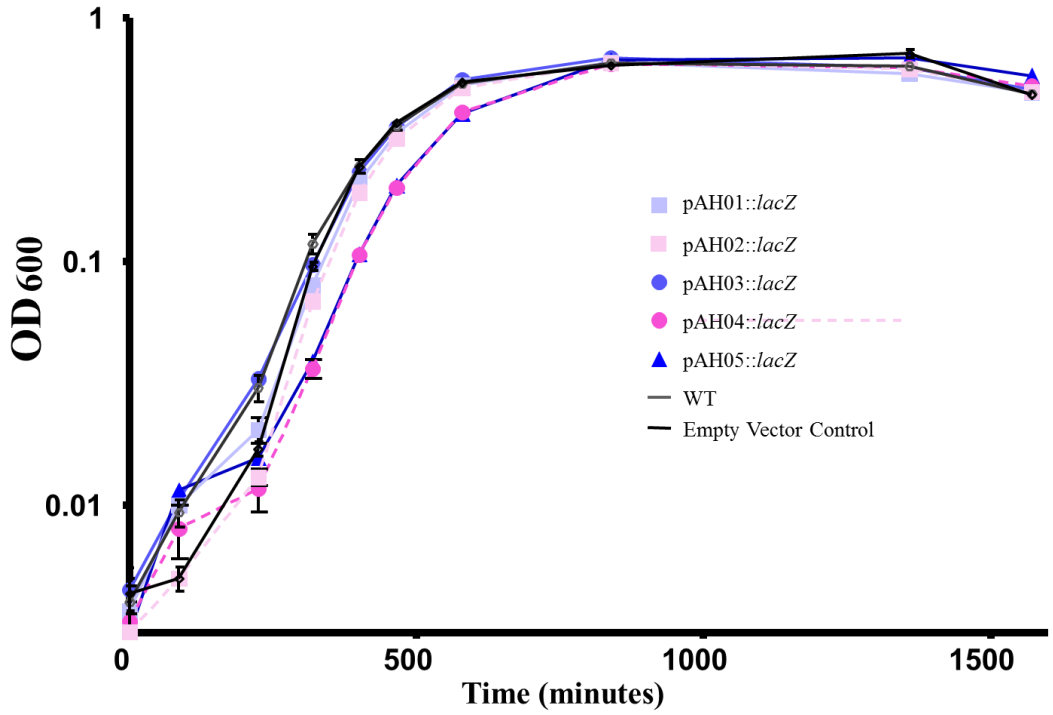

Figure 2.7 – *Shewanella oneidensis* MR-1 growth curve in SBM + 20 mM lactate/40 mM fumarate aerobically

S. oneidensis MR-1 (WT) is shown by the solid black line; Empty vector control is shown by the solid grey line. Transformed MR-1 strains carrying vectors pAH01-pAH05 are shown in color - plasmids with the P_{lac} promoter are shades of blue (solid lines) and plasmids with the $P_{A1/O4/O3}$ promoter are shown in shades of red (dashed lines). Symbol shape represents the RBS on the plasmids – square = $RBS_{lac+7bp}$; circle = RBS_{lac}; triangle = RBS_{II}.

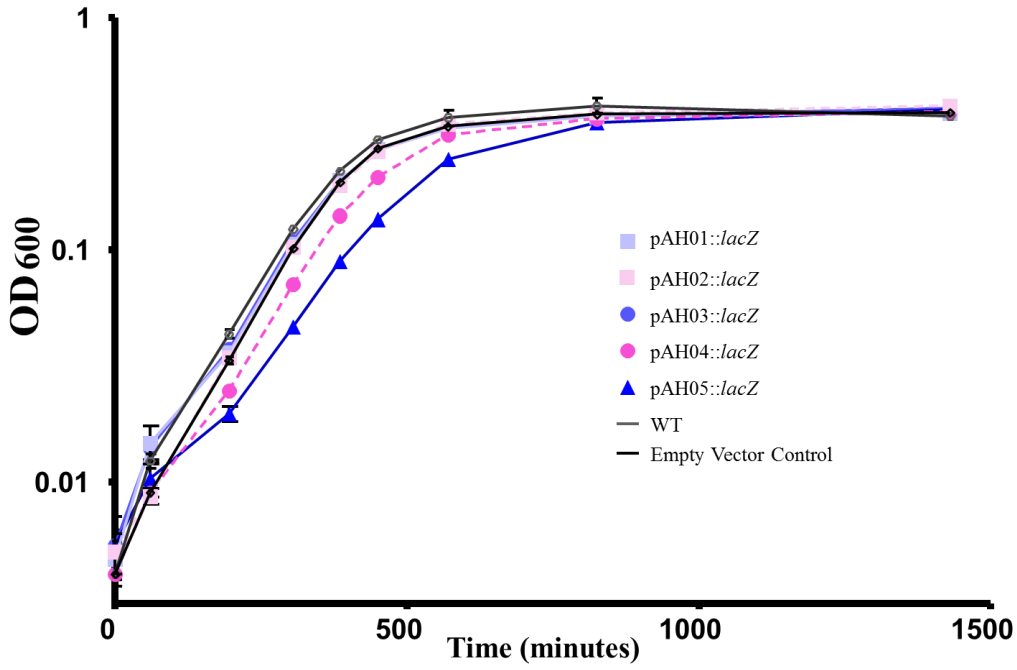

Figure 2.8 – *Shewanella oneidensis* MR-1 growth curve in SBM + 20 mM lactate + 40 mM fumarate anaerobically

S. oneidensis MR-1 (WT) is shown by the solid black line; Empty vector control is shown by the solid grey line. Transformed MR-1 strains carrying vectors pAH01-pAH05 are shown in color - plasmids with the P_{lac} promoter are shades of blue (solid lines) and plasmids with the $P_{A1/O4/O3}$ promoter are shown in shades of red (dashed lines). Symbol shape represents the RBS on the plasmids – square = $RBS_{lac+7bp}$; circle = RBS_{lac}; triangle = RBS_{II}.

Determination of Protein Production Levels:

In order to get an accurate estimate of the strength of the promoter and RBS combinations, the full length, 3,075 base pair sequence of *lacZ* from *E. coli* MG1655 was inserted into the multiple cloning site (MCS) of plasmids pBBR1-AH1 through pBBR1-AH6 for use as a reporter gene as explained previously. Miller assays were conducted to determine the level of β-galactosidase resulting from the plasmids under different growth conditions in *Shewanella oneidensis* MR-1. Expression levels of mid log growth cultures were compared in complex versus minimal media and aerobically versus anaerobically (with lactate as a carbon source and fumarate as the terminal electron acceptor). These conditions are commonly used in labs studying *Shewanella* and so will be the most predictive for future applications. Also, the suite of expression vectors was tested in *E. coli* for comparison to extensive literature on protein production in response to these promoter and RBS segments.

There are many ways to evaluate both transcript and protein levels, but for this study, Miller units have been used as convenient units to describe protein activity normalized to culture OD. These measurements are useful for our purposes as they allow us to compare plasmid performance at a very sensitive level across a wide range of activity. Due to the differences in host strains, growth conditions, plasmid copy number, media, temperature and spectrophotometers, the Miller Unit measurement can vary between labs. Miller reports a fully induced, single copy native *lac* promoter yields around 1,000 MUs and an uninduced culture at 1 MU (Miller, 1992).

Results from Miller assays from cultures under different conditions are shown in Table 2.3. The combinations of promoters and ribosome binding sites resulted in about a 10 fold range of β-galactosidase being produced, ranging from the lowest (pAH01) to the highest (pAH05). The expression levels from plasmids with the A1/O4/O3 promoter (pAH02 and pAH04) are higher than those from plasmids with the *lac* promoter (pAH01, pAH03 and pAH05) when combined with the same RBS. Miller units increase as the RBS spacing becomes more optimal with $RBS_{lac+7bp}$ leading to the lowest expression and

RBS_{II} leading to the highest expression. The expression levels of all plasmids are higher when cultures are grown in minimal medium. Plasmids with the *lac* promoter have higher Miller units when cultures are grown anaerobically, possibly due to the different conformation of CRP binding sites present in the promoter regions. *S. oneidensis* has been shown to elevate expression of genes activated by CRP binding when grown anaerobically (as opposed to *E.coli* strains that use CRP to activate alternate catabolism pathways) (Saffarini et al., 2003). Plasmids with the A1/O4/O3 promoter produce slightly less β-galactosidase anaerobically.

Table 2.3 – β-galactosidase assay results

Numbers shown are Miller Units +/- standard error from at least two experiments on biological triplicates. Vectors with different promoter/RBS combinations are listed in rows and the host and growth conditions are listed in columns. Light to dark shading of each column represents strength of vectors in specified host and growth conditions.

	E. coli **UQ950**	***S. oneidensis*** **MR-1**	***S. oneidensis*** **MR-1**	***S. oneidensis*** **MR-1**	
	Aerobic –LB	**Aerobic – LB**	**Aerobic – SBM**	**Anaerobic - SBM**	
pAH01::*lacZ* (P_{lac}+$RBS_{lac+7bp}$)	**189 +/- 9**	**357 +/- 21**	**742 +/- 90**	**1313 +/- 136**	**Low**
pAH02::*lacZ* ($P_{A1/O4/O3}$+$RBS_{lac+7bp}$)	**1015 +/- 23**	**992 +/- 24**	**2121 +/- 77**	**2011 +/- 84**	
pAH03::*lacZ* (P_{lac}+RBS_{lac})	**306 +/- 26**	**508 +/- 100**	**1658 +/- 109**	**2595 +/- 176**	
pAH04::*lacZ* ($P_{A1/O4/O3}$+RBS_{lac})	**1467 +/- 28**	**1814 +/- 108**	**4629 +/- 421**	**4025 +/- 370**	
pAH05::*lacZ* (P_{lac}+RBS_{II})	**2346 +/- 40**	**3497 +/- 192**	**6660 +/- 425**	**8497 +/- 662**	**High**

Conclusion:

The results presented in this chapter show that indeed the promoters and ribosome binding sites chosen have dramatic and consistent impacts on the expression level of *lacZ* and that those pieces behave in a similar fashion in both *E. coli* and *Shewanella*. We have learned that fine tuning of expression is possible by manipulation of either transcription or translation and have the confidence that almost any level of expression is attainable by swapping out the modular parts of this system. In future lab studies, any promoter or RBS could be amplified to include proper restriction sites and easily inserted into expression vectors. We have explored the upper limit of expression by failing to construct a plasmid driving *lacZ* expression with the strongest promoter and RBS combination (pAH06), demonstrating that the metabolic burden incurred by having this strong region in multiple copies in the cell is too costly to be tenable. After gaining confidence that these expression vectors lead to specific levels of reporter protein production, the next step is to make these vectors more versatile and controllable. This is shown in the next chapter with the addition of repressor protein, making the expression vectors inducible. Therefore, expression at levels determined in this chapter could be turned on by adding an induction signal to any cells bearing these plasmids regardless of host background.

Chapter 3
Controlling Expression Vectors Using Repressor Protein LacI

Introduction:

The primary goal of this project is to understand and control plasmid-based gene expression in *S. oneidensis*. In the previous chapter, a suite of expression vectors (pAH01 through pAH06) were presented that result in a range of protein production levels depending on the promoter and ribosome binding site. Although these vectors will themselves be useful, utility may be improved by having gene expression inhibited during normal cell growth and then turned on at a desired point. For example, when producing a product that is harmful to the bacteria, cultures are typically grown to a high cell density before the toxic product is made. *Shewanella* and other dissimilatory metal reducing bacteria are often found associated with surfaces or in biofilms. For potential biotreatment applications, it may be beneficial to allow cells to attach to a surface prior to inducing expression of genes to remediate contaminated areas. Or for electrode studies in the laboratory, cells can attach to the electrode prior to turning on pathways that may affect electron transport processes. The subject of this chapter is the creation of plasmids and a *S. oneidensis* host strain that will allow plasmid based expression to be turned on and off.

Molecular biologists have exploited the extensive knowledge of *E. coli* metabolism in order to control other bacteria in the lab. As explained in the previous chapter, lactose catabolism is accomplished in *E. coli* by turning on gene expression from the *lac* operon. Jacob and Monod discovered that this gene expression was dependent on both the absence of glucose and the presence of lactose. After extensive mutational analysis, they determined that the operon is negatively regulated by the repressor protein LacI binding to the operator region in the promoter upstream of *lacZYA*. This negative regulation

allows *E. coli* strains to avoid wastefully producing high levels of lactose catabolism enzymes when that carbon source is not available. *lacI* is constitutively expressed by a weak promoter in *E. coli*, resulting in only about 5-10 LacI proteins being made per cell per generation. Muller-Hill and Gilbert identified mutants displaying strong repression phenotypes and examined them to reveal two common mutations in the *lacI* promoter region that lead to an overproduction of LacI (Muller-Hill et al.,1968). The first, referred to as $lacI^{q}$, is a single transition mutation (C>T) in the -35 region of the *lacI* promoter which causes approximately 10 times more LacI production than in wild type *E. coli*. The other ($lacI^{qs}$) mutant is a 15 base pair deletion which creates a new and much stronger -35 region resulting in protein production levels around 170 times higher than wild-type.

When *E. coli* is undergoing the shift from growing on glucose to growing on lactose, a signal must be transmitted inactivating the repressor protein. Due to the poor repression by LacI, low levels of the enzymes transcribed by the *lac* operon are always present and β-galactosidase (LacZ) is able to transglycosylate incoming lactose to form allolactose. It is allolactose that binds to the LacI protein, causing a conformational change which impairs the ability of the protein to bind DNA. LacI then releases the operator sequence and allows active transcription of the genes necessary for lactose catabolism. This repressor protein and repressor protein deactivator system has been utilized to control protein production in the laboratory. Instead of providing cultures lactose, isopropyl β-D-1-thiogalactopyranoside (IPTG) is used as a non-metabolizable analogue. IPTG binds LacI, mimicking the allosteric effect caused by LacI binding to allolactose. IPTG is a common choice in molecular biology as it is not toxic to cells, inexpensive, and levels will remain constant throughout the experiment. Addition of IPTG results in almost immediate transcription increase from the now unrepressed *lac* operon and is referred to as induction.

LacI will function as a repressor not only for the *lac* promoter used in the plasmids pAH01, pAH03, and pAH05 but also for the A1/O4/O3 promoter used in plasmids pAH02, pAH04 and pAH06. The *lac* promoter has two native LacI operator sites, one

close to the transcription start site and one approximately 82 base pairs upstream. LacI binds the operator sequence as a dimer and if two operator sites are occupied by LacI dimers, those dimers can bind to form a tetramer. This causes DNA looping which contributes to the repression of the *lac* operon. $P_{A1/O4/O3}$ also has two LacI operator sequences, one in the same position as *E. coli*'s native transcription start site operator and the other approximately 22 base pairs upstream, located between the two conserved hexamers. There must be sufficient LacI repressor protein to bind the operator sequences present in the promoter regions of P_{lac} and $P_{A1/O4/O3}$ to achieve complete repression. Since the copy numbers of pBBR1-MCS2 and its derivatives are assumed to be similar to *E. coli* at around 20-50 plasmids per cell (Kovach et al., 1995), the $lacI^q$ promoter was chosen to ensure that there is stochastically abundant repressor protein available to block transcription from each plasmid promoter. A high ratio of LacI protein to operator sequence should decrease any undesirable pre-induction expression of the *lacZ* gene from the expression plasmids (Glascock and Weickert, 1998).

Strategies for $lacI^q$ Insertion:

This project involved two strategies of *lacZ* repression from the expression vectors. The first was inserting the promoter and coding sequence for $lacI^q$ onto the backbone of the vectors described in the previous chapter, creating vectors pAH11 through pAH16 (see Figure 3.1). This ensures ample repressor protein as every plasmid will be producing LacI in vast stoichiometric excess of what is needed to block transcription from each operator. Forward orientation of the $lacI^q$ gene was desirable to avoid read-through that could potentially interfere with the *mob* genes running in the opposite direction caused by the very strong $lacI^q$ promoter. $LacI^q$ was separated from the modular promoter/RBS region by ~1350 base pairs, which includes the Km^R open reading frame, in order to decrease the likelihood that strong transcription of $lacI^q$ would affect expression levels of *lacZ* established in the previous chapter. Addition of $lacI^q$ onto the plasmid backbone enables these vectors to be inducible regardless of host background.

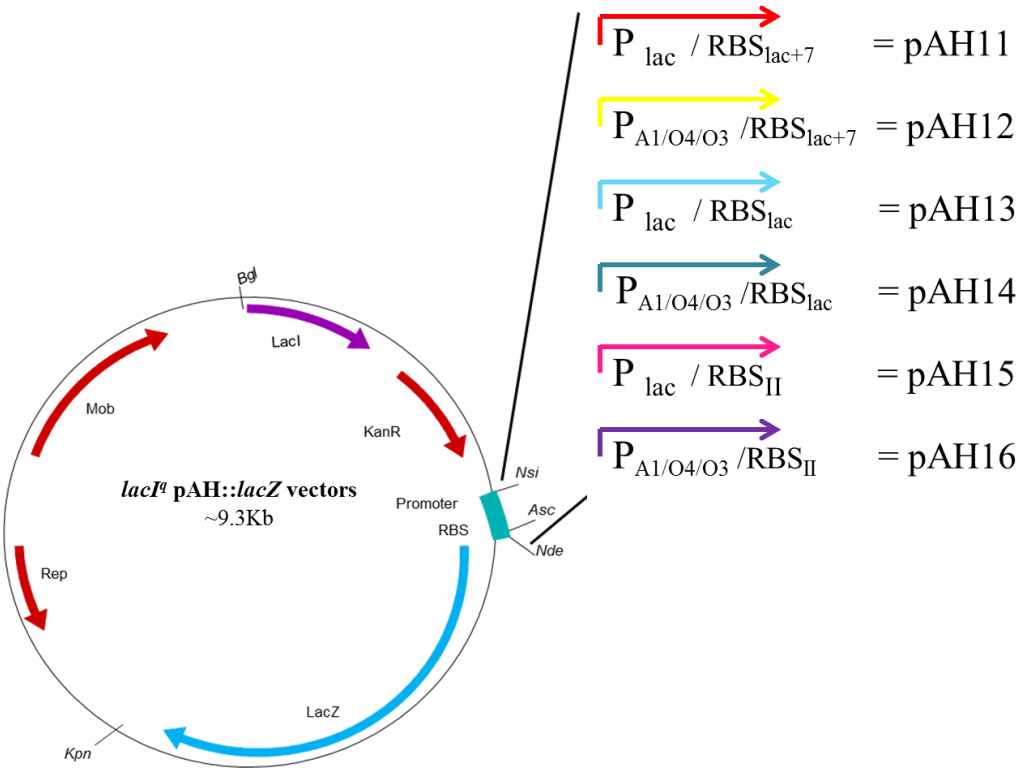

Figure 3.1 – Inducible expression vectors with *lacI*^{q}

The *lacI*^{q} promoter and coding sequence from *E. coli* was inserted into the backbone of pAH01 through pAH06 to create pAH11 through pAH16, respectively. These six new expression vectors drive variable strength inducible/repressible expression of *lacZ*.

