Transcriptional Regulation of *Shewanella oneidensis* Using Native TMAO-Inducible Promoters in a Plasmid-Free System

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Chapter 1
Introduction

As our world continues developing, we are faced with problems related to energy, pollution, and water quality. Growing populations and dwindling resources will only amplify the urgency of these issues. We need solutions for these global challenges and biotechnological advances may lead to effective strategies. The utility of microbes in biotechnology has already been realized in applications ranging from biofuel production to medical treatments. Recent advances in genomics and synthetic biology will likely facilitate the continued use of microbes in addressing global problems. However, not all bacteria are suitable for large-scale biotechnological applications. An ideal candidate would be easily cultured, have a sequenced genome, and be genetically tractable. Members of the genus *Shewanella* have attracted attention due to their unique respiratory capabilities and potential use in biotechnological applications.

Shewanella species are gram negative facultative anaerobes that are generally found in aquatic environments. They are most well known for their ability to use an extensive number of terminal electron acceptors, enabling their survival in diverse environments. Of the roughly 20 known compounds Shewanellae can respire, many are insoluble and some are toxic pollutants. Most *Shewanella* species are also capable of growing at low temperatures. Although many thrive at ~4°C, their optimal temperature is usually higher. Their respiratory diversity coupled with their ability to grow at low temperatures allows them to occupy many habitats (1).

Shewanella oneidensis MR-1 has emerged as a model organism due to its robust growth, sequenced genome, genetic tractability, and its metabolic diversity. It was isolated from Lake Oneida in 1988 and has since been the focus of many studies (1). We now have a better understanding of how electrons from carbon oxidation travel from the inside of the cell to an extracellular substrate, a process known as extracellular electron transport (EET). Identification of the genes and proteins involved in the many respiratory pathways of *S. oneidensis* have helped understand the physiology of Shewanellae and other dissimilatory metal reducing bacteria.

The ability of *S. oneidensis* to respire insoluble substrates led to the discovery of its ability to interface with an electrode. *S. oneidensis* can oxidize a carbon source and direct electrons outside of the cell to an electrode, generating current in the process. There is also interest in the reverse process in which electrons from the electrode are transferred into the cell. Their ability to interact with an electrode presents opportunities for using *S. oneidensis* in applications related to microbial fuel cells and electrosynthesis.

In order to maximize the performance of *S. oneidensis* in biotechnological applications, genetic manipulation will likely be necessary. For example, increasing the flux through extracellular electron transport pathways and upregulating genes needed for electrode colonization may optimize its performance. Tools for precisely regulating gene expression can be used to accomplish this. The work presented in this thesis demonstrates the utility of an inducible promoter system native to *S. oneidensis* that can be used to rapidly induce gene expression in response to trimethylamine *N*-oxide (TMAO). The ability to replace a native promoter with the inducible promoter, within the genome, rather than relying on a plasmid, is also demonstrated.

Chapter Two
TMAO in the Environment and as an Electron Acceptor for *Shewanella oneidensis*

Introduction

Trimethylamine *N*-oxide (TMAO) is a small molecule that participates in a variety of functions throughout different domains of life. One of its most well known and studied roles is as an osmolyte in marine organisms (2-4). Living in a high salinity environment necessitates a strategy for preventing water loss. Different molecules including urea, amino acids, sugars, and methylamines help maintain osmotic homeostasis. TMAO has been found in animals ranging from sharks to mollusks and can accumulate to high concentrations in their tissues (up to 500 mM in some organisms) (2,3).

In addition to its role as an osmolyte, TMAO also stabilizes proteins in the presence of high urea concentrations (4). Similar to TMAO, urea helps maintain osmotic pressure in marine organisms. However, high concentrations of urea can lead to protein denaturation through its interactions with protein backbones, making the unfolded state more thermodynamically favorable (5). Unlike urea, TMAO is a protective osmolyte and pushes the equilibrium towards the folded state. TMAO stabilizes proteins by altering the surrounding water structure (4).