The second strategy was inserting the promoter and coding sequence of *lacI*^{q} into the genome of *S. oneidensis* MR-1 via homologous recombination (see Figure 3.2). The resulting strain, JG2929 was transformed with the expression vectors pAH01 through pAH06. The LacI protein coded for in the genome travels to repress the P_{lac} and $P_{A1/O4/O3}$ promoters found on the plasmids. Chromosomal insertion was accomplished using a pSMV3 based addition vector constructed by Aunica Kane using the same design concept as described by Teal (Teal et al., 2006). pSMV3 is a suicide plasmid containing both a kanamycin resistance gene as well as a gene that catalyzes the lethal polymerization of sucrose (*sacB*) (Sukovich et al., 2010). *S. oneidensis* lacks the *pir* genes which enable pSMV3 replication and therefore the resistance coded on the plasmid must be integrated in the host genome in order for the host cells to survive under Km selection. Also on the

plasmid is an upstream region homologous to the end of the *S. oneidensis* glutamine synthetase gene (*glmS*) which is separated by a SpeI recognition site from a downstream region homologous to the intergenic region following *glmS*. This *glmS* intergenic region has been shown to be a Tn7 transposon attachment site, allowing insertions without undesirable pleiotropic effects (Teal et al., 2006; Waddell & Craig, 1989). The gene intended for scar-free integration (in this case $lacI^q$) is inserted into the vector SpeI site. The first recombination event inserts the entire suicide plasmid and SpeI inserted gene just after *glmS*. Cells that have undergone a single recombination are selected by plating on LB + Km. Overnight growth in LB and plating on LB + 5% sucrose medium allows selection for a second recombination event occurring between the upstream and downstream regions which excises the suicide plasmid sequence, leaves the SpeI inserted gene, and results in colonies that have lost Km resistance and can grow in the presence of sucrose. Since the genomic insertion results in a single copy of $lacI^q$ per chromosome responsible for blocking all transcription from the multi-copy expression vectors, there may be some operator regions not bound by LacI and therefore available for transcription prior to induction.

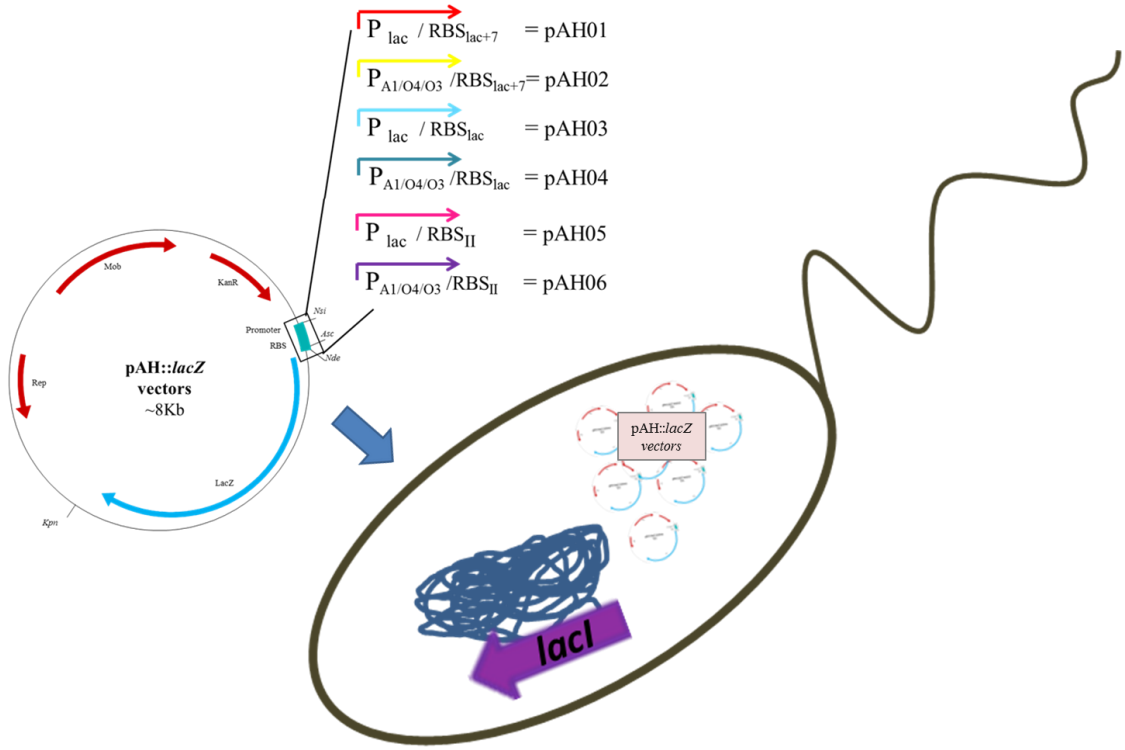

Figure 3.2 – Inducible expression via genomic insertion of *lacI*^{q}

The *lacI*^{q} promoter and coding sequence from *E. coli* (represented by purple arrow) was inserted into the genome of *S. oneidensis* MR-1 in order to repress vector expression of *lacZ*. The single copy genomic insertion must produce enough repressor protein to occupy each LacI binding site on the promoters of the multi-copy plasmids. Plasmids pAH01 through pAH06 were used to drive production of LacZ for β-galactosidase assays in the *S. oneidensis lacI*^{q} host strain.

S. oneidensis MR-1 transformed with *lacI*^{q} expression vectors pAH11 through pAH16 and *S. oneidensis* JG2929 transformed with expression vectors pAH01 through pAH06 were evaluated and characterized. The growth of these isolates and their protein production levels with and without IPTG induction were assessed under relevant laboratory conditions. Construction and evaluation of strains and plasmids will be explained in the rest of the chapter.

Materials and Methods:

Culture conditions:

Strains and plasmids used in this study are listed in Table 3.1. *E. coli* strains were streaked from 15% glycerol freezer vial stocks onto Luria-Bertani (LB) plates and single colonies picked for inoculation into 2 mL LB, supplemented with 50 µg/mL kanamycin (Km) for plasmid bearing strains. Cultures were grown overnight (~14 hours) at 37^{o} C with constant shaking at 225 rpm. *Shewanella* strains were grown in the same way at 30^{o} C. For growth in minimal media, *Shewanella* Basal Media (SBM) was used as described previously (Hau et al., 2008) with 0.05% casamino acids, 20 mM sodium lactate, and 40 mM sodium fumarate added.

Table 3.1 – Strains and plasmids used in this study

E. coli strains:	**Features:**	**Reference/ source:**
UQ950	DH5α λ(*pir*); F-Δ(*argF-lac*)169 Φ80d*lacZ*58(ΔM15) *glnV*44(AS) *rfbD*1 *gyrA*96(NalR) *recA*1 *endA*1 *spoT*1 *thi*-1 *hsdR*17 *deoR* λpir+; Used for cloning, plasmid preps and expression assays	D. Lies, Caltech/ Saltikov & Newman, 2003
WM3064	*thrB*1004 *pro thi rpsL hdsS lacZ* ΔM15 RP4--1360 Δ(*araBAD*)567 Δ*dapA* 1341::[*erm pir(* wt)]; Donor strain for conjugation	W. Metcalf, Univ of Illinois, Urbana
BL21 (DE3)	F^{-} *ompT gal dcm lon hsdS*$_B$($r_B^- m_B^-$) λ(DE3 [*lacI*^{q} *lac*UV5-T7 gene 1 *ind1 sam7 nin5*]); Source of *lacI*^{q} promoter and coding sequence	A. Khodursky, U of MN
Shewanella oneidensis strains:		
MR-1	Wild type strain (JG274) ; Used as host strain for expression assays	Nealson, et al., 1999
JG2929	JG274 with chromosomal insertion of *lacI*^{q} at *glmS* addition site	This study
Plasmids:		
pBBR1-MCS2	5.1-kb broad range host plasmid, Km^r, lacZα; Backbone of pAH vectors	Kovach, 1999
pucBB:*lacZ*	Source of full length *lacZ* from *E. coli* MG1655	D. Bond, U of MN
pSMV3::$p_{A1/O4/O3}$ -*gfp* mut3*	Source of $p_{A1/O4/O3}$ and RBS_{II}	J. Gralnick, U of MN/ Teal, 2006
pAH01	5.1-kb broad-range expression vector, Km^r, pro_{lac} + $RBS_{lac+7bp}$	This study
pAH02	5.1-kb broad-range expression vector, Km^r, $pro_{A1/O4/O3}$ + $RBS_{lac+7bp}$	This study
pAH03	5.1-kb broad-range expression vector, Km^r, pro_{lac} + RBS_{lac}	This study
pAH04	5.1-kb broad-range expression vector, Km^r, $p_{A1/O4/O3}$ + RBS_{lac}	This study
pAH05	5.1-kb broad-range expression vector, Km^r, pro_{lac} + RBS_{II}	This study
pAH06	5.1-kb broad-range expression vector, Km^r, $pro_{A1/O4/O3}$ + RBS_{II}	This study
pAH11	6.3-kb broad-range expression vector, *laclq* , Km^r, pro_{lac} + $RBS_{lac+7bp}$	This study
pAH12	6.3-kb broad-range expression vector, laclq, Km^r, $pro_{A1/O4/O3}$ + $RBS_{lac+7bp}$	This study
pAH13	6.3-kb broad-range expression vector, *laclq* , Km^r, pro_{lac} + RBS_{lac}	This study
pAH14	6.3-kb broad-range expression vector, *laclq* , Km^r, $p_{A1/O4/O3}$ + RBS_{lac}	This study
pAH15	6.3-kb broad-range expression vector, *laclq* , Km^r, pro_{lac} + RBS_{II}	This study
pAH16	6.3-kb broad-range expression vector, *laclq* , Km^r, $pro_{A1/O4/O3}$ + RBS_{II}	This study

Reagents:

GoTaq Green Mix was purchased from Promega, all restriction enzymes, T4 ligase, and Antarctic phosphatase were purchased from New England Biolabs (NEB) and *pfu* ultra

high fidelity polymerase was purchased from Agilent. The protocols provided by the suppliers were followed. Plasmid isolation, gel purifications, and PCR clean up were performed using Invitrogen's PureLink HiPure Plasmid Miniprep Kit, PureLink Gel Extraction Kit, and PureLink PCR Purification Kit. Digested vectors were concentrated using Zymo Clean and Concentrate Kit. All oligonucleotides and primers were ordered from Integrated DNA Technologies (IDT) and all promoters and RBSs were sequenced by the University of Minnesota Genomics Center (UMGC). Primers used in this study are listed in Table 3.2.

Table 3.2 – Primers/ oligos used in this study

Name:	5' - 3' sequence:	Target:
AH7F	TCAGTCAGGGCGCGCCGTCACGACGTTGTAAAACG	*lac* promoter from pBBR1-MCS2
AH8R	TCAGTCAGGGCGCGCCATATTATACGCAAGGCGACAAG	*lac* promoter from pBBR1-MCS2
AH9F	TCAGTCAGGGCGCGCCTCGCGCATTACCTCACTAAG	A1/O4/O3 promoter from JG1592
AH11R	CAGTCAGGGCGCGCCTGTGAAATTGTTATCCGCTCAC	separate promoters from RBSs
AH16F	CAGTCAGGGCGCGCCCACACATCTAG	RBS_{II}
AH17R	CGCGGTGCCGGTACCCATATGTAATTTCTCC	RBS_{II}
AH19F	CAGTCAGGGCGCGCCCACACAGGAAACACATATGGGTACCACGTCCG	RBS_{lac}
AH20R	CGGACGTGGTACCCATATGTGTTTCCTGTGTGGGCGCGCCCTGACTG	RBS_{lac}
AH21F	CAGTCAGGGCGCGCCCACACAGGAAACACATTCAACATATGGGTACCACGTCCG	$RBS_{lac+7bp}$
AH22R	CGGACGTGGTACCCATATGTTGAATGTGTTTCCTGTGTGGGCGCGCCCTGACTG	$RBS_{lac+7bp}$
AH26F1	TATGACCATGATTACGGATTCACTG	Heterostagger PCR for *lacZ*
AH27F2	TGACCATGATTACGGATTCACTG	Heterostagger PCR for *lacZ*
AH28R	GATACTGAGGTACCTTATTTTTGACACCAGAC	Heterostagger PCR for *lacZ*
AH59F	ATATCATAGATCTGAAGCGGCATGC	$lacI^q$ promoter and coding sequence (adds BglII restriction site)
AH60R	AATCAAAGATCTTCACTGCCCGC	$lacI^q$ promoter and coding sequence (adds BglII restriction site)
AH61F	ATATCATACTAGTGAAGCGGCATGC	$lacI^q$ promoter and coding sequence (adds SpeI restriction site)
AH62R	AATCAAACTAGTTCACTGCCCGC	$lacI^q$ promoter and coding sequence (adds SpeI restriction site)

Construction of *lacI*^{q} Vectors:

LacI^q *insert-*

LacI^q and the 200 base pairs upstream of the gene start site (including the native "q" promoter region) was amplified from *E. coli* BL21(DE3) given to the Gralnick lab by Arkady Khodursky using primers AH59F and AH60R (see table 3.1) to create a ~1200 base pair product flanked by BglII sites on both ends. The expression plasmids described in the previous chapter were digested with BglII, treated with NEB Antarctic Phosphatase, separated by gel electrophoresis, excised and purified to decrease undigested or re-ligated vectors. Correct orientation of *lacI*^{q} was confirmed with PCR using primers AH59F and AH11R.

pAH01-pAH06-

Construction of expression plasmids described in the previous chapter.

pAH11-

The *lacI*^{q} insert was digested with BglII and ligated into BglII digested pAH01 to create pAH11.

pAH12-

The *lacI*^{q} insert was digested with BglII and ligated into BglII digested pAH02 to create pAH12.

pAH13-

The *lacI*^{q} insert was digested with BglII and ligated into BglII digested pAH03 to create pAH13.

pAH14-

The *lacI*^{q} insert was digested with BglII and ligated into BglII digested pAH04 to create pAH14.

pAH15-

The *lacI*^{q} insert was digested with BglII and ligated into BglII digested pAH05 to create pAH15.

pAH16-

The *lacI*^{q} insert was digested with BglII and ligated into BglII digested pAH06 to create pAH16.

Chromosomal Insertion of *lacI*^{q} to *S. oneidensis* MR-1 (Construction of Strain JG2929):

Addition of *lacI*^{q} to the genome of *S. oneidensis* MR-1 was accomplished via two homologous recombination steps as described earlier. The gene and the upstream 200 base pairs were amplified from *E. coli* BL21(DE3) using primers AH61F and AH62R. This produced an approximately 1200 base pair product flanked by SpeI sites on both sides. The *lacI*^{q} PCR product was digested and ligated into the SpeI site of the *glmS* addition suicide vector and transformed into competent *E. coli* WM3064 cells. *S. oneidensis* MR-1 was transformed via conjugation with WM3064 and then struck out onto LB+ Km plates. Kanamycin resistant colonies were picked and grown up in LB

broth overnight, then transferred to fresh LB and grown overnight again. This culture was spread onto LB+ 5% sucrose plates and colonies that were able to grow under these conditions picked and struck back onto LB and LB+ Km plates. Isolates picked from the sucrose plate that are no longer Km resistant were PCR verified and sequenced to confirm proper insertion of *lacI*^{q}, yielding strain JG2929.

Transformation of Strains:

S. oneidensis MR-1 isolates were transformed with plasmids pAH11-pAH16 via conjugation with *E. coli* WM3064. JG2929 isolates were transformed with plasmids pAH01-pAH06 via conjugation with *E. coli* WM3064. LB plates plus diaminopimelic acid (DAP) were cross-struck with donor strain (WM3064) and recipient strain (*S. oneidensis*) and incubated overnight. Overnight growth was streaked out onto new LB+ Km plates. Only *S. oneidensis* cells successfully transformed with plasmids conferring kanamycin resistance can grow as there is no DAP on the plate to support growth of the DAP auxotroph donor strain.

Growth curves:

Overnight cultures from *S. oneidensis* MR-1 wild type and transformed strains bearing plasmids pAH11-pAH16 as well as wild-type and transformed JG2929 strains harboring plasmids pAH01-pAH06 were pelleted, washed once, and resuspended in 1 mL LB. For aerobic growth curves, resuspended cultures were back diluted into 48-well plates filled with either 500 µl LB or SBM+ 20 mM lactate + 40 mM fumarate to achieve an absorbance at 600 nm of ~0.005-0.01. Plates were shaken at 30^{o} C and absorbance read on a SpectraMax5.1. For anaerobic investigations of *S. oneidensis*, this resuspension was back diluted into anaerobic Balch tubes containing 10 mL SBM+20 mM lactate + 40 mM fumarate to achieve an absorbance at 600 nM of ~0.01-0.02. Tubes were incubated at 30^{o} C and absorbance read on a Thermo Electron Corp Spectronic 20D+ spectrophotometer. Media was supplemented with 50 ug/mL Km for plasmid bearing strains. Cultures were induced with 1 mM IPTG during mid-exponential phase. All growth curves were performed with biological triplicates.