The stabilizing effects of TMAO may also be important for animals that live in more extreme environments. For example, a study examining the osmolyte composition of deep sea-dwelling animals found that the concentration of TMAO increased with depth. The researchers concluded that TMAO might help protect proteins under high pressures (6). TMAO may also help animals survive cold water temperatures (7). Seasonal fluctuations in TMAO concentrations were observed in rainbow smelt blood, with higher concentrations occurring in the winter. During the winter, animals are faced with freezing temperatures and must have a way to protect their proteins. It is possible that TMAO is synthesized in response to cold temperatures and influences the freezing point depression (7).

Despite the protective role TMAO plays in certain animals, high TMAO concentrations in humans are correlated with increased risk of certain diseases. In humans, TMAO is produced from choline and L-carnitine, which are primarily obtained from the diet and are abundant in foods such as red meat. Gut microflora are responsible for the conversion of choline and L-carnitine to trimethylamine (TMA), which is then oxidized to TMAO by hepatic flavin monooxygenase (8). High levels of TMAO are thought to lead to cardiovascular disease by

effecting hormone and lipid homeostasis (8). Diseases such as renal failure, diabetes and colorectal cancer have also been correlated with high TMAO concentrations (4).

In humans, TMAO is a waste product from bacterial metabolism of choline and L-carnitine. However, for some bacteria, TMA and TMAO are important nutrients. For example, *Alphaproteobacteria Candidatus* Pelagibacter ubique HTCC1062 and *Betaproteobacteria Methylophilales* sp. HTCC2181 generate ATP from TMAO oxidation (9,10). In these organisms, the methyl groups of TMAO are oxidized to CO_2, yielding ATP in the process. The TMAO derived carbon is not incorporated into biomass. TMAO and TMA can also serve as a nitrogen sources for some bacteria, such as the marine heterotroph *Ruegeria pomeroyi* DSS-3 (2). Identification of a TMAO specific transporter in *R. pomeroyi* that is widespread in marine organisms suggests it is an important nutrient. In addition to its use for energy generation and nitrogen, it is also an important electron acceptor for many bacteria, including species of *Escherichia, Vibrio,* and *Shewanella* (11-19).

TMAO respiration in *E. coli, V. parahaemolyticus,* and *S. oneidensis* occurs via homologous pathways. A three-component system is responsible for sensing and responding to TMAO that enters the periplasm (figure 1). TorT, which is found in all three species, has been shown to bind to TMAO in *Escherichia* and *Vibrio* species, but has not been as thoroughly characterized in *Shewanella* species (14,15). In *Escherichia* and *Vibrio* species, TorT binds TMAO, forming a complex that activates TorS, a histidine kinase. The crystal structure of the *V. parahaemolyticus* TorT-TorS complex with and without a TMAO analog was recently solved (14). The results suggest TorT and TorS interact in the presence and absence of TMAO, but TorS is only activated when TMAO is included in the complex (14). The *E. coli* TorT is hypothesized to act in the same way (15).

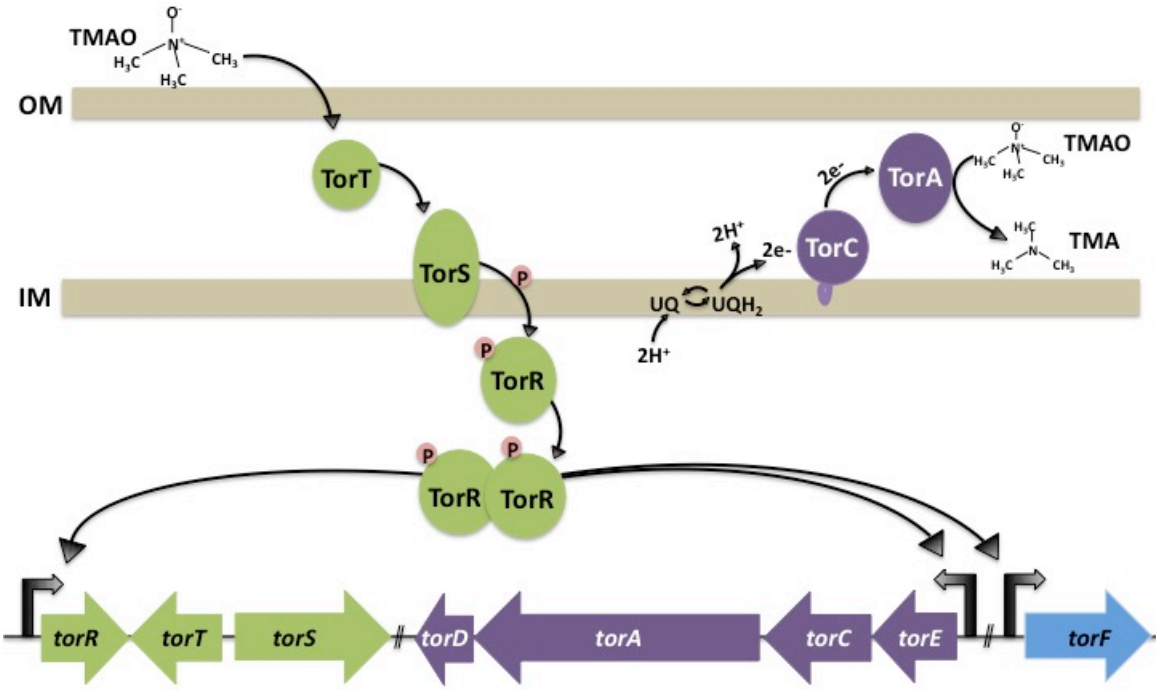

Figure 1. The Tor pathway for TMAO respiration and the genes regulated by the Tor three-component system.

The *S. oneidensis* TorT protein sequence has 34% and 35% percent identity with the *E. coli* and *V. parahaemolyticus* TorT protein, respectively. Several important residues within the binding pocket of TorT have been identified and are widely conserved across species, including *S. oneidensis* (figure 2). Three residues (Trp45, Tyr71, Asp42) interact with the oxygen atom in TMAO and three aromatic residues (Tyr44, Trp140, Tyr252) interact with its quaternary amine (14). Also conserved within the TorT binding pocket are the glycine and proline residues in the GPXXXGG motif that gives rise to a loop that connects two TorT domains (14). In addition to the mentioned residues, Baraquet et al. identified a negative mutant in which the leucine at position 80 is substituted for a proline (15). Although this residue is conserved in *V. parahaemolyticus* and other *Shewanella* species, it is not conserved in *S. oneidensis,* raising the question of whether TorT has maintained its TMAO sensing role.