Miller Assays and IPTG Induction:

Overnight cultures from *S. oneidensis* wild-type and transformed strains bearing plasmids pAH11-pAH16 as well as wild-type and transformed JG2929 strains harboring plasmids pAH01-pAH06 were spun down and the cell pellets washed twice in 1 mL SBM. Pellets were resuspended in 1 mL SBM. For aerobic investigations, the washed and resuspended culture was back diluted into 2 mL of either LB or SBM + 20 mM lactate + 40 mM fumarate to achieve an OD600 of ~0.005-0.01. For anaerobic investigations, this resuspension was back diluted into anaerobic Balch tubes containing 10 mL SBM+20 mM lactate/40 mM fumarate. All cultures were grown in shaking incubators with 50 µg/ml kanamycin as a selective agent. Cultures were grown to mid-exponential phase before induction with 1 mM IPTG. Cells were harvested for β-galactosidase assays at late log/early stationary phase. All β-galactosidase assays were performed with cultures from biological triplicates with OD_{600} absorbance recorded from a Thermo Electron Corp Genesys 10uv spectrophotometer for aerobic cultures and a Spectronic 20D+ for anaerobic tubes. Miller Assays to quantify β-galactosidase levels were conducted as described in the previous chapter.

Results and Discussion:

Vector Impact on *S. oneidensis* MR-1 Growth Rates:

Aerobic growth in both rich and minimal media as well as anaerobic growth in minimal media was evaluated. As can be seen by Figures 3.3, 3.4, and 3.5, *S. oneidensis* MR-1 cultures show no significant growth impairment when carrying plasmids with $lacI^q$ as compared to wild-type MR-1 whether cells were grown in rich or minimal media and in the presence or absence of oxygen. IPTG induction (and therefore reporter gene expression) has no obvious effect on cell growth, suggesting that the burden caused by $lacI^q$ expression from the plasmid is minimal but that enough LacI is blocking the overexpression of *lacZ* that impacted growth in the previous chapter.

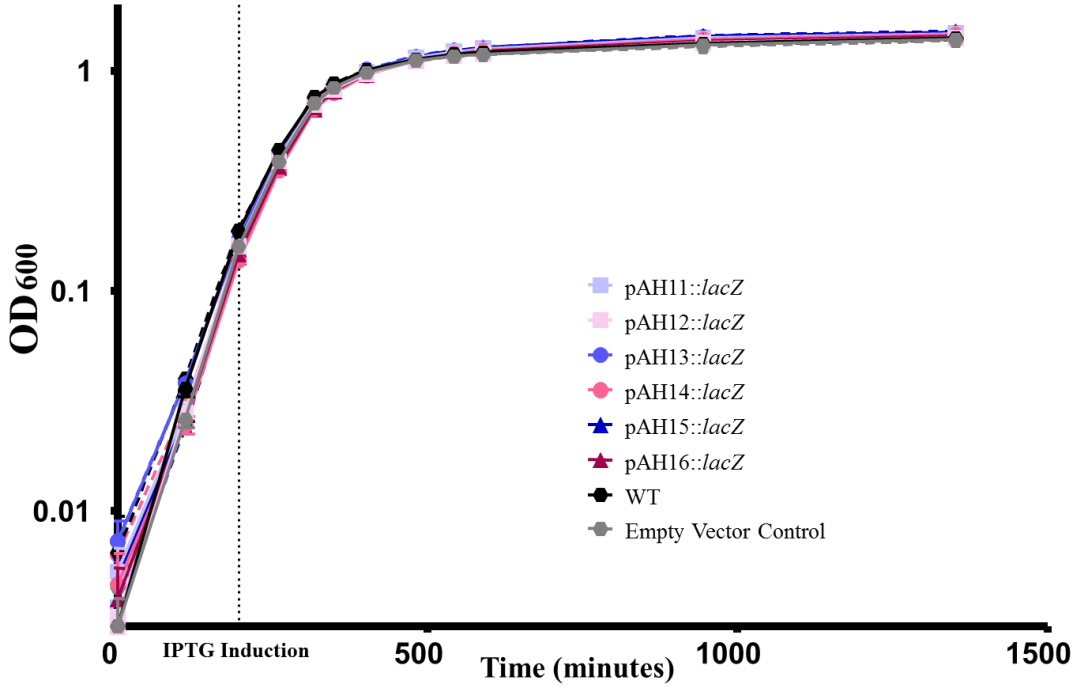

Figure 3.3 – *Shewanella oneidensis* MR-1 growth curve in LB aerobically
Solid lines represent growth of uninduced cultures; dashed lines represent cultures induced with 1mM IPTG at the point indicated by the dotted vertical line. *S. oneidensis* MR-1 (WT) is shown in black; empty vector control is shown in grey. Transformed MR-1 strains carrying vectors pAH11-pAH16::*lacZ* (vectors with *lacI*^{q} on backbone) are shown in color - plasmids with the P_{lac} promoter are shades of blue and plasmids with the $P_{A1/O4/O3}$ promoter are shown in shades of red. Symbol shape represents the ribosome binding site on the plasmids – square = $RBS_{lac+7bp}$; circle = RBS_{lac}; triangle = RBS_{II}.

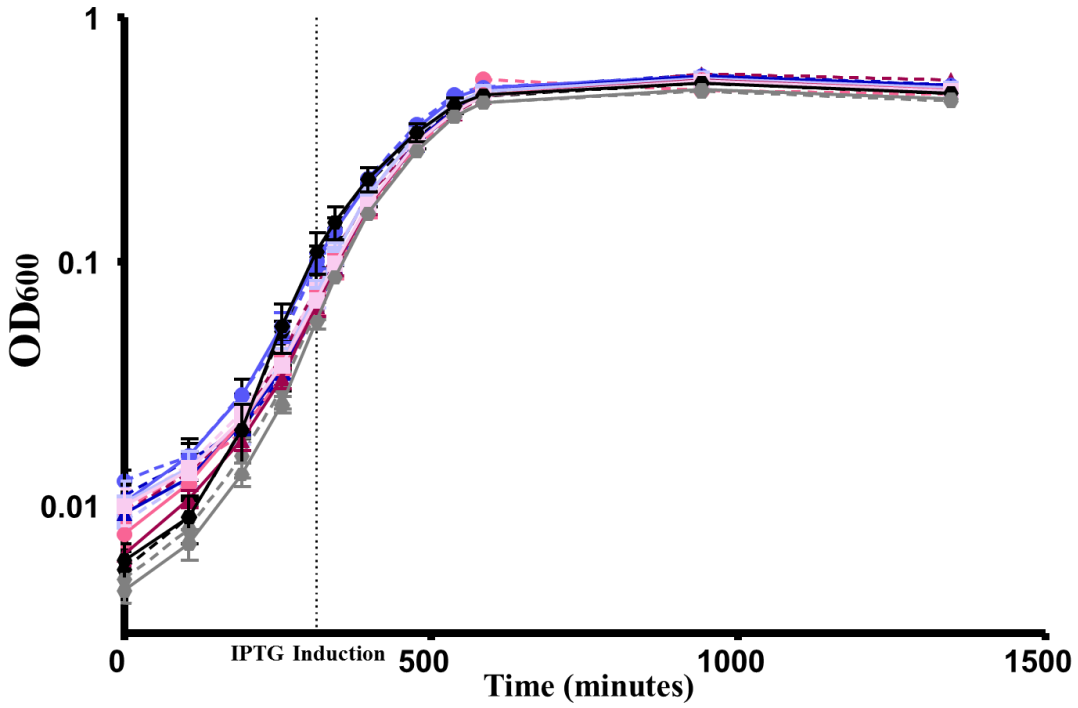

Figure 3.4 – *Shewanella oneidensis* MR-1 growth curve in SBM + 20 mM Lactate + 40 mM fumarate aerobically

Solid lines represent growth of uninduced cultures; dashed lines represent cultures induced with 1mM IPTG at the point indicated by the dotted vertical line. *S. oneidensis* MR-1 (WT) is shown in black; empty vector control is shown in grey. Transformed MR-1 strains carrying vectors pAH11-pAH16::*lacZ* (vectors with $lacI^q$ on backbone) are shown in color - plasmids with the P_{lac} promoter are shades of blue and plasmids with the $P_{A1/O4/O3}$ promoter are shown in shades of red. Symbol shape represents the ribosome binding site on the plasmids – square = $RBS_{lac+7bp}$; circle = RBS_{lac}; triangle = RBS_{II}.

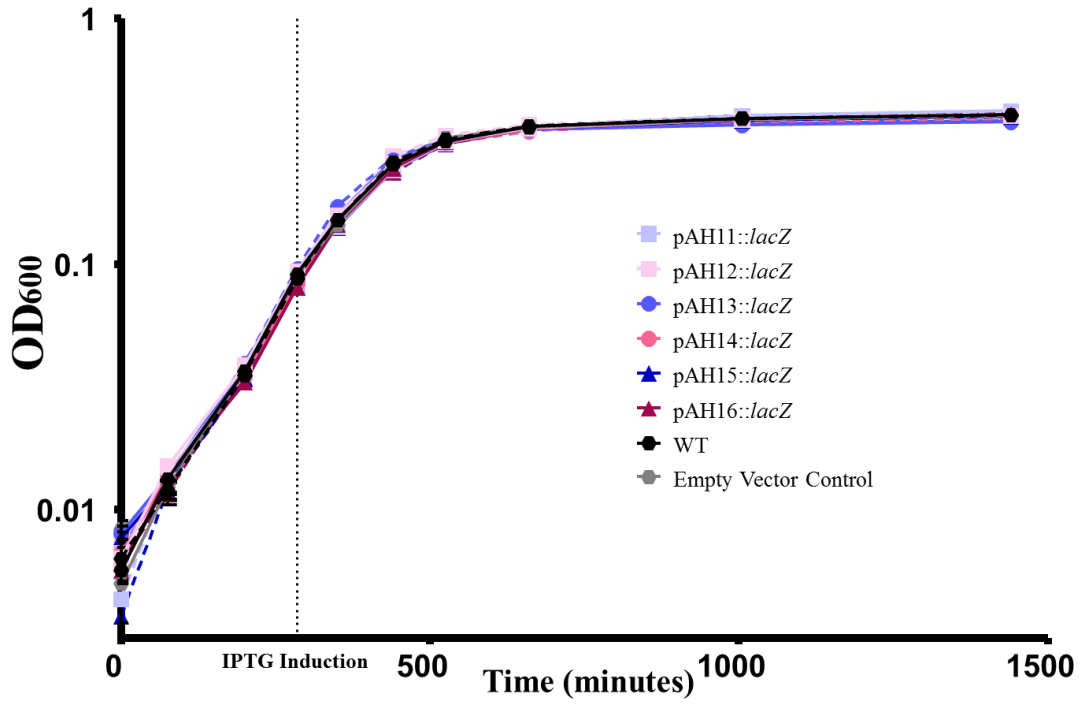

Figure 3.5– *Shewanella oneidensis* MR-1 growth curve in SBM + 20 mM Lactate + 40 mM fumarate anaerobically

Solid lines represent growth of uninduced cultures; dashed lines represent cultures induced with 1mM IPTG at the point indicated by the dotted vertical line. *S. oneidensis* MR-1 (WT) is shown in black; empty vector control is shown in grey. Transformed MR-1 strains carrying vectors pAH11-pAH16::*lacZ* (vectors with *lacI*^{q} on backbone) are shown in color - plasmids with the P_{lac} promoter are shades of blue and plasmids with the $P_{A1/O4/O3}$ promoter are shown in shades of red. Symbol shape represents the ribosome binding site on the plasmids – square = $RBS_{lac+7bp}$; circle = RBS_{lac}; triangle = RBS_{II}.

Vector Impact on *S. oneidensis* JG2929 Growth Rates:

The newly created *S. oneidensis* strain JG2929 (with *lacI*^{q} on the chromosome) grows the same as wild-type MR-1, both in rich and minimal media and aerobically as well as anaerobically (data not shown). In both LB and SBM aerobically, the metabolic load caused by carrying expression plasmids has no negative effect on growth rate in the JG2929 background, even after IPTG induction, again suggesting that even in single copy, the level of LacI is controlling growth impairing expression of *lacZ* (see Figures 3.6 and 3.7). Under anaerobic conditions in SBM, IPTG induction immediately affects the growth of the most highly expressing strain (see pAH06::*lacZ* in Figure 3.8).

pAH06::*lacZ* was unstable and unusable in a wild-type *S. oneidensis* background, but was stable and inducible when JG2929 was used as a host.

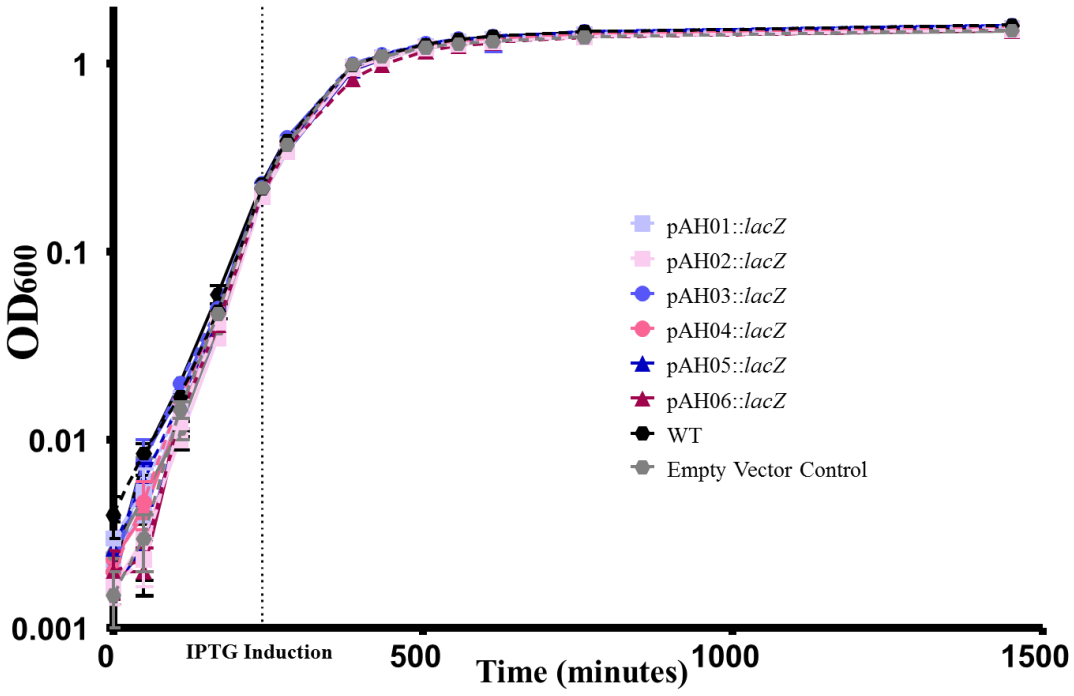

Figure 3.6 – *Shewanella oneidensis* JG2929 growth curve in LB aerobically
Solid lines represent growth of uninduced cultures; dashed lines represent cultures induced with 1mM IPTG at the point indicated by the dotted vertical line. *S. oneidensis* JG2929 (MR-1 with chromosomal *lacI*^{q} insertion) is shown in black; empty vector control is shown in grey. Transformed JG2929 strains carrying vectors pAH01-pAH06::*lacZ* are shown in color - plasmids with the P_{lac} promoter are shades of blue and plasmids with the $P_{A1/O4/O3}$ promoter are shown in shades of red. Symbol shape represents the ribosome binding site on the plasmids – square = $RBS_{lac+7bp}$; circle = RBS_{lac}; triangle = RBS_{II}.

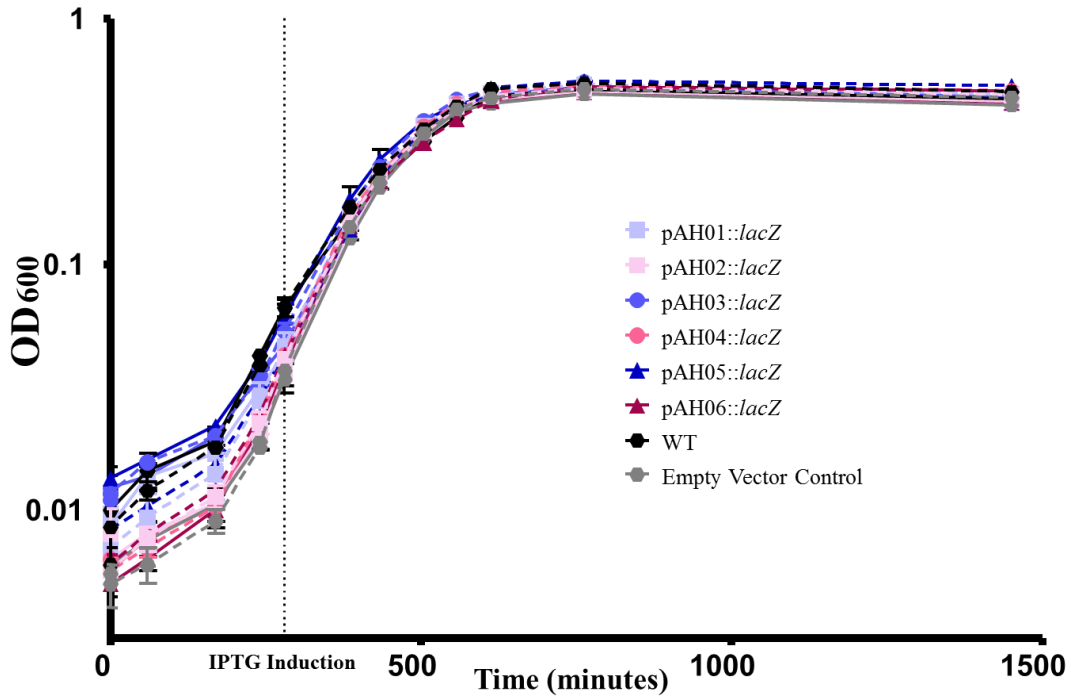

Figure 3.7– *Shewanella oneidensis* JG2929 growth curve in SBM + 20 mM lactate + 40 mM fumarate aerobically

Solid lines represent growth of uninduced cultures; dashed lines represent cultures induced with 1mM IPTG at the point indicated by the dotted vertical line. *S. oneidensis* JG2929 (MR-1 with chromosomal *lacI*^{q} insertion) is shown in black; empty vector control is shown in grey. Transformed JG2929 strains carrying vectors pAH01-pAH06::*lacZ* are shown in color - plasmids with the P_{lac} promoter are shades of blue and plasmids with the $P_{A1/O4/O3}$ promoter are shown in shades of red. Symbol shape represents the ribosome binding site on the plasmids – square = $RBS_{lac+7bp}$; circle = RBS_{lac}; triangle = RBS_{II}.

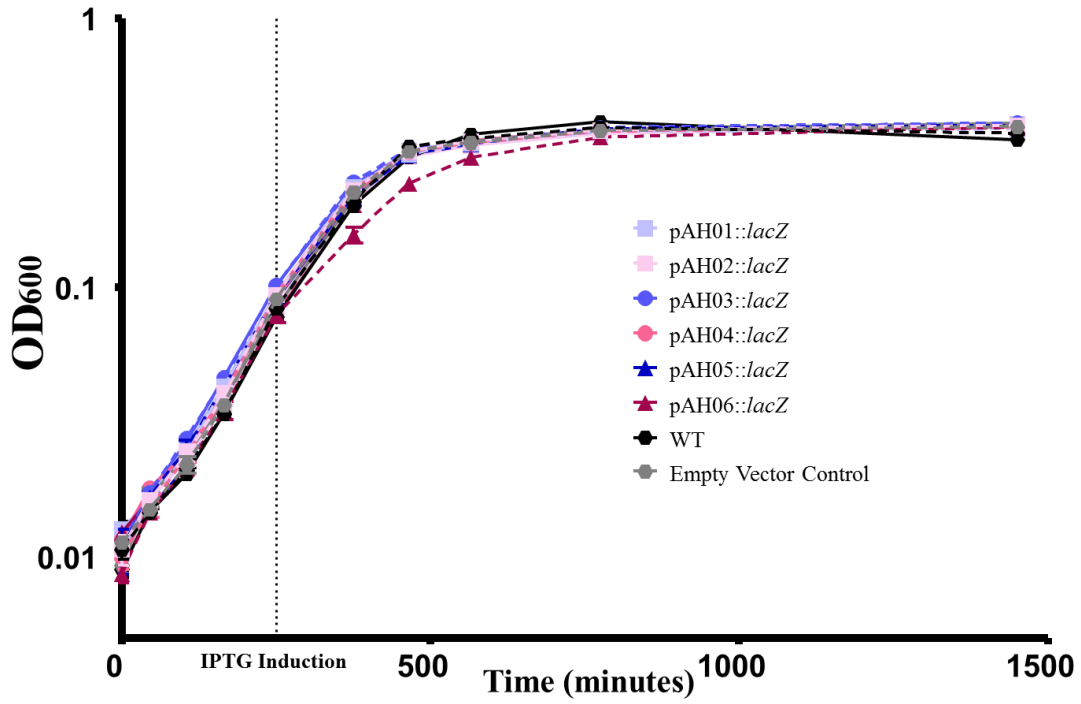

Figure 3.8 – *Shewanella oneidensis* JG2929 growth curve in SBM + 20 mM lactate + 40 mM fumarate anaerobically

Solid lines represent growth of uninduced cultures; dashed lines represent cultures induced with 1mM IPTG at the point indicated by the dotted vertical line. *S. oneidensis* JG2929 (MR-1 with chromosomal *lacI*^{q} insertion) is shown in black; empty vector control is shown in grey. Transformed JG2929 strains carrying vectors pAH01-pAH06::*lacZ* are shown in color - plasmids with the P_{lac} promoter are shades of blue and plasmids with the $P_{A1/O4/O3}$ promoter are shown in shades of red. Symbol shape represents the

Miller Assay Results for *lacI*^{q} vectors in *S. oneidensis* MR-1:

Miller assays were performed on cultures grown aerobically in both rich and minimal media and on cultures grown anaerobically in minimal media. Results from β-galactosidase assays for *S. oneidensis* MR-1 harboring *lacI*^{q} expression vectors pAH11::*lacZ* through pAH16::*lacZ* are shown in Table 3.3. As demonstrated in the previous chapter, promoter and ribosome binding site choice are responsible for the level of reporter gene expression. The LacI repressor system affects the rate of transcription levels but not translation levels, thus stronger RBS segments yield more pre-induction expression when coupled with the same promoter.

Table 3.3 – β-galactosidase assay results for *lacI*^{q} plasmids in *S. oneidensis* MR-1
Numbers shown are Miller Units +/- standard error from at least two experiments on biological triplicates. Values are given for uninduced cultures and cultures induced with 1mM IPTG. Also shown in parenthesis is the approximate fold change of expression post induction. Light to dark shading of each column represents the vector strength in the specified host and growth conditions.

	Aerobic - LB	**Aerobic - SBM**	**Anaerobic - SBM**	
	Uninduced	Uninduced	Uninduced	
	Induced (~fold induction)	Induced (~fold induction)	Induced (~fold induction)	
pAH11::*lacZ* (P_{lac}+$RBS_{lac+7bp}$)	10.1 +/-0.6	6.3 +/-0.3	21.5 +/-2.0	**Low**
	69.7 +/- 2.3 (7)	46.3 +/- 0.3 (7)	127.2 +/- 11.3 (6)	
pAH12::*lacZ* ($P_{A1/O4/O3}$+RBS_{lac+7})	26.9 +/-1.4	22.6 +/-0.4	19.7 +/-2.7	
	1,062.2 +/- 89.7 (39)	698.3 +/- 109.1 (31)	1,139.3 +/- 18.3 (58)	
pAH13::*lacZ* (P_{lac}+RBS_{lac})	80.0 +/-5.6	38.7 +/-1.8	121.7 +/-13.8	
	454.8 +/- 31.2 (6)	301.0 +/- 4.7 (8)	950.8 +/- 80.0 (8)	
pAH14::*lacZ* ($P_{A1/O4/O3}$+RBS_{lac})	23.5 +/-2.1	41.7 +/-9.9	49.9 +/-4.7	
	1,275.0 +/- 22.8 (55)	2,661.5 +/- 122.3 (64)	2,873.1 +/- 222.2 (58)	
pAH15::*lacZ* (P_{lac}+RBS_{II})	323.1 +/-12.7	299.3 +/-42.7	732.7 +/-11.2	
	2,002.3 +/- 52.9 (6)	2,502.3 +/- 36.3 (8)	3,197.7 +/- 212.3 (4)	
pAH16::*lacZ* ($P_{A1/O4/O3}$+RBS_{II})	953.3 +/-84.7	480.3 +/-11.4	921.8 +/-32.9	**High**
	17,427 +/- 398 (18)	17,413 +/- 160 (36)	12,346 +/- 145 (13)	

All promoter/RBS combinations resulted in lower Miller units when *lacI*^{q} was incorporated onto the plasmid backbone as compared to the same combinations without *lacI*^{q} as presented in the previous chapter, even upon full induction and regardless of growth conditions. The only exception is aerobically in LB, pAH12 has slightly higher expression levels than pAH02 (1062.2+/-89.7 vs. 992+/-24). The lower expression levels were expected for two reasons. First, the insertion of a strong and constitutive promoter driving *lacI* expression to each plasmid decreases the cellular machinery available for transcription of the reporter gene. Second, there is always some competition between RNA polymerase and LacI binding which may slow transcription, even when the amount of IPTG added should be sufficient to bind all available LacI.

The pattern of expression seen in the *lacI*^{q} plasmids is similar to the pattern established in the previous chapter for the same promoter and RBS combinations without *lacI*^{q}. In both LB aerobically and SBM anaerobically, the pattern of expression for *lacI*^{q} plasmids is pAH11 < pAH13 < PAH12 < pAH14 < pAH15 < pAH16. In SBM aerobically, pAH15 is

slightly lower than pAH14. In the previous chapter, aerobic levels were pAH01 < pAH03 < pAH02 <pAH04 < pAH05 while anaerobic levels were pAH01 < pAH02 < pAH03 < pAH04 < pAH05. The difference in pattern can be attributed to the promoter response to repression and induction. Aerobically, plasmids pAH11, pAH12, and pAH13 have lower expression when *S. oneidensis* strains are grown in minimal media versus rich media, while plasmids pAH14 and pAH15 have higher expression levels and pAH16 results in the same level in both rich and minimal media. This is contrary to results in the previous chapter where all expression increased in minimal media. All *lacI*^{q} plasmids produced higher levels of β-galactosidase under anaerobic conditions than aerobic conditions with the exception of pAH16. This is also contrary to the previous chapter in which P_{lac} vectors resulted in higher expression anaerobically but $P_{A1/O4/O3}$ vectors had slightly lower expression. Again, this can be attributed to the interaction of repressor protein with the promoter regions.

This suite of *lacI*^{q} plasmids results in a wide range of expression levels. In rich media, the Miller units for the strongest promoter/RBS combination (pAH16) are ~250 fold those of the lowest expression vector (pAH11), ~375 fold in minimal media aerobically, and ~97 fold anaerobically. Results indicate repression expands the functional range of the vectors due to the ability to include the strongest $P_{A1/O4/O3}$+RBS_{II} combination (in pAH16) which was unstable without repression. Although pAH16::*lacZ* results in high pre-induction levels (480-953), the induced levels are incredibly high (12,346-14,427). The most useful vector across all conditions may be pAH14::*lacZ* since it reliably has the greatest fold induction – that is the pre-induction expression levels are low (23.5-49.9) but the post-induction expression is fairly high (1,275-2,873) because of the well repressed promoter ($P_{A1/O4/O3}$) and a medium strength RBS (RBS_{lac}). Generally, the amount of β-galactosidase being produced before induction reflects the strength of the expression after induction, but expression plasmids containing $P_{A1/O4/O3}$ (pAH12, pAH14 and pAH16) have much higher fold induction under all conditions. This suggests that the orientation of the two operator regions leads to more successful sequestration by LacI in the absence

of induction than in those vectors containing P_{lac}. Trends of fold induction for each vector hold across conditions with few exceptions.

Miller Assay Results for vectors in *S. oneidensis* JG2929:

Results from β-galactosidase assays for *S. oneidensis* JG2929 harboring expression vectors pAH01::*lacZ* through pAH06::*lacZ* are shown in Table 3.4. These are the same expression vectors described in the Chapter 2, now controlled by the genomic insertion of $lacI^q$ into strain JG2929. In induced SBM cultures, both aerobically and anaerobically, the same expression vectors result in lower Miller units in the JG2929 background than in MR-1. Again, this suggests that LacI is still causing some slight repression, even when the amount of IPTG added should bind all available LacI. In LB aerobically, the vectors with P_{lac} result in lower Miller units while the vectors with $P_{A1/O4/O3}$ result in higher Miller units in JG2929 versus MR-1. This may be because the cells are allowed to grow to a high density before the burden of overproducing *lacZ* is induced.

Table 3.4 – β-galactosidase assay results for expression plasmids in *S. oneidensis* JG2929
Numbers shown are Miller Units +/- standard error from at least two experiments on biological triplicates. Values are given for uninduced cultures and cultures induced with 1mM IPTG. Also shown in parenthesis is the approximate fold change of expression post induction. Light to dark shading of each column represents the vector strength in the specified host and under the listed growth conditions.

	Aerobic - LB	**Aerobic - SBM**	**Anaerobic - SBM**	
	Uninduced	Uninduced	Uninduced	
	Induced (~fold induction)	Induced (~fold induction)	Induced (~fold induction)	
pAH01::*lacZ*	3.3 +/-0.3	14.7 +/-4.7	58.6 +/-15.2	**Low**
(P_{lac}+$RBS_{lac+7bp}$)	93.7 +/- 3.9 (28)	83.3 +/- 2.5 (6)	484.5 +/- 16.4 (5)	
pAH02::*lacZ*	11.6 +/-4.6	30.3 +/-3.4	90.2 +/-18.9	
($P_{A1/O4/O3}$+RBS_{lac+7})	1,026 +/- 242 (93)	947.7 +/- 79.1 (31)	1,661 +/- 127.8 (12)	
pAH03::*lacZ*	19.7 +/-1.5	51.7 +/-8.3	139.1 +/-36.1	
(P_{lac}+RBS_{lac})	458.3 +/- 18.9 (23)	325.4 +/- 45 (6)	1,546.9 +/- 79.3 (7)	
pAH04::*lacZ*	14.0 +/-3.2	57.1 +/-9.8	168.6 +/-56.0	
($P_{A1/O4/O3}$+RBS_{lac})	2,442 +/- 194 (174)	2,789 +/- 47 (49)	4,112 +/- 121 (14)	
pAH05::*lacZ*	229.7 +/-24.9	564.7 +/-75.5	711.4 +/-166.5	
(P_{lac}+RBS_{II})	2,723.7 +/- 443 (12)	2,797 +/- 432 (5)	6,395 +/- 720 (7)	
pAH06::*lacZ*	466.3 +/-41.2	855.3 +/-20.6	759.3 +/-151.2	**High**
($P_{A1/O4/O3}$+RBS_{II})	13,997 +/- 2451 (30)	38,108 +/- 95 (45)	14,756 +/- 2,087 (19)	

The response of the vectors to LacI changed the expression pattern slightly from that established in the previous chapter. In a JG2929 background, the expression pattern is pAH01<pAH03 < pAH02 < pAH04 < pAH05 < pAH06 for all conditions. That matches the pattern of expression for LB and SBM aerobically from the previous chapter, but anaerobically, pAH02 < pAH03 in an unrepressed MR-1 background. This change in pattern matches the change seen in the *lacI*^{q} expression vectors, further indicating that the promoter, RBS, and response to repression and induction all impact final expression levels. Miller units are lower for induced plasmids pAH01, pAH02, and pAH03 but higher for pAH04, pAH05, and pAH06 in minimal media as compared to rich in the JG2929 background. This is consistent with results seen for the *lacI*^{q} plasmids, but contrary to the previous chapter in which all plasmids had higher expression in minimal media in the MR-1 background.

The fold induction across all conditions is not as consistent when relying on single copy genomic *lacI*^{q} in JG2929 than when *lacI*^{q} is saturating as is the case with the *lacI*^{q} vectors (Table 3.3 vs. Table 3.4). In JG2929 cultures grown aerobically in SBM, the fold induction of each plasmid is similar to the results seen when plasmids contained *lacI*^{q} on the backbone. In LB aerobically, the fold induction numbers are generally much higher for plasmids in JG2929 versus *lacI*^{q} plasmids in MR-1 while growth in SBM anaerobically results in lower fold induction for plasmids in JG2929 versus *lacI*^{q} plasmids in MR-1. All plasmids in the JG2929 background displayed higher Miller units after induction anaerobically than aerobically. This is again contrary to the last chapter in which only the P_{lac} vectors increased expression anaerobically but consistent with results obtained from *lacI*^{q} vectors.

Once again, the vectors with $P_{A1/O4/O3}$ have higher fold induction in the JG2929 background than do vectors with the P_{lac}. Even with the lesser amount of LacI produced by JG2929 (as compared to *lacI*^{q} vectors), $P_{A1/O4/O3}$ is more effectively repressed than P_{lac}. Also, the non-induced Miller units reflect the ‘leakiness’ of the system and the strength of the RBS. Once again, LacI repression allows use of the strongest promoter/

RBS combination and increases the range of induction significantly. Using a *S. oneidensis* background strain with chromosomal *lacI*^{q}, the difference between the strongest vector (pAH16) and the weakest (pAH11) is ~149 fold in LB aerobically, ~450 fold in SBM aerobically, and ~30 fold in SBM anaerobically.

Conclusion:

Utilizing the repressor protein LacI to control expression is successful both when inserted in single copy on the genome of *S. oneidensis* MR-1 and when inserted into the vector backbone and therefore in multi-copy in the cell. When cultures are grown in minimal media, both the un-induced and induced expression levels are generally lower in the *lacI*^{q} vectors as compared to the same promoter/RBS combinations in JG2929. As there is around 50-100 times more LacI in the system when expressed from the multi-copy plasmid, it was expected that reporter gene expression from *lacI*^{q} vectors should be impaired, although the difference was predicted to be even more substantial. Expression from the same promoter/RBS combinations in unrepressed *S. oneidensis* MR-1 grown either aerobically or anaerobically in SBM almost always resulted in higher Miller units than induced JG2929 cultures or induced MR-1 cultures harboring *lacI*^{q} vectors. These results show that RNA polymerase and LacI always compete for promoter binding to some extent, even when the amount of IPTG added should be sufficient to sequester all LacI protein.

When grown in LB, the protein production levels in un-induced JG2929 cultures are lower than those resulting from un-induced *lacI*^{q} vectors while the induced culture results are mixed. Induced JG2929 cultures grown in LB with $P_{A1/O4/O3}$ containing vectors result in higher Miller units than the same constructs in MR-1, while the induced MR-1 cultures harboring *lacI*^{q} vectors almost always result in lower Miller units than MR-1 harboring non- *lacI*^{q} vectors regardless of promoter. The difference of behavior for expression and induction in LB probably reflects the increased energy availability and faster growth rate in this rich medium and the fact that overexpression of *lacZ* was not induced until cells reached mid-exponential phase.