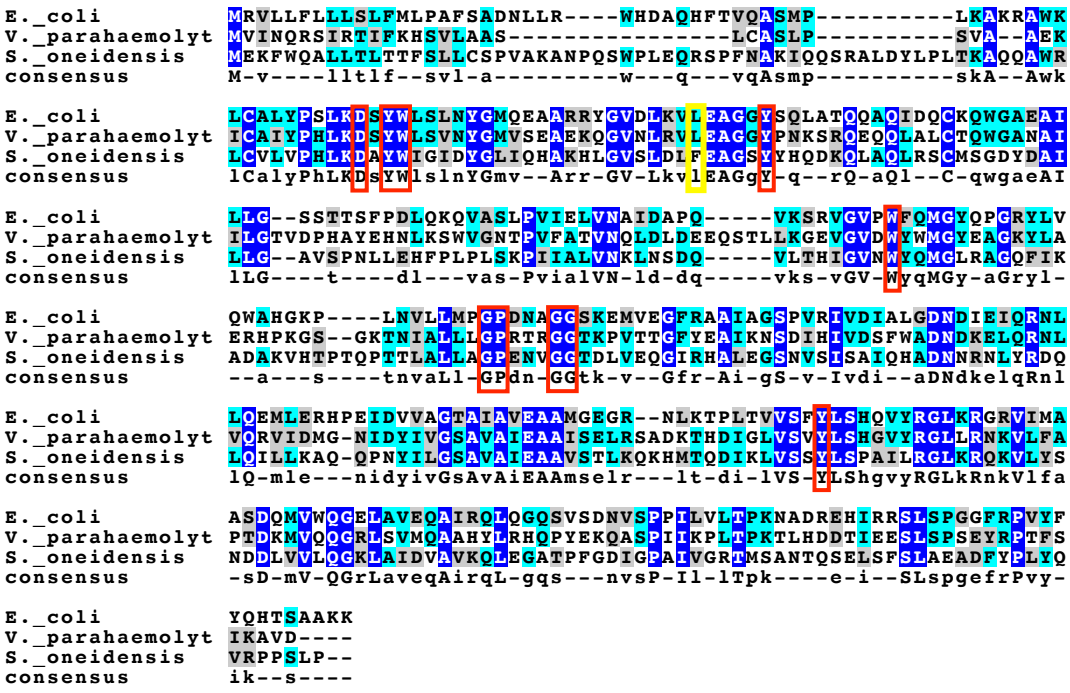

Figure 2. Multiple sequence alignment of *E. coli, V. parahaemolyticus,* and *S. oneidensis* TorT sequences. Residues important for TMAO binding are boxed in red. The residue Baraquet et al. found necessary for TMAO binding is boxed in yellow.

Activated TorS phosphorylates and activates TorR, which dimerizes and binds to hexameric repeats upstream of *torR*, resulting in negative autoregulation. TorR also binds to repeats upstream of the *torECAD* operon, activating its expression (12,19). *torE* encodes a short protein of unknown function that is similar to proteins in the NapE superfamily of bacterial nitrate reductase proteins. It is not found in *E. coli* and *V. parahaemolyticus*. *torC* encodes a pentaheme *c*-type cytochrome that obtains electrons from the ubiquinone pool (11). Unlike *S. oneidensis,* the *E. coli* TorC is reduced by electrons from the menaquinone pool (20). Once reduced, TorC is oxidized by TorA, the molybdenum containing periplasmic reductase. *torD* encodes a TorA specific chaperone. Once these proteins properly located, TMAO is respired to TMA within the periplasm.

TorR also regulates expression of *torF,* which encodes a protein of unknown function (12). Hexameric repeats are found within the promoter region of *torF,* and it is highly induced in the presence of TMAO and dimethyl sulfoxide (DMSO) (12,21). *torF* encodes a protein of 248 amino acids that is annotated as a porin and predicted to localize to the outer membrane. *Vibrio*

and *Escherichia* species do not have a *torF* homolog. *torF* is not conserved in all *Shewanella* species, and homologs are found in some *Colwellia* and *Ralstonia* species.

The Tor pathway is not the only route by which TMAO reduction occurs. Some organisms have a single pathway that is used for respiration of both TMAO and DMSO (22). Similar to TMAO, DMSO is a small molecule that is abundant in the ocean and can serve as an electron acceptor for certain bacteria. Due to the similar structures of TMAO and DMSO, some bacteria, such as certain *Rhodobacter* species, use that same reductase to respire both compounds (22). Other bacteria, including *E. coli* and *S. oneidensis*, have a distinct pathway for DMSO reduction. Although *E. coli* has the Tor pathway, the DMSO reductase has a weak affinity for TMAO (22). Growth of *S. oneidensis* on TMAO using the DMSO reductase has been alluded to, but not rigorously demonstrated (13).

This chapter addresses questions related to TMAO respiration in *S. oneidensis.* The roles of TorT, TorF, and the DMSO reductase in this process were examined by constructing mutant strains. The data illustrate the necessity of TorT for TMAO respiration, the promiscuity of the DMSO reductase for TMAO, and the need for additional studies to understand the role of TorF.

Materials and Methods

Reagents

Phusion High- Fidelity DNA polymerase, enzymes, Antarctic phosphatase, and T4 ligase were purchased from New England Biolabs (Ipswich, MA). Reactions were set up according to the manufacturer's protocol. Gel extraction and plasmid mini-prep kits were purchased from Qiagen (Valencia, CA). The PureLink PCR clean up kit was purchased from Invitrogen (Carlsbad, CA). Primers and gene blocks were ordered from Integrated DNA Technologies via the University of Minnesota Genomics Center (UMGC). Sanger sequencing was carried out at the UMGC facility.