$P_{A1/O4/O3}$ has LacI binding sites arranged close together, making it very difficult for RNA polymerase to bind the promoter when LacI is present, and is therefore very tightly repressed. Upon induction, across all conditions, the fold increase of expression is much higher for vectors containing this promoter compared to those containing P_{lac}. The rate of expression for un-induced cultures depends largely on the RBS chosen. Also importantly, introducing LacI allows control of the most highly expressing vector containing $P_{A1/O4/O3}$+RBS_{II} which was unstable in *S. oneidensis* MR-1 without repression. The knowledge of how each promoter and RBS respond to repression and induction in different conditions will allow informed selection of the most appropriate combinations for specific applications.

Chapter 4

Rational Engineering of Expression Vectors to Increase Extracellular Flavin Levels in *S. oneidensis* Strains

Introduction:

Members of the *Shewanella* genus can be found around the world - anywhere there is a sediment-aquatic interface. This is a rapidly changing environment; quickly swinging from oxic to anoxic. Bacteria that survive here must have adaptations allowing flexibility enough to survive such dramatic and rapid environmental shifts. *Shewanella* species are part of a unique group of microorganisms called dissimilatory metal reducing bacteria. Although *S. oneidensis* MR-1 can respire oxygen, in anoxic conditions, electrons are shuttled from carbon catabolism to a vast array of terminal electron acceptors, including insoluble substrates such as metal oxides. Many metals, most notably iron, though plentiful in the environment are not typically available for microbial respiration as they are insoluble at physiologically relevant pH levels. This ability to move electrons to insoluble substrates expands respiratory options allowing the survival of *Shewanella* species in such varied environments.

Microbial respiration of soluble terminal electron acceptors involves moving electrons through the electron transport chain in the inner membrane and then onto the accepting substrate inside the cell. The difficulty in respiring insoluble substrates is in moving electrons across the inner membrane, through the periplasmic space, and across the outer membrane where they can be deposited onto the external substrate allowing metabolism to continue. *S. oneidensis* MR-1 uses a network of cytochromes to accomplish this (Shi et al., 2007). As modeled in Figure 4.1, electrons come out of the inner membrane quinone pool and are transferred to CymA, a tetraheme periplasmic cytochrome. CymA interacts

with the periplasmic facing, outer membrane decaheme cytochrome MtrA. MtrA forms an association with outside facing, outer membrane decaheme cytochrome MtrC which is stabilized by outer membrane barrel protein MtrB. The arrangement of these cytochromes provides a pathway of closely positioned heme centers for electron travel out of the cell. MtrC can then transfer electrons to the acceptor.

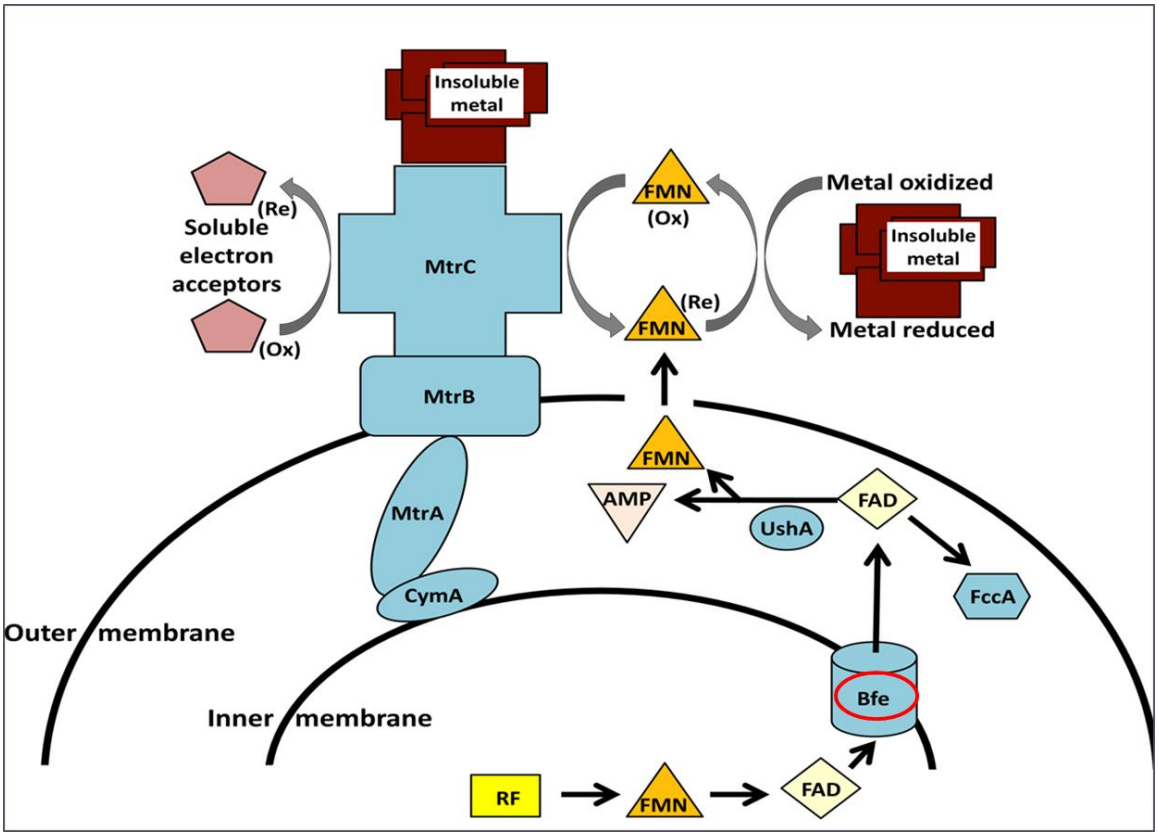

Figure 4.1 – Electron transport model in *S. oneidensis* MR-1.
Figure edited from N. Kotloski. Shown are proteins and molecules important in extracellular electron transport. Electrons from oxidation of lactate are transferred from CymA through the Mtr pathway. Flavin adenine dinuleotide (FAD) is transported across the inner membrane by Bfe (circled in red) and either used as a cofactor for fumarate reductase (FccA) or cleaved by UshA into flavin mononucleotide (FMN) and adenosine monophosphate (AMP) in the periplasm. FMN is secreted and can shuttle electrons between cells and insoluble substrates.

Importance of Flavins in Extracellular Electron Transport:

There are several competing but potentially overlapping theories for how *S. oneidensis* MR-1 is able to accomplish the transport of electrons from outer membrane cytochromes to final respiratory acceptors. Direct physical contact between outer membrane

cytochromes in the metal-reducing (Mtr) pathway and insoluble substrate accounts for some observed reduction. Although there may be large quantities of metal oxides in the proximity of dissimilatory metal reducing bacteria, the physical properties of tiny cells reducing amorphous substrate surface areas force alternate strategies besides direct contact. In complex environments such as sediments or soils, it is easy to imagine the porosity or inconsistency of substrate distribution challenging access of cells to substrate. Reports have been made about electrically conductive pili aiding electron long-range electron transfer but studies showing Fe(III)oxide and electrode reduction without bacterial or pili contact demonstrate that there must be a soluble factor at least partially responsible for either reducing or chelating the substrate (Lies et al., 2005). Additionally, Marsili et al. observed that replacing spent bioreactor medium with fresh medium causes current to drop dramatically but replacement with a filtered medium in which *S. oneidensis* was previously grown immediately restored current. This observation led to the conclusion that the cells are secreting a soluble compound which aids electron transfer (Marsili et al., 2008). Other bacterial species have been shown to utilize humic substances, sulfur compounds, or phenazines as electron shuttles while analysis of supernatants of *S. oneidensis* cultures reveal the secretion of highly soluble, redox-active compounds called flavins (Marsili et al., 2008; von Canstein et al., 2008). These reduced flavins can diffuse to the metal oxide, deposit electrons to become oxidized, and then be reduced again at the cell surface by the outer membrane cytochrome MtrC. The crystal structure of a MtrC homolog (MtrF) reveals a domain structure common in flavin binding proteins and a series of hemes arranged to allow reduction of both soluble and insoluble substrates simultaneously (Clarke et al., 2011). *mtr* mutants are deficient in flavin reduction, further supporting the theory that flavins are again reduced at the cell surface (Coursolle et al., 2010). Outer membrane cytochrome reduction of oxidized flavins can be repeated and, if the concentration of extracellular flavins is sufficient, can result in very rapid reduction of substrates.

Riboflavin (vitamin B2) biosynthesis (Figure 4.2) occurs in most microorganisms and plants beginning with one guanosine triphosphate (GTP) and one ribulose-5-phospahte

molecule. A series of enzymatic steps catalyzed by *ribA* and *ribD* take GTP to 5-amino-6-(D-ribitylamino) uracil. Ribulose-5-phospahte is converted to 1-deoxy-L-glycero tetrulose phosphate by *ribBA (ribBX)* or *ribB*. The entry steps into both branches of the riboflavin synthesis pathway, catalyzed by GTP cyclohydrolase II RibA and 3,4-dihydroxy-2-butanone 4-phosphate (DHBP) synthase RibB, are the rate limiting steps in the process (Humbelin et al., 1999). The 5-amino-6-(D-ribitylamino) uracil and 1-deoxy-L-glycero tetrulose phosphate are combined into riboflavin by the action of *ribH* and *ribE*. Riboflavin is then converted to flavin mononucleotide (FMN) and then to flavin adenine dinucleotide (FAD) by *ribF*. A bacterial flavin exporter (SO_0702) transports FAD across the inner membrane and into the periplasm (Kotloski & Gralnick, 2013). FAD is used as a cofactor for the periplasmic fumarate reductase FccA or cleaved by UshA into FMN and adenine monophosphate (AMP) (Covington et al., 2010). FMN is the primary secreted flavin by *S. oneidensis* MR-1 and can carry two electrons per flavin molecule to reduce iron oxide (von Canstein et al., 2008). The extracellular concentration of flavins is easily measured by florescence spectroscopy of the supernatant.

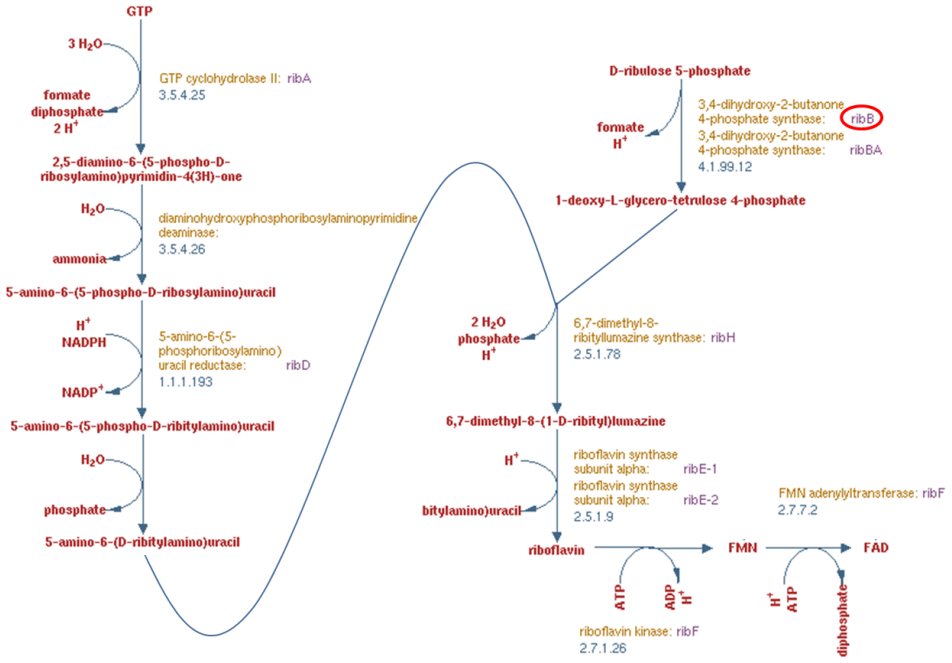

Figure 4.2 – Flavin synthesis pathway in *S. oneidensis* MR-1.
Figure edited from Biocyc.org. Entry steps into the GTP and R5P branches, catalyzed by *ribA* and *ribB* (circled in red) respectively, are the rate limiting steps in the pathway.

As flavins play a major role in *S. oneidensis* MR-1 anaerobic respiration of insoluble substrates, it is not surprising that the genome contains multiple copies of genes involved in riboflavin biosynthesis including a duplication of *ribE* and a fusion gene *ribBA*. A recent study revealed that the *ribBA* gene annotated in *S. oneidensis* genome has only partial function as shown by the ability to complement an *E. coli ribB* deletion mutant but not a *ribA* deletion mutant (Brutinel et al., 2013). In *S. oneidensis,* a *ribB* deletion mutant produces similar levels of extracellular flavins as wild-type MR-1. This contributes further evidence that RibBA can provide the enzymatic activity encoded by *ribB* necessary for riboflavin biosynthesis. In many bacterial species, *ribB* is regulated by an upstream riboswitch element that binds to FMN (Winkler et al., 2002). The FMN riboswitch is found in the 5' untranslated region of *S. oneidensis* MR-1 *ribB* mRNA and upon binding FMN forms a structure that is proposed to cause both premature transcription termination and sequestration of the *ribB* Shine-Dalgarno sequence (Vitreschak et al., 2002).

Flavin Expression Vectors:

The goal of the project described in this chapter was to increase extracellular respiration rates by overexpressing genes involved in riboflavin synthesis and export using the expression plasmids and host strains described in Chapters 2 and 3. Expression of flavin genes under control of the non-native, inducible promoters and ribosome binding sites contained in the vectors should allow controllable and predictable flavin increases at different levels. Overexpression of *ribB* is hypothesized to increase flavin biosynthesis while avoiding internal regulation issues associated with the *ribB* RFN element. Overexpression of *bfe* is hypothesized to increase transport of FMN across the inner membrane, possibly decreasing intracellular flavin levels and leading to the production of more flavins.

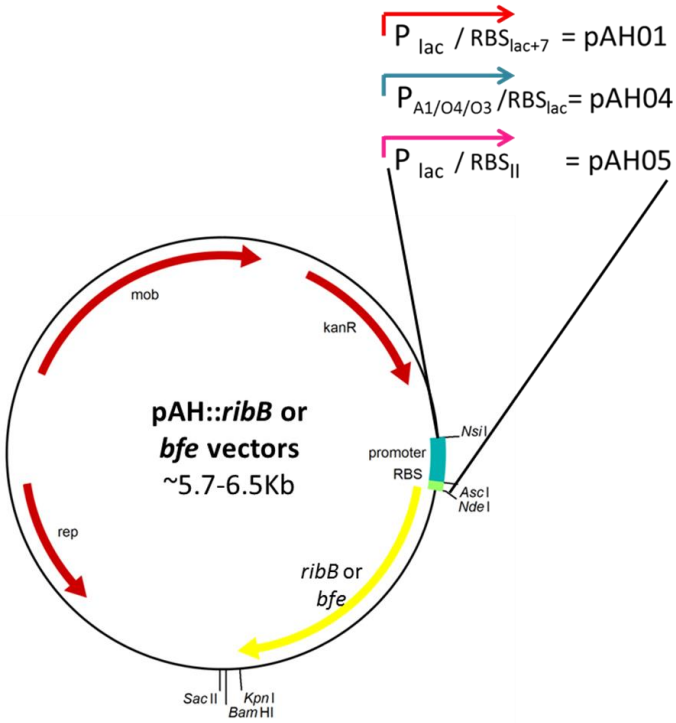

Figure 4.3 – Expression vectors containing either *ribB* or *bfe*
The coding sequence for either DHBP synthase (*ribB*) or bacterial flavin exporter (*bfe*) was inserted into pAH01 (low strength), pAH04 (medium strength), and pAH05 (high strength). Promoters and ribosome binding sites (RBS) are described in Chapter 2. Both promoters (P_{lac} and $P_{A1/O4/O3}$) are repressible by LacI.

Expression vectors were selected based on β-galactosidase assay results from the previous two chapters. pAH01, pAH04, and pAH05 were used to drive either *ribB* or *bfe* expression at predicted low, medium, and high levels, respectively. The choice of these vectors allows the opportunity to further evaluate all of the parts described in the previous chapters including the P_{lac} and $P_{A1/O4/O3}$ promoters and the RBS_{lac}, $RBS_{lac+7bp}$, and RBS_{II} ribosome binding sites (see figure 4.3). Another vector, named pAH17, was constructed to evaluate the effects of expressing *ribB* and *bfe* simultaneously. In this vector, the expression of *ribB* is driven by the P_{lac} promoter/RBS_{II} combination followed by RBS_{II} plus the coding region of *bfe* (see Figure 4.4). pAH17 was designed to maximize expression of both genes without causing deleterious effects on cell growth. The effect of overexpression from all plasmids was evaluated by bulk florescence assays in *S. oneidensis* in both the wild-type and the genomic *lacI*^{q} strain (JG2929), both aerobically and anaerobically. Although using the previously characterized vectors to overexpress

either *ribB* or *bfe* is hypothesized to increase overall levels of extracellular flavins, the previous characterizations were done by assaying directly the protein resulting from *lacZ* overexpression. Bulk florescence assays measure the amount of flavins produced and exported, the result of a pathway with many more steps involved than just those accomplished via the products of *ribB* and *bfe*, and will therefore reflect not only the effect caused by overexpression of those two genes but will also reflect the limitations of flavin production and export imposed by the rest of the pathway.

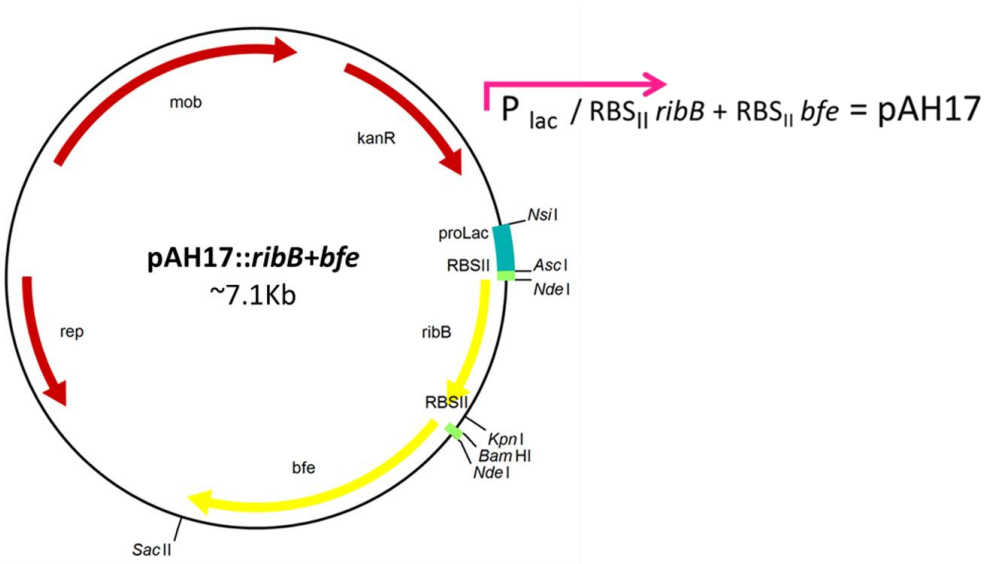

Figure 4.4 – Combinatorial expression vector containing both *ribB* and *bfe*
Expression of DHBP synthase (*ribB*) is driven by P_{lac}+RBS_{II} followed by an approximately 75 base pair space then RBS_{II} + bacterial flavin exporter (*bfe*). Promoters and ribosome binding sites (RBS) are described in Chapter 2. P_{lac} is repressible by LacI.

Understanding and controlling the rate of flavin production and secretion in *S. oneidensis* MR-1 could improve future uses of this microbe in biotechnology applications. It is expected that genetic manipulation resulting in maximal synthesis and export of flavins will enhance extracellular respiration rates of insoluble substrates. This enhancement could improve bioprecipitation of toxic metals or current generation from microbial fuel cells.

Materials and Methods:

Culture conditions:

Strains and plasmids used in this study are listed in Table 4.1. *E. coli* strains were streaked from 15% glycerol freezer vial stocks onto Luria-Bertani (LB) plates and single colonies picked for inoculation into 2 mL LB, supplemented with 50 µg/mL kanamycin (Km) for plasmid bearing strains. Cultures were grown overnight (~14 hours) at 37^{o} C with constant shaking at 225 rpm. *Shewanella* strains were grown in the same way at 30^{o} C. For growth in minimal media, *Shewanella* Basal Media (SBM) was used as described previously (Hau et al., 2008) with 0.01% casamino acids, 20 mM sodium lactate, and 40 mM sodium fumarate added.

Table 4.1 – Strains and plasmids used in this study

E. coli strains:	Features:	Reference/ source:
UQ950	DH5α λ(*pir*); F-Δ(*argF-lac*)169 Φ80d*lacZ*58(ΔM15) *glnV*44(AS) *rfbD*1 *gyrA*96(NalR) *recA*1 *endA*1 *spoT*1 *thi*-1 *hsdR*17 *deoR* λpir+; Used for cloning, plasmid preps and expression assays	D. Lies, Caltech/ Saltikov & Newman, 2003
WM3064	*thrB*1004 *pro thi rpsL hsdS lacZ* ΔM15 RP4–1360 Δ(*araBAD*)567 Δ*dapA*1341::[*erm pir(* wt)]; Donor strain for conjugation	W. Metcalf, Univ of Illinois, Urbana
Shewanella oneidensis strains:		
MR-1	Wild type strain (JG274) ; Used as host strain for expression assays	Nealson, et al., 1999
JG2929	JG274 with chromosomal insertion of $lacI^{q}$ at *glmS* addition site	This study
Plasmids:		
pAH01	5.1-kb broad-range expression vector, Km^{r}, pro_{lac} + $RBS_{lac+7bp}$	This study
pAH04	5.1-kb broad-range expression vector, Km^{r}, $p_{A1/O4/O3}$ + RBS_{lac}	This study
pAH05	5.1-kb broad-range expression vector, Km^{r}, pro_{lac} + RBS_{II}	This study
pAH17	5.1-kb broad-range expression vector, Km^{r}, $p_{A1/O4/O3}$ + RBS_{II}::*ribB*, RBS_{II}::*bfe*	This study

Reagents:

GoTaq Green Mix was purchased from Promega while all restriction enzymes, T4 ligase, and Antarctic phosphatase were purchased from New England Biolabs (NEB), and *pfu* ultra high fidelity polymerase was purchased from Agilent. The protocols provided by the suppliers were followed. Plasmid isolation, gel purifications, and PCR clean up were performed using Invitrogen's PureLink HiPure Plasmid Miniprep Kit, PureLink Gel Extraction Kit, and PureLink PCR Purification Kit. Digested vectors were concentrated

using Zymo Clean and Concentrate Kit. All oligonucleotides and primers were ordered from Integrated DNA Technologies (IDT) and all sequencing done by the University of Minnesota Genomics Center (UMGC). Primers used in this study are listed in Table 4.2.

Table 4.2 – Primers/ oligos used in this study

Name:	5' - 3' sequence:	Target:
AH49F	TGTACGCATATGAATCAGTCTTTACTTGC	SO_0142 - *ribB* from *S. oneidensis* MR-1 (adds upstream NdeI restriction site)
AH50R	ATTATAATGGTACCTTAGCCTACGTTGGC	SO_0142 - *ribB* from *S. oneidensis* MR-1 (adds downstream KpnI restriction site)
AH51F	TATCACATATGAAAGACAGACACGGGC	SO_0702 - *bfe* from *S. oneidensis* MR-1 (adds upstream NdeI restriction site)
AH52R	TATTCAGGTACCCTAAAGGGTGTCGGC	SO_0702 - *bfe* from *S. oneidensis* MR-1 (adds downstream KpnI restriction site)
AH67F	TATCGTAGGATCCCACACATCTAGAATTAAAGAG	RBS_{II}+ *bfe* from pAH02::*bfe* (adds upstream BamHI restriction site)
AH68R	ACTAGTCCGCGGCTAAAGGGTGTCG	RBS_{II}+ *bfe* from pAH02::*bfe* (adds downstream SacII restriction site)

Construction of Flavin Expression Vectors:

RibB insert-

The coding region for *ribB* (SO_0142) was amplified from *S. oneidensis* MR-1 using primers AH49F and AH50R (see table 4.1) to create a 654 base pair product flanked by NdeI on the upstream side and KpnI on the downstream side. This insert was digested was NdeI and KpnI, enzymes removed using the PureLink PCR Clean up kit, and then ligated into the digested expression vectors. Orientation of *ribB* was confirmed by PCR and sequenced by Sanger sequencing at UMGC.

bfe insert-

The coding region for *bfe* (SO_0702) was amplified from *S. oneidensis* MR-1 using primers 51F and 52R (see table 4.1) to create a 1349 base pair product flanked by NdeI on the upstream side and KpnI on the downstream side. This insert was digested with NdeI and KpnI, enzymes removed using the PureLink PCR Clean up kit, and then ligated into the digested expression vectors. Orientation of *bfe* was confirmed by PCR and the sequence was confirmed by Sanger sequencing at UMGC.

pAH02, pAH03, pAH04-

Construction of expression plasmids is described in Chapter 2. Plasmids pAH02, pAH03 and pAH04 were digested with KpnI and NdeI, treated with Antarctic phosphatase, separated by gel electrophoresis, excised and purified to decrease undigested or re-ligated

vectors. Either the *ribB* or the *bfe* insert was ligated into the digested vectors to create pAH02::*ribB*, pAH03::*ribB*, pAH04::*ribB*, pAH02::*bfe*, pAH03::*bfe*, and pAH04::*bfe*.

pAH17 -

RBS_{II} plus the coding region for *bfe (SO_0702)* was amplified using primers 67F and 68R (see table 4.1) from pAH02::*bfe* to create a 1382 base pair product flanked by BamHI on the upstream side and SacII on the downstream side. Both the RBS_{II}+ *bfe* insert as well as pAH02::*ribB* were digested with BamHI and SacII, gel purified, and then ligated together according to the manufacturer's T4 ligase protocol. *ribB* is immediately downstream of pA1/O4/O3+RBS_{II}, followed by ~72bps, then the RBS_{II} segment immediately upstream of *bfe*. Orientation and sequence was confirmed by Sanger sequencing at UMGC.

Transformation of Strains:

S. oneidensis MR-1 and JG2929 were transformed with plasmids pAH02 through pAH04 and pAH17 via conjugation with *E. coli* WM3064. LB plates plus diaminopimelic acid (DAP) were cross-struck with donor strain (WM3064) and recipient strain (MR-1 or JG2929) and incubated overnight. Overnight growth was streaked out onto new LB+ Km plates. Only *S. oneidensis* cells successfully transformed with plasmids conferring kanamycin resistance can grow as there is no DAP on the plate to support growth of the DAP auxotroph donor strain.

Bulk Florescence Assays and IPTG Induction:

Overnight SBM cultures from *S. oneidensis* MR-1 and JG2929 wild type and transformed strains were spun down and the cell pellets washed twice in 1 mL SBM. Pellets were resuspended in 1 mL SBM. For aerobic investigations, the washed and resuspended culture was back diluted into 2 mL of either LB or SBM to achieve an OD_{600} of ~0.005-0.01. For anaerobic investigations, this resuspension was back diluted into anaerobic Balch tubes containing 10 mL SBM. All cultures were grown in shaking incubators with 50 µg/ml Km as a selective agent for plasmid bearing strains. JG2929 cultures were grown to mid-exponential phase before induction with 1 mM IPTG. Cells were harvested

at late log/early stationary phase and centrifuged for two minutes at 8,000 rpm. 300 µl of the supernatant was transferred to a black, 96-well plate and florescence read on a SpectraMax5.1 plate reader with an excitation wavelength of 440 nm and emission at 525 nm. All bulk florescence assays were performed with cultures from biological triplicates with OD_{600} absorbance recorded from a Thermo Electron Corp Genesys 10uv spectrophotometer for aerobic cultures and a Spectronic 20D+ for anaerobic tubes.

Results and Discussion:

Bulk Fluorescence Assay Results in *S. oneidensis* MR-1:

Bulk florescence assays were performed on cultures grown aerobically and anaerobically in minimal media. Plasmids in the wild-type *S. oneidensis* MR-1 host result in constitutive expression of *ribB* and *bfe* at varying strengths from pAH01, pAH04 and pAH05 and pAH17. Putting these same plasmids into the host strain JG2929 (*S. oneidensis* MR-1 with constitutive, genomic $lacI^q$ insertion) requires induction with IPTG to relieve repression and allow full transcription. Promoter and ribosome binding choice are responsible for the level of gene expression as demonstrated previously. pAH01 was chosen to evaluate the effects of slight overexpression of flavin synthesis or export genes. pAH04 and pAH05 were selected with the intent of driving strong expression of flavin genes under aerobic or anaerobic conditions, respectively. It was demonstrated in Chapter 2 that plasmids containing the P_{lac} promoter (pAH01 and pAH05) were significantly upregulated in *S. oneidensis* growing under anoxic conditions. The LacI repressor protein interferes with transcription but not translation of intact transcripts. Therefore, stronger RBS segments lead to more pre-induction expression when coupled with the same promoter. pAH05 contains RBS_{II}, which was shown to result in stronger ribosome binding than RBS_{lac} (found in pAH04) and $RBS_{lac+7bp}$ (found in pAH01).

Results from bulk florescence assays for *S. oneidensis* MR-1 harboring expression vectors pAH01::*ribB*, pAH04::*ribB*, pAH05::*ribB*, pAH01::*bfe*, pAH04::*bfe*, and an empty vector control, as well as *ribB* and *bfe* deletion mutants are shown in Table 4.3.

Note that there is no value given for pAH05::*bfe* or pAH17::*ribB*+*bfe* as both of these plasmids are unstable in *S. oneidensis* MR-1. Wild-type MR-1, the empty vector control, Δ*ribB*, and Δ*bfe* strains produce approximately twice as many extracellular flavins anaerobically versus aerobically. Flavin levels resulting from pAH01::*ribB* also doubled anaerobically, whereas levels from pAH05::*ribB* and pAH04::*ribB* are almost the same when grown anaerobically as compared to aerobically. However, both pAH01::*bfe* and pAH04::*bfe* have much higher (1.5 and 2.3 fold, respectively) relative florescence aerobically than the same plasmids anaerobically. Although overexpression of *ribB* and *bfe* both lead to increased extracellular flavins, it is clear that overexpressing an enzyme in a pathway (*ribB*) and overexpressing a transporter (*bfe*) have very different consequences. Anaerobically, strains overexpressing *ribB* or *bfe* resulted in approximately 1.5 to 2.2 fold the relative florescence of the wild-type strain. Although the range of relative florescence was narrow, the resulting pattern of pAH05 > pAH04 > pAH01 was as expected. Aerobically, overexpression of *ribB* resulted in a range of florescence from approximately 1.8 to 4.2 fold the relative florescence of the wild-type strain with pAH04::*ribB* producing the highest amount of extracellular flavins. Overexpression of *bfe* aerobically resulted in an increase of approximately 7 fold wild-type level for pAH01::*bfe* and 12 fold wild-type level for pAH04::*bfe*. This may be due to the cell gaining more energy through aerobic growth and therefore being able to either produce more flavins to export or maintain membrane integrity despite exporting many more flavin molecules. The *S. oneidensis* Δ*ribB* strain has a slight reduction in extracellular flavins anaerobically but produces the same amount as wild-type aerobically. This is due to the ability of *ribBA* to complement a *ribB* deletion and the aerobic versus anaerobic difference may reflect regulation effects. The Δ*bfe* strain has a much more obvious phenotype and flavins released into the supernatant are decreased by almost 90% as compared to wild-type, both aerobically and anaerobically.

Table 4.3 – Bulk florescence assay results for plasmids in MR-1

Numbers shown are Relative Florescence Units (florescence value/OD_{600}) +/- standard error. Readings are taken with an excitement wavelength of 440 and emission at 525 and results are from two separate experiments using biological triplicates. SBM contains 20 mM lactate and 40 mM fumarate. Light to dark shading indicates impact of either *ribB* or *bfe* overexpression vectors on flavin production.

	Anaerobic in SBM with 0.01% casamino acids		
	RFU	**~ times WT**	low
pAH01*::ribB*	617.7 +/-14	1.49	
pAH04*::ribB*	719.7 +/-16.1	1.74	
pAH05*::ribB*	732.7 +/-12.6	1.77	high
pAH01*::bfe*	851.5 +/-20.4	2.05	
pAH04*::bfe*	894 +/-27.5	2.16	
JG274 (MR-1 WT)	414.5 +/-13.4	1.00	
pAH05::empty vector	407 +/-11.7	0.98	
JG1604 (MR-1 Δ*ribB*)	351.3 +/-12.9	0.85	
JG1758 (MR-1 Δ*bfe*)	46.7 +/-9.4	0.11	
	Aerobic in SBM with 0.01% casamino acids		
	RFU	**~ times WT**	low
pAH01*::ribB*	313.2 +/-21.9	1.78	
pAH04*::ribB*	733.3 +/-75.4	4.17	
pAH05*::ribB*	677.7 +/-31.0	3.85	high
pAH01*::bfe*	1240.0 +/-149.6	7.05	
pAH04*::bfe*	2123.0 +/-183.6	12.06	
JG274 (MR-1 WT)	176.0 +/-16.4	1.00	
pAH05::empty vector	168.2 +/-13.1	0.96	
JG1604 (MR-1 Δ*ribB*)	180.7 +/-5.2	1.03	
JG1758 (MR-1 Δ*bfe*)	23.7 +/-1.2	0.13	

Bulk Fluorescence Assay Results in *S. oneidensis* JG2929:

Results from bulk florescence assays for *S. oneidensis* JG2929 with genomic *lacI*^{q} harboring expression vectors pAH01::*ribB*, pAH04::*ribB*, pAH05::*ribB*, pAH01::*bfe*, pAH04::*bfe*, pAH05::*bfe*, *and* pAH17::*ribB*+*bfe* are shown in Table 4.4. Maximal expression in this system is dependent on promoter and RBS strength as well as the response to IPTG induction. Aerobically, JG2929 and the empty vector control produce about one third of the extracellular flavins compared to those strains anaerobically. Uninduced plasmids containing *ribB* have similar RFU levels as JG2929 and the empty vector control, both aerobically and anaerobically. The effect of overexpressing a transporter protein is evident from the uninduced florescence levels of plasmids driving *bfe*, which range from 1.2 to 1.5 fold anaerobically and 1.8 to 3.9 fold of JG2929 levels

aerobically. As demonstrated in the previous chapter, the vector with $P_{A1/O4/O3}$ (pAH04) is better repressed than vectors containing P_{lac} (pAH01 and pAH05). Fully induced relative florescence units are higher anaerobically for pAH01::*ribB*, pAH04::*ribB*, pAH05::*ribB*, pAH01::*bfe*, and pAH04::*bfe* than aerobically. However, florescence is approximately 1.5 fold higher for pAH05::*bfe* aerobically. This differs from results seen from pAH01::*bfe* and pAH04::*bfe* in an MR-1 background which were higher aerobically and can be attributed to the promoter response to repression.

Table 4.4 – Bulk florescence assay results for plasmids in *S. oneidensis* JG2929 (genomic *lacI*^{q})

Numbers shown are Relative Florescence Units (florescence value/OD_{600}) +/- standard error. Readings are taken with an excitement wavelength of 440 and emission at 525 and results are from two separate experiments using biological triplicates. Values are given for uninduced cultures and cultures induced with 1mM IPTG. Also shown is approximate fold change of expression post induction. SBM contains 20 mM lactate and 40 mM fumarate. Light to dark shading indicates impact of either *ribB* or *bfe* overexpression vectors on flavin production.