Culturing conditions

Strains used in this study are listed in table 1. All *E. coli* strains were grown at 37°C on Luria-Bertania (LB) media in the presence of 50 µg/mL kanamycin (Km). *E. coli* WM3064 strains were also supplemented with diaminopimelic acid (DAP). *S. oneidensis* strains were grown at 30°C on either LB or *Shewanella* Basal Medium (SBM). One liter of SBM contains: 0.225 g K_2HPO_4, 0.225 g KH_2PO_4, 0.46 g NaCl, 0.225 g $(NH_4)_2SO_4$, 0.117 10 g $MgSO_4 \cdot 7H_2O$, 100mM HEPES buffer. SBM was supplemented with vitamins, minerals, and casamino acids

(CAA, Fisher Scientific) for a final concentration of 5 mL/L vitamins and minerals and 0.05% CAA. One liter of mineral mix contained: 3 g $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g $CoCl_2 \cdot 6H_2O$, 0.13 g $ZnCl_2$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.01 g $AlK(SO_4)_2 \cdot 12H_2O$, 0.01 g H_3BO_3, 0.025 g Na_2MoO_4, 0.024 g $NiCl_2 \cdot 6H_2O$, and 0.025 g $Na_2WO_4 \cdot 2H_2O$. One liter of vitamin mix contains: 0.002 g biotin, 0.002 g folic acid, 0.01 g pyridoxine HCl, 0.005 g thiamine, 0.005 g nicotinic acid, 0.005 g pantothenic acid, 0.0001 g B-12, 0.005 g p-aminobenzoic acid, and 0.005 g thioctic acid. Lactate was added to SBM medium for a final concentration of 20 mM. When grown anaerobically, fumarate or TMAO were added to a final concentration of 40 mM. Glycerol stocks stored at -80°C were used to streak LB agar plates and obtain isolated colonies. Medium for overnight liquid cultures was inoculated with a single colony and grown overnight, shaking at 225 rpm, at the appropriate temperature.

Table 1. Strains and plasmids used in this chapter

***E. coli* Strains**	**Genotype/Characteristic**	**Reference**
UQ950	DH5α λ(*pir*) cloning host; F^-Δ(*argF-lac*)169 Φ80d*lacZ58*ΔM15 *glnV*44(AS) *rfbD1 gyrA96*(Nal^R) *recA1 endA1 spoT1 thi-1 hsdR17 deoR λpir*$^+$	(23)
WM3064	Donor strain for conjugation; *thrB1*004 *pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD)567 ΔdapA*1341::[*erm pir*(wt)]	(23)
***S. oneidensis* Strains**	**Genotype/Characteristic**	**Reference**
JG274	WT MR-1	(24)
JG3278	Δ*torECADF*	This Study
JG3293	Δ*torF*	This Study
JG3396	Δ*torT*	This Study
JG3462	Δ*torT*, genomic P*torF::lacZ*	This Study
JG3544	Δ*torECADF*Δ*dmsEFABGH (*Δ*tor*Δ*dms*)	This Study
Plasmids	**Genotype/Characteristic**	**Reference**
pEB007	Used to insert promoter-*lacZ* fusions into the genome at the *glmS* site	Evan Brutinel
pSMV3	Used to generate gene deletions.	(23)

Generation of mutants

In frame deletion mutants of *torT, torF,* and *torECADF* were generated by homologous recombination. Briefly, 1 kb regions upstream and downstream of the portion to be deleted were amplified, ligated together, and inserted into pSMV3, which can replicate in *E. coli,* but not

Shewanella. Additionally, pSMV3 contains a kanamycin cassette and *sacB*, which results in the lethal polymerization of sucrose. *E. coli* UQ950 was transformed with the resulting plasmid and PCR was used to screen for colonies that maintained plasmid with the proper insert. The plasmid was extracted, sequence verified and used to transform WM3064, a DAP auxotroph and the plasmid donor strain. *Shewanella* from an LB +Km plate and WM3064 from an LB +DAP plate were cross struck on an LB + DAP plate to allow for plasmid uptake in *Shewanella*. The plate was incubated at 30°C for at least 6 hours. A portion of cells was transferred to LB + Km to select for the first recombination event. An isolated colony was used to streak for single colonies on a fresh LB + Km plate, one of which was grown overnight in LB liquid media for the purpose of recovery. Dilutions of the liquid culture were plated on 5% sucrose and grown at room temperature until colonies appeared. Colonies from the sucrose plate were screened to check for the deletion and plated on LB and LB + Km to screen for colonies that successfully underwent a second recombination event and resulted in the desired deletion. Glycerol stocks were made and the upstream and downstream junction was sequenced to verify the deletion.