	Anaerobic in SBM with 0.01% casamino acids			
	RFU - Uninduced	~ fold induction	~ times WT	low
	Induced			
pAH01*::ribB* (P_{lac}+$RBS_{lac+7bp}$)	391.5 +/-3.9	1.17	1.06	
	458 +/- 14.9			
pAH04*::ribB* ($P_{A1/O4/O3}$+RBS_{lac})	370.2 +/-18.1	1.80	1.55	
	667.8 +/- 22.5			
pAH05*::ribB* (P_{lac}+RBS_{II})	415.8 +/-9.1	1.53	1.47	
	635.2 +/- 21.6			high
pAH01*::bfe* (P_{lac}+$RBS_{lac+7bp}$)	539.8 +/-9.8	1.18	1.47	
	635.3 +/- 12.3			
pAH04*::bfe* ($P_{A1/O4/O3}$+RBS_{lac})	563.2 +/-7.9	1.22	1.60	
	689.8 +/- 20.4			
pAH05*::bfe* (P_{lac}+RBS_{II})	638.2 +/-4.9	1.14	1.68	
	726.3 +/- 15.4			
pAH17*::ribB+bfe* (P_{lac}+RBS_{II}::ribB+RBS_{II}::bfe)	738.7 +/-49.6	1.18	2.02	
	874 +/- 30.5			
JG2929 (MR-1+*lacI*^{q})	425.7 +/-2.6	1.01	1.00	
	431.8 +/- 5.7			
pAH05::empty vector	412.3 +/-15.2	0.99	0.95	
	409.3 +/- 7.6			
	Aerobic in SBM with 0.01% casamino acids			
	RFU - Uninduced	~ fold induction	~ times WT	low
	Induced			
pAH01*::ribB* (P_{lac}+$RBS_{lac+7bp}$)	124.5 +/-7.5	1.19	1.18	
	148.3 +/- 6.3			
pAH04*::ribB* ($P_{A1/O4/O3}$+RBS_{lac})	127.5.2 +/-7.1	3.39	3.43	
	432.7 +/- 15.4			
pAH05*::ribB* (P_{lac}+RBS_{II})	138.7 +/-6.5	2.60	2.86	high
	360.7 +/- 13.1			
pAH01*::bfe* (P_{lac}+$RBS_{lac+7bp}$)	245.7 +/-16.8	1.56	3.04	
	383.3 +/- 27.4			
pAH04*::bfe* ($P_{A1/O4/O3}$+RBS_{lac})	228.3 +/-19.5	2.40	4.34	
	547.0 +/- 27.8			
pAH05*::bfe* (P_{lac}+RBS_{II})	503.3 +/-26.9	2.04	8.17	
	1029.0 +/- 44.4			
pAH17*::ribB+bfe* (P_{lac}+RBS_{II}::ribB+RBS_{II}::bfe)	754.9 +/-33.2	1.49	8.90	
	1121.3 +/- 78.3			
JG2929 (MR-1+*lacI*^{q})	127.3 +/-5.0	0.99	1.00	
	126.0 +/- 2.6			
pAH05::empty vector	129.5 +/-5.3	1.02	1.05	
	132.2 +/- 5.3			

Fully induced JG2929 cultures produced a similar pattern but a lesser amount of relative florescence than the same plasmids in the non-repressing MR-1 background. Aerobically, MR-1 and JG2929 (without vectors) result in similar relative florescence levels, but anaerobically, JG2929 produces only about 75% of the extracellular flavins found in the wild-type MR-1 strain. Chromosomal insertion of *lacI*^{q} allows expression from plasmids that are unstable in *S. oneidensis* MR-1. Expression from pAH05::*bfe* and pAH17::*ribB+bfe* result in the highest levels of florescence for plasmids in JG2929, but levels for unrepressed pAH04::*bfe* and pAH01::*bfe* in MR-1 were higher demonstrating that even when maximally induced, some repression still occurs. Combinatorial expression of both *ribB* and *bfe* (in pAH17) resulted in higher RFU than expression of each gene alone. This effect was not as strong as expected, however, as single expression of *bfe* from pAH05 (the strongest promoter/RBS combination tested in JG2929) resulted in only marginally lower bulk florescence values. Also surprisingly, aerobic florescence levels for vectors pAH01::*bfe* and pAH04::*bfe* in unrepressed MR-1 were both higher than pAH05::*bfe* and pAH17::*ribB+bfe* after induction in JG2929.

Conclusion:

The expected expression pattern based on β-galactosidase assay results established in the Chapters 2 and 3 generally held for Tables 4.3 and 4.4 but overexpression of *S. oneidensis* native genes does not result in the same dramatic range of expression or fold induction previously presented with reporter gene *lacZ*. Even with massive overexpression of the enzyme catalyzing one of the rate limiting steps of riboflavin synthesis (*ribB*), maximal increase in flavins depends on precursors and other enzyme levels involved in the pathway. Overexpression of *bfe* results in dramatic increase of exported flavins, especially aerobically, but is limited by the cellular production of flavins and is detrimental to cell vitality at high levels. Bulk fluoresce assays indicate that the knowledge gained from β-galactosidase assays can be applied to control expression of native genes but that other factors such as internal regulation or precursor limitation may complicate predictions.

Chapter 5

Conclusions and Future Directions

The goal of this project was to evaluate the effect of different strength promoters and ribosome binding sites (RBS) on protein expression in *Shewanella oneidensis* MR-1. We have demonstrated that these promoters and RBS produce the same pattern of response in *S. oneidensis* MR-1 documented in *Escherichia coli* grown in LB aerobically. A suite of plasmids was constructed that can be used to produce low, medium, or high levels of protein. Promoters and RBS have been characterized in conditions relevant to both laboratory study and biotechnology applications of MR-1, including growth in various media, use of different terminal electron acceptors, and with or without repressor protein. These expression vectors have been designed so that the promoters and RBS can be exchanged for alternative promoters and RBS using simple molecular techniques.

A subsequent set of plasmids was constructed adding the *E. coli* promoter and coding region for transcription repressor protein $LacI^q$ to the previously characterized vectors enabling inducible expression with IPTG. Gene expression in the uninduced cultures is not entirely abolished; however the reduction of expression is still effective as uninduced protein production levels are slight compared to induced cultures. Vectors with the *lacI*^{q} insertion allow inducible expression regardless of host strain. Protein expression resulting from the same promoter and RBS combinations are lower for *lacI*^{q} vectors than for vectors without *lacI*^{q}, but the same patterns apply. The number and arrangement of LacI operator sites within the promoter region determine the efficacy of repression and induction. RBS affinity affects the rate of translation and those segments are therefore unaffected by the transcription repressing protein. *E. coli lacI*^{q} was also inserted into the genome of *S. oneidensis* MR-1, creating strain JG2929. This strain allows repression of plasmid borne genes without *lacI*^{q} while allowing maximum transcription when induced.

Uninduced and induced expression levels are similar whether *lacI*^{q} is present in multicopy on the plasmid or in single copy on the host genome.

Finally, the utility of expression vectors of variable strength was demonstrated by driving expression of native *S. oneidensis* genes involved in flavin biosynthesis and export. Based on earlier characterization of promoter and RBS strength, as well as the response to induction, vectors were designed to modulate expression of *ribB* (riboflavin synthesis pathway) and *bfe* (FAD export protein) separately and together. Overexpression of either gene individually increased extracellular flavin levels. Combinatorial expression of both *ribB* and *bfe* increased extracellular flavin levels more than either gene alone, but the effect was not additive as expected.

Extracellular flavin concentrations were detected using fluorescence spectroscopy. This measures bulk fluorescence and is therefore indiscriminate toward the type of flavin present. Culture supernatants from plasmid harboring strains will be analyzed by high performance liquid chromatography to ensure gene overexpression does not alter the flavin profiles normally observed in *S. oneidensis* MR-1. In addition, Fe(III) oxide reduction assays will be performed to determine the effect of different levels of increased extracellular flavins on the rate of insoluble metal reduction. After evaluating Fe(III) oxide reduction rates, optimal promoter and RBS combinations will be used to engineer strains for increased current production in bioreactors. The ultimate goal of this project is to apply the knowledge acquired in regard to controlling gene expression to rationally design strains appropriate for specific conditions.

References

Antoine, R., & Locht, C. (1992). Isolation and molecular characterization of a novel broad-host-range plasmid from *Bordetella bronchiseptica* with sequence similarities to plasmids from Gram-positive organisms. *Molecular Microbiology, 6*(13), 1785–1799.

Brewster, R. C., Jones, D. L., & Phillips, R. (2012). Tuning promoter strength through RNA polymerase binding site design in *Escherichia coli*. *PLoS Computational Biology, 8*(12).

Brutinel, E. D., Dean, A. M., & Gralnick, J. A. (2013). Description of a riboflavin biosynthetic gene variant prevalent in the phylum Proteobacteria. *Journal of Bacteriology*, *195*(24), 5479–86.

Brutinel, E. D., & Gralnick, J. A. (2012). Shuttling happens: soluble flavin mediators of extracellular electron transfer in *Shewanella*. *Applied Microbiology and Biotechnology, 93*(1), 41–8.

Caccavo, F., Lonergan, D. J., Lovley, D. R., Davis, M., Stolz, J. F., & McInerney, M. J. (1994). *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. *Applied and Environmental Microbiology, 60*(10), 3752–9.

Chen, H., Bjerknes, M., Kumar, R., & Jay, E. (1994). Determination of the optimal aligned spacing between the Shine-Dalgarno sequence and the translation initiation codon of *Escherichia coli* mRNAs. *Nucleic Acids Research, 22*(23), 4953–7.

Clarke, T. A., Edwards, M. J., Gates, A. J., Hall, A., White, G. F., Bradley, J., Reardon, C.L., Shi, L., Beliaev, A.S., Marshall, M.J., Wang, Z., Watmough, N.J., Fredrickson, J.K., Zachara, J.M., Butt, J.N. & Richardson, D. J. (2011). Structure of a bacterial cell surface decaheme electron conduit. *Proceedings of the National Academy of Sciences of the United States of America, 108*(23), 9384–9.

Coppi, M. V, Leang, C., Sandler, S. J., & Lovley, D. R. (2001). Development of a Genetic System for *Geobacter sulfurreducens*. *Applied and Environmental Microbiology, 67*(7).

Coursolle, D., Baron, D. B., Bond, D. R., & Gralnick, J. A. (2010). The *Mtr* respiratory pathway is essential for reducing flavins and electrodes in *Shewanella oneidensis*. *Journal of Bacteriology, 192(2),* 467-74.

Coursolle, D., & Gralnick, J. A. (2010). Modularity of the Mtr respiratory pathway of *Shewanella oneidensis* strain MR-1. *Molecular Microbiology, 77*(4), 995-1008.

Covington, E. D., Gelbmann, C. B., Kotloski, N. J., & Gralnick, J. A. (2010). An essential role for UshA in processing of extracellular flavin electron shuttles by *Shewanella oneidensis*. *Molecular Microbiology*, *78*(2), 519–32.

Deuschlel, U., Kammerer, W., Gentz, R., & Bujard, H. (1986). Promoters of *Escherichia coli*: a hierarchy of in vivo strength indicates alternate structures. *EMBO Journal*, *5*(11), 2987–2994.

Eberhard, W. G. (1989). Why do bacterial plasmids carry some genes and not others? *Plasmid*, *21*(3), 167–74.

Gao, H., Wang, X., Yang, Z. K., Chen, J., Liang, Y., Chen, H., Palzkill, T. & Zhou, J. (2010). Physiological roles of ArcA, Crp, and EtrA and their interactive control on aerobic and anaerobic respiration in Shewanella oneidensis. *PloS One*, *5*(12), e15295.

Glascock, C., & Weickert, M. (1998). Using chromosomal *lacI*^{Q1} to control expression of genes on high-copy-number plasmids in *Escherichia coli*. *Gene*, *223*, 221–231.

Golitsch, F., Bücking, C., & Gescher, J. (2013). Proof of principle for an engineered microbial biosensor based on *Shewanella oneidensis* outer membrane protein complexes. *Biosensors & Bioelectronics*, *47*, 285–91.

Gralnick, J. a, & Newman, D. K. (2007). Extracellular respiration. *Molecular Microbiology*, *65*(1), 1–11.

Hau, H. H., Gilbert, A., Coursolle, D., & Gralnick, J. A. (2008). Mechanism and Consequences of anaerobic respiration of cobalt by *Shewanella oneidensis* strain MR-1. *Applied and Environmental Microbiology*, *74*(22), 6880–6.

Hau, H. H., & Gralnick, J. A. (2007). Ecology and biotechnology of the genus *Shewanella*. *Annual Review of Microbiology*, *61*, 237–58.

Heidelberg, J.F., Paulsen, I.T., Nelson, K.E., Gaidos, E.J., Nelson, W.C., Read, T.D., Eisen, J.A., Seshadr, R., Ward, N., Methe1, B., Clayton, R.A., Meyer, T., Tsapin, A., Scott, J., Beanan, M., Brinkac, L., Daugherty, S., DeBoy, R.T., Dodson, R.J., Durkin, A.S., Haft, D.H., Kolonay, J.F., Madupu, R., Peterson, J.D., Umayam, L.A., White, O., Wolf, A.M., Vamathevan, J., Weidman, J., Impraim, M., Lee1, K., Berry, K., Lee1, C., Mueller, J., Khouri, H., Gill, J., Utterback, T.R., McDonald, L.A., Feldblyum, T.V., Smith, H.O., Venter, J.C., Nealson, K.H., & Fraser, C.M. (2002). Genome sequence of the dissimilatory metal ion–reducing bacterium *Shewanella oneidensis*. *Nature Biotechnology*, *20*, 1118-1123.

Hümbelin, M., Griesser, V., Keller, T., Schurter, W., Haiker, M., Hohmann, H.P., Ritz, H., Richter, G., Bacher, A., van Loon, A.P.G.M. (1999). GTP cyclo-hydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase are rate-limiting enzymes in riboflavin synthesis of an industrial *Bacillus subtilis* strain used for riboflavin production. *Journal of Industrial Microbiology and Biotechnology*, 22, 1–7.

Koch, B., Jensen, L. E., & Nybroe, O. (2001). A panel of Tn7-based vectors for insertion of the gfp marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomal site. *Journal of Microbiological Methods*, *45*(3), 187–95.

Kotloski, N. J., & Gralnick, J. A. (2013). Flavin electron shuttles dominate extracellular electron transfer by *Shewanella oneidensis*. *mBio*, *4*(1).

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M., Roop, R. M., & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene*, *166*(1), 175–6.

Lanzer, M., & Bujard, H. (1988). Promoters largely determine the efficiency of repressor action. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(23), 8973–7.

Lies, D. P., Hernandez, M. E., Kappler, A., Mielke, R. E., Gralnick, J. A., & Newman, D. K. (2005). *Shewanella oneidensis* MR-1 uses overlapping pathways for iron reduction at a distance and by direct contact under conditions relevant for biofilms. *Applied and Environmental Microbiology*, *71*(8), 4414–26.

Lovley, D. R., Holmes, D. E., & Nevin, K. P. (2004). Dissimilatory Fe(III) and Mn(IV) reduction. *Advances in Microbial Physiology*, *49*, 219–86.

Marsili, E., Baron, D. B., Shikhare, I. D., Coursolle, D., Gralnick, J. A., & Bond, D. R. (2008). *Shewanella* secretes flavins that mediate extracellular electron transfer. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(10), 3968–73.

Marx, C. J., & Lidstrom, M. E. (2001). Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria. *Microbiology (Reading, England)*, *147*(8), 2065–75.

Miller, J. H. (1992). *A Short Course in Bacterial Genetics*. Plainview, NY: Cold Spring Harbor Laboratory Press.

Muller-Hill, B., Crapo, L., & Gilbert, W. (1968). Mutants that make more lac repressor. *Proceedings of the National Academy of Sciences of the United States of America*, *59*, 1259–1264.

Myers, C. R., & Nealson, K. H. (1988). Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. *Science*, *240*(4857), 1319–21.

Nealson, K. H., Belz, A., & McKee, B. (2002). Breathing metals as a way of life: geobiology in action. *Antonie van Leeuwenhoek*, *81*(1-4), 215–22.

Pinchuk, G. E., Geydebrekht, O. V, Hill, E. A., Reed, J. L., Konopka, A. E., Beliaev, A. S., & Fredrickson, J. K. (2011). Pyruvate and lactate metabolism by *Shewanella oneidensis* MR-1 under fermentation, oxygen limitation, and fumarate respiration conditions. *Applied and Environmental Microbiology*, *77*(23), 8234–40.

Richter, K., Schicklberger, M., & Gescher, J. (2012). Dissimilatory reduction of extracellular electron acceptors in anaerobic respiration. *Applied and Environmental Microbiology*, *78*(4), 913–21.

Saffarini, D. A., Schultz, R., & Beliaev, A. (2003). Involvement of cyclic AMP (cAMP) and cAMP receptor protein in anaerobic respiration of *Shewanella oneidensis*. *Journal of Bacteriology*, *185*(12), 3668–71.

Salis, H. M. (2011). The ribosome binding site calculator. *Methods in Enzymology*, *498*, 19–42.

Saltikov, C. W., & Newman, D. K. (2003). Genetic identification of a respiratory arsenate reductase. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(19), 10983–8.

Shi, L., Squier, T. C., Zachara, J. M., & Fredrickson, J. K. (2007). Respiration of metal (hydr)oxides by *Shewanella* and *Geobacter*: a key role for multihaem c-type cytochromes. *Molecular Microbiology*, *65*(1), 12–20.

Sukovich, D. J., Seffernick, J. L., Richman, J. E., Hunt, K. A., Gralnick, J. A., & Wackett, L. P. (2010). Structure, function, and insights into the biosynthesis of a head-to-head hydrocarbon in *Shewanella oneidensis* strain MR-1. *Applied and Environmental Microbiology*, *76*(12), 3842–9.