Identification of P*torECAD* and P*torF*

The promoter regions of P*torECAD* and P*torF* had been characterized previously and led to the identification of the transcriptional start sites and TorR binding sites (12). Guided by that work, the transcriptional start site (TSS) and the upstream region were isolated and used in this work. P*torF* consisted of the region 120 bp upstream of the *torF* TSS and P*torECAD* consisted of 160 bp upstream of the *torECAD* TSS.

Plasmid construction of promoter-*lacZ* fusions and insertion into the genome

A pSMV3 derivative modified by Evan Brutinel, pEB007, was used to insert the promoter-*lacZ* fusions in to the genome upstream of *glmS.* Insertion into the genome occurs via homologous recombination, facilitated by regions flanking the promoter-*lacZ* fusions that are homologous to the sequence flanking the insertion site. A gene block of the *torF* promoter was ordered, and the *torECAD* promoter was obtained using colony PCR. The promoters were cloned into pEB007 using restriction sites upstream of *lacZ.* The lac RBS was maintained in the plasmid, so only the sequence upstream of the transcriptional start sites was cloned into the plasmid. The promoters were inserted into the genome using the process for gene deletion outlined above.

Miller assays

Miller assays were used to assay promoter activity and strength. Overnight LB cultures that had been inoculated from single colonies, were spun down and resuspended to an OD of 1.0 in LB. 10 ml cultures of medium were inoculated with 75 µl of the resuspension. TMAO was added to the culture to a final concentration of 1 mM. The cultures were grown at 30°C while shaking until they reached an OD of about 0.1. At this point, the OD was recorded, and 100 µl of the culture were added to a solution containing 900 µl of Z-buffer (60 mM Na_2HPO_4-$7H_2O$, 40 mM NaH_2PO_4 .H_2O, 10 mM KCl, 1 mM $MgSO_4$ -$7H_20$), 3.5 µl of 2-mercaptoethanol, 20 µl of sodium dodecyl sulfate, and 20 µl of chloroform. 200 µl of 4mg/mL *ortho*-Nitrophenyl-β-galactoside was added to begin the reaction. Once the solution was visibly yellow, 250 µl of 2M Na_2CO_3 was added to stop the reaction. After spinning the mixtures at maximum speed for four minutes, the absorbance at 420 nm was recorded. Miller units were calculated as follows, where OD is the OD_{600}, V is volume of cells, t is time in minutes, and A is the absorbance at 420 nm:

$$MU = \frac{1000}{Time * Volume * OD_{600}} * A_{420}$$

Fold inductions were calculated relative to the Miller unit corresponding to no added TMAO.

Growth characterization of mutants

SBM with 20 mM lactate and 40 mM TMAO was inoculated with washed cells from LB overnight cultures to an OD_{600} of ~0.05. Growth of the biological triplicates was followed over time by monitoring the OD_{600}.

Results and Discussion

The DMSO reductase can reduce TMAO in *S. oneidensis*

A strain deficient in TMAO respiration was needed for experiments discussed in subsequent chapters. Deletion of *torECAD* was thought to be sufficient for preventing growth on TMAO. However, when grown on TMAO, Δ*torECAD* reaches the same yield as WT, albeit more slowly (figure 3).