Teal, T. K., Lies, D. P., Wold, B. J., & Newman, D. K. (2006). Spatiometabolic stratification of *Shewanella oneidensis* biofilms. *Applied and Environmental Microbiology*, *72*(11), 7324–30.

Vitreschak, A. G., Rodionov, D. A., Mironov, A. A., & Gelfand, M. S. (2002). Regulation of riboflavin biosynthesis and transport genes in bacteria by transcriptional and translational attenuation. *Nucleic Acids Research*, *30*(14), 3141–51.

Von Canstein, H., Ogawa, J., Shimizu, S., & Lloyd, J. R. (2008). Secretion of flavins by *Shewanella* species and their role in extracellular electron transfer. *Applied and Environmental Microbiology*, *74*(3), 615–23.

Waddell, C. S., & Craig, N. L. (1989). Tn7 transposition: recognition of the attTn7 target sequence. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(11), 3958–62.

Webster, D. P., TerAvest, M. A., Doud, D. F. R., Chakravorty, A., Holmes, E. C., Radens, C. M., Sureka, S., Gralnick, J. A., & Angenent, L. T. (2014). An arsenic-specific biosensor with genetically engineered *Shewanella oneidensis* in a bioelectrochemical system. *Biosensors & Bioelectronics*, *62*, 320–4.

Winkler, W. C., Cohen-Chalamish, S., & Breaker, R. R. (2002). An mRNA structure that controls gene expression by binding FMN. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(25), 15908–13.

Appendix

Introduction:

Understanding the process of dissimilatory metal reduction by bacteria can be aided by the study of multiple organisms that potentially accomplish extracellular electron transport in different ways. Bacteria in the *Geobacter* genus are important metal reducers found commonly in redox active, anaerobic environments (reviewed by Lovley et al., 2004). *Geobacter sulfurreducens*, a hydrogen and acetate oxidizer and iron oxide reducer originally isolated from hydrocarbon contaminated sediment, is the most well studied species in this genus (Caccavo et al., 1994). Genetic systems for manipulation of *Geobacter sulfurredcuens* have been established in order to accomplish gene deletion and overexpression (Coppi et al., 2001). Coppi identified suicide plasmids and incompatibility group IncQ plasmids capable of stable replication in *G. sulfurreducens* for use as complementation or expression vectors. Although genetic manipulation can be accomplished, further investigation of expression levels based on plasmid promoter and ribosome binding site selection would allow more precise manipulation of *Geobacter* isolates in the future, allowing gene expression to be tuned as required.

Some of the *lacZ* expression vectors characterized in this thesis in *S. oneidensis* were evaluated in *G. sulfurreducens* PCA anaerobically in a minimal medium with acetate added as a carbon source and fumarate added as an electron acceptor. The response to promoters and ribosome binding site selection was investigated by transforming *G. sulfurreducens* PCA with non-inducible vectors pAH01::*lacZ* through pAH06::*lacZ* and measuring production of β-galactosidase. The response to LacI repression and IPTG induction was investigated by transforming *G. sulfurredcuens* PCA with $lacI^q$ containing vectors pAH15::*lacZ* and pAH16::*lacZ* and measuring the resulting production of β-galactosidase.

Materials and Methods:

Culture Conditions:

G. sulfurreducens PCA was used as the wildtype strain acquired from Daniel Bond's lab at the University of Minnesota. *E. coli* WM3064 was used as a donor strain for plasmid conjugation and is described in Chapter 1. *E. coli* was streaked from 15% glycerol freezer vial stocks onto Luria-Bertani (LB) plates and single colonies picked for inoculation into 2 mL LB. Cultures were grown overnight (~14 hours) at 37^{o} C with constant shaking at 225 rpm. *G. sulfurreducens* strains were streaked from 10% DMSO freezer vial stocks and grown on NB (previously described by Coppi, 2001) plates with 20 mM sodium lactate and 40 mM sodium fumarate added (NBFA). All *G. sulfurreducens* work was done anaerobically, either in a 30^{o}C dwScientific MACS-MG-500 anaerobic workstation with 80% N_2 and 20% CO_2 or in Balch tubes with 80% N_2 and 20% CO_2 headspace. Cultures were grown overnight (~14 hours) in 10 mL liquid NBFA in Balch tubes and incubated at 30^{o} C with constant shaking at 225 rpm. *E. coli* cultures were grown on plates and in liquid media supplemented with 50 µg/mL kanamycin (Km) for plasmid bearing strains and *G. sulfurreducens* cultures were grown on plates and in liquid media supplemented with 200 µg/mL Km for plasmids bearing strains.

Transformation of Strains:

G. sulfurreducens PCA was transformed with plasmids pAH01-pAH06 and pAH15-pAH16 via filter conjugation with diaminopimelic acid (DAP) auxotroph *E. coli* WM3064. Both strains were grown from overnight cultures to reach and OD_{600} of ~0.4 and then concentrated together onto a sterilized filter. The filter was rinsed twice with sterile NBFA to remove any residual Km from the *E. coli* culture. This filter was placed on an NBFA plate in the anaerobic chamber and after 4-8 hours, growth was scraped from the filter and streaked out onto NBFA + 200 µg/mL kanamycin (Km) and incubated for approximately one week until visible colonies formed. Only recipient *G. sulfurreducens* cells successfully transformed with plasmids conferring resistance to Km can grow as there is no DAP on the plate to support growth of the *E. coli* DAP auxotroph donor strain.

Expression Vector Construction:

pAH01-

Contains P_{lac} and $RBS_{lac+7bp}$ – construction detailed in Chapter 2.

pAH02-

Contains $P_{A1/O4/O3}$ and $RBS_{lac+7bp}$ – construction detailed in Chapter 2.

pAH03-

Contains P_{lac} and RBS_{lac} – construction detailed in Chapter 2.

pAH04-

Contains $P_{A1/O4/O3}$ and RBS_{lac} – construction detailed in Chapter 2.

pAH05-

Contains P_{lac} and RBS_{II} – construction detailed in Chapter 2.

pAH06-

Contains $P_{A1/O4/O3}$ and RBS_{II} – construction detailed in Chapter 2.

pAH15-

Contains *lacI*^{q} on the vector backbone and P_{lac} and RBS_{II} – construction detailed in Chapter 3.

pAH16-

Contains *lacI*^{q} on the vector backbone and $P_{A1/O4/O3}$ and RBS_{II} – construction detailed in Chapter 3.

lacZ insert-

The *lacZ* insert (coding for β-galactosidase production) was amplified via heterostagger PCR and ligated into vectors as described in Chapter 1.

Miller Assays:

Overnight cultures from *G. sulfurreducens* PCA wild type and transformed strains bearing plasmids pAH01-pAH06, pAH15, and pAH16 were spun down and the cell pellets washed twice in 1 mL NBFA. Pellets were resuspended in 1 mL NBFA and the pelleting and resuspension were done in the anaerobic chamber. This resuspension was back diluted into anaerobic Balch tubes containing 10mL NBFA with N_2/CO_2 headspace to achieve an OD_{600} of ~0.01. Medium was supplemented with 200 µg/mL Km for

plasmid bearing strains. All β-galactosidase assays were performed with cultures from biological triplicates and performed two or three separate times. OD_{600} absorbance was recorded from a Genesys 10uv spectrophotometer for anaerobic tubes made by Thermo Electron Corporation.

β-galactosidase assays were performed according to Miller's 1992 Cold Spring Harbor Protocol (Miller, 1992) with some modifications. 1 mL of *G. sulfurreducens* culture was harvested in mid log phase (OD_{600} between 0.300-0.400, precise readings recorded) and cells pelleted by centrifugation at 8,000 rpm for 3 minutes and put in a -20^{o}C freezer until the assay was performed. For inducible vectors, 1 mM anaerobic IPTG was added when cells were growing exponentially, around an OD_{600} of 0.08. To assay, cell pellets were resuspended in centrifuge tubes containing 3.5 µL β-mercaptoethanol, 50 µL chloroform, 20 µL sodium dodecyl sulfate (SDS), and 900 µL Z-buffer (60 mM $Na_2HPO_4{\cdot}7H_20$, 40 mM $NaH_2PO_4{\cdot}H_20$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_20$) and then vortexed. 200 µL of colorless 4 mg/mL ortho-nitrophenylβ-galactoside (ONPG) was added to begin the reaction. The reaction was stopped when tubes became visibly yellow by adding 250 µL 1M $NaCO_3$ and vortexing. The time taken to achieve the color change was recorded. Permeabilized cells are then pelleted for 4 minutes at 8,000 rpm and 300 µl of the supernatant transferred to a clear 96 well plate. The absorbance of the supernatant at 420 nm was determined by a SpectraMax 5.1 plate reader. A cell free blank (using 100 µL of NBFA) was run through the same procedure and the absorbance reading of the blank subtracted from the sample readings.

The equation used to determine Miller Units is as follows:

$$MU = 1000 * \left(\frac{Abs420}{t * v * Abs600}\right)$$

Where *Abs420* measures the color generated by cleavage of ONPG, *t* is time in minutes, *v* is volume of cell culture in milliliters, and *Abs600* is the optical density of the culture.

Results and Discussion:

Miller Assays:

G. sulfurreducens β-galactosidase production was evaluated using non-inducible vectors (Figure A.1). Plasmids pAH01::*lacZ* and pAH02::*lacZ* (both containing $RBS_{lac+7bp}$) resulted in basically no β-galactosidase production. Plasmids pAH04::*lacZ* and pAH06::*lacZ* (both containing PA1/O4/O3) resulted in the highest level of β-galactosidase production. Using this suite of non-inducible plasmids in *G. sulfurreducens* resulted in a wide range of expression levels (from about three Miller Units for pAH03 to 120 Miller Units for pAH06) but had a slightly different pattern of expression and vastly reduced Miller Units as compared to *E. coli* and *S. oneidensis* results shown in Chapter 2. $P_{A1/O4/O3}$ is a stronger promoter than P_{lac} in all species but the impact of promoter choice seems to be the most important factor in *G. sulfurreducens* regardless of the ribosome binding site (RBS). In *S. oneidensis* and *E. coli*, using the very strong RBS_{II} results in higher β-galactosidase expression than either promoter combined with weaker RBS_{lac} whereas in *G. sulfurreducens*, using $P_{A1/O4/O3}$ combined with the RBS_{lac} still results in higher expression than when using the P_{lac} promoter combined with RBS_{II}. The lower level of β-galactosidase production in *G. sulfurreducens* allows the highest expression vector (pAH06::*lacZ*) to be stable in a non-repressed background unlike in *S. oneidensis* or *E. coli* where the high level of expression caused that plasmid to mutate.

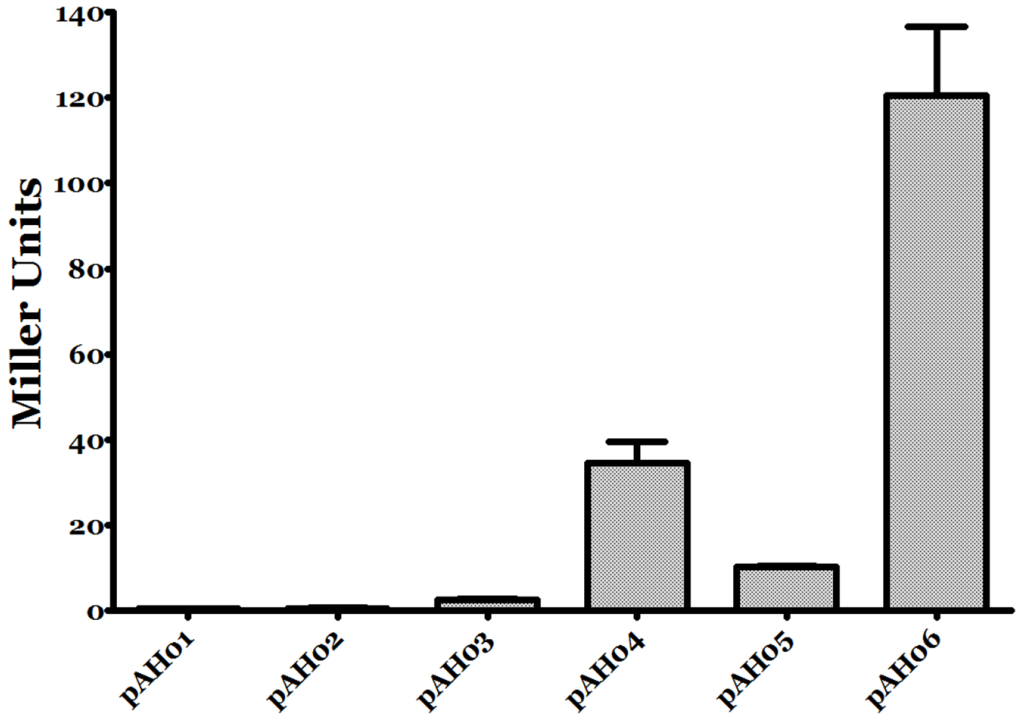

Figure A.1 – *G. sulfurreducens* Miller assay with non-inducible *lacZ* vectors
Vectors contain lacZ in the multiple cloning site. Assays were performed using biological triplicates on at least two separate occasions

G. sulfurreducens β-galactosidase production was also evaluated using inducible vectors (Figure A.2). These vectors contain the promoter and coding sequence for *lacI*^{q} on the backbone and therefore produce LacI repressor protein to control *lacZ* expression from vector promoters P_{lac} and $P_{A1/O4/O3}$. Isopropyl β-D-1-thiogalactopyranoside (IPTG) is added to bind LacI and alleviate repression. pAH15 uses P_{lac} and RBS_{II} to drive transcription and translation of *lacZ* whereas pAH16 uses $P_{A1/O4/O3}$ and RBS_{II}. $P_{A1/O4/O3}$ is a more inducible and repressible promoter than P_{lac} as demonstrated in Chapter 3 and seen in the difference of fold induction in Figure A.2 (~2 for pAH15 vs. ~10 for pAH16). The fully induced Miller Units for pAH15 and pAH16 are higher than for the same promoter/RBS combinations without *lacI*^{q} (pAH05 and pAH06). This is contrary to results shown for *S. oneidensis* where all Miller Units were lower when LacI was part of the system. For *G. sulfurreducens*, it may be that by allowing cells to grow to a robust density before induction with IPTG, more cellular resources can be devoted to production of β-galactosidase.

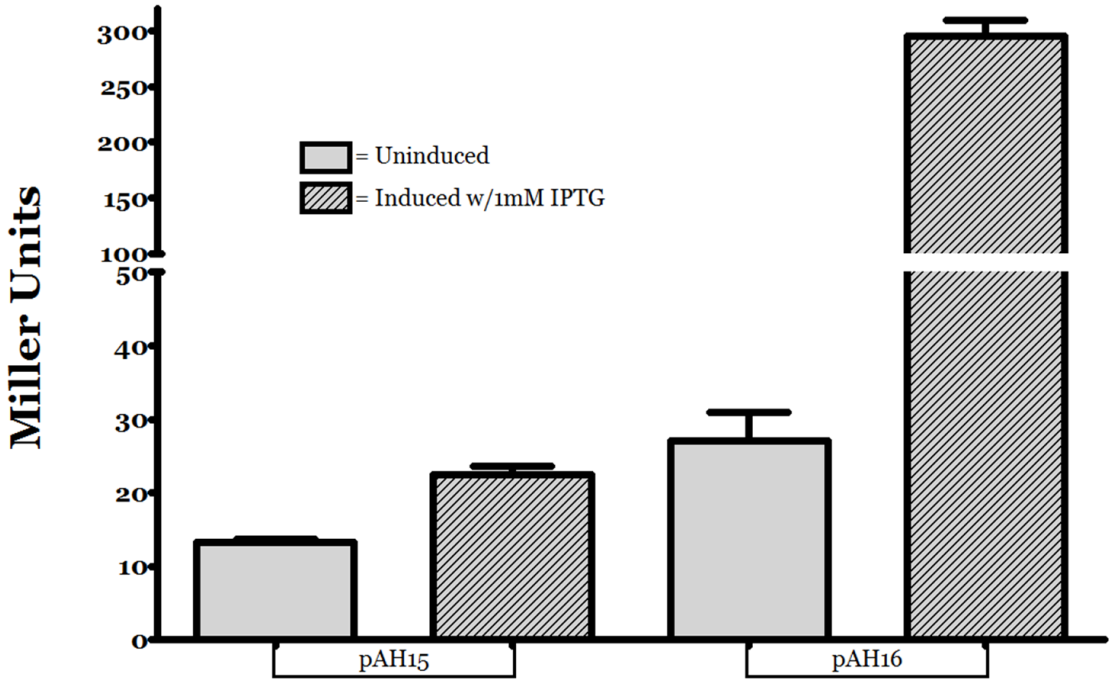

Figure A.2 – *G. sulfurreducens* Miller assay with *lacI*^{q} vectors

Vectors contain *lacI*^{q} on the backbone and *lacZ* in the multiple cloning site. Assays were performed twice using biological triplicates and expression was induced in early exponential phase by addition of 1 mM IPTG.

Conclusion:

A range of expression levels and inducible expression can be achieved in G. sulfurreducens using the vectors constructed in this study. $P_{A1/O4/O3}$ is a stronger promoter than P_{lac} and RBS_{II} is a stronger RBS than RBS_{lac} in *G. sulfurreducens* as demonstrated in both *E. coli* and *S. oneidensis*. Protein production levels resulting from plasmid *lacZ* expression are approximately 100 fold lower in *G. sulfurreducens* and may reflect poor response to plasmids, promoters, RBS, or may reflect that *G. sulfureducens* is not as robust of a protein producer as *S. oneidensis*. Future expression vector construction work may benefit from the knowledge that *G. sulfurreducens* is sensitive to less optimal RBS spacing that *S. oneidensis* can overcome as well the knowledge that $p_{A1/O4/O3}$ is a strong promoter and that LacI repression and IPTG induction can be successfully utilized in *G. sulfurreducens.*