Most literature discussing TMAO respiration in *S. oneidensis* only discusses the Tor pathway. However, growth of a *torA* mutant on TMAO has been reported in addition to a reductase with activity toward TMAO and DMSO in *S. oneidensis* (13). Deletion of the DMSO

and TMAO reductases inhibits growth with TMAO as the sole electron acceptor. Deletion of the DMSO reductase does not result in a growth defect, indicating the Tor pathway is the primary mode of TMAO reduction in *S. oneidensis.*

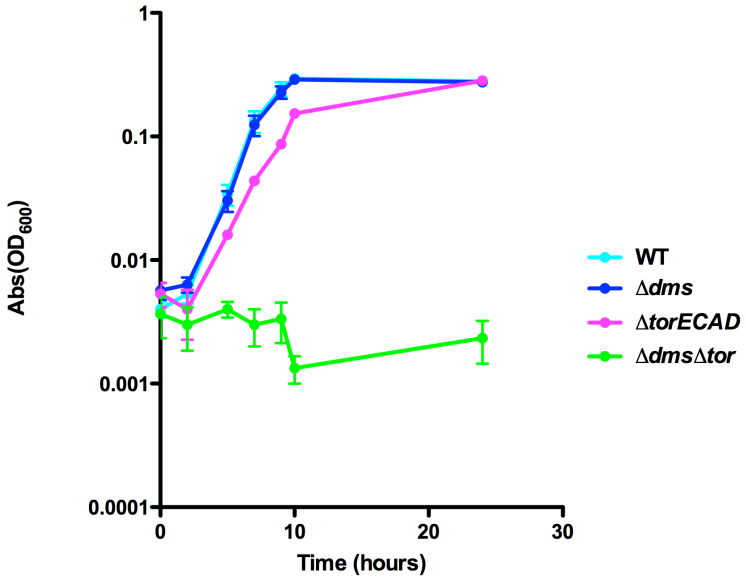

Figure 3. Anaerobic growth in SBM with 20 mM lactate and 40 mM TMAO.

TorT is essential for sensing TMAO and activating *tor* gene expression in *S. oneidensis*

Previous studies in *S. oneidensis* have noted the importance of TorT for TMAO respiration, however the lack of conservation of an apparently crucial residue in *E. coli* warranted further investigation (11). Additionally, studies in *S. oneidensis* examining its growth on TMAO without *torT* have not been presented. To investigate the role of TorT in *S. oneidensis,* P*torF* and P*torECAD* transcriptional activation and growth on TMAO were assessed in a *torT* mutant. The mutant has a similar growth rate to Δ*torECAD*, and a *ΔdmsΔtorT* mutant does not grow with TMAO as the terminal electron acceptor (figure 4). Miller assay results demonstrate that TorT is required for activation of both promoters (figure 5). The results presented here and the conservation of residues needed for TMAO binding suggest TorT functions the same in *S. oneidensis* as in *E. coli* and *V. parahaemolyticus.*

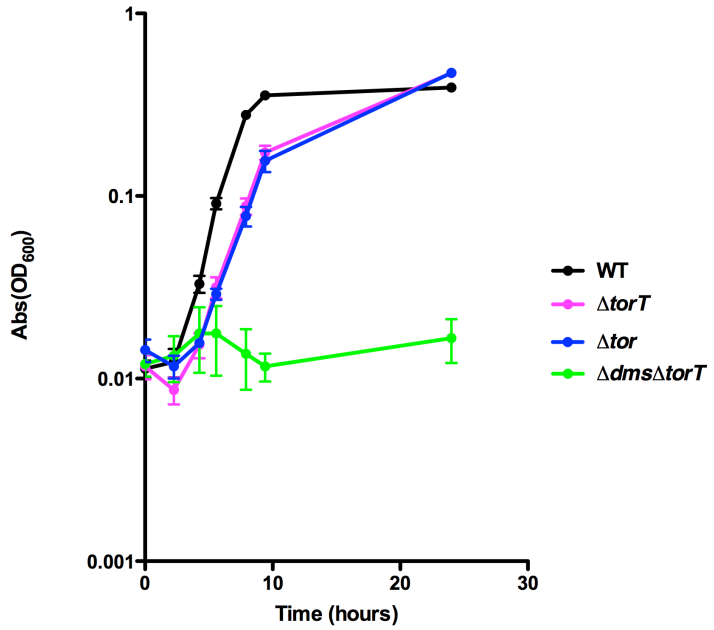

Figure 4. Anaerobic growth in SBM with 20 mM lactate and 40 mM TMAO.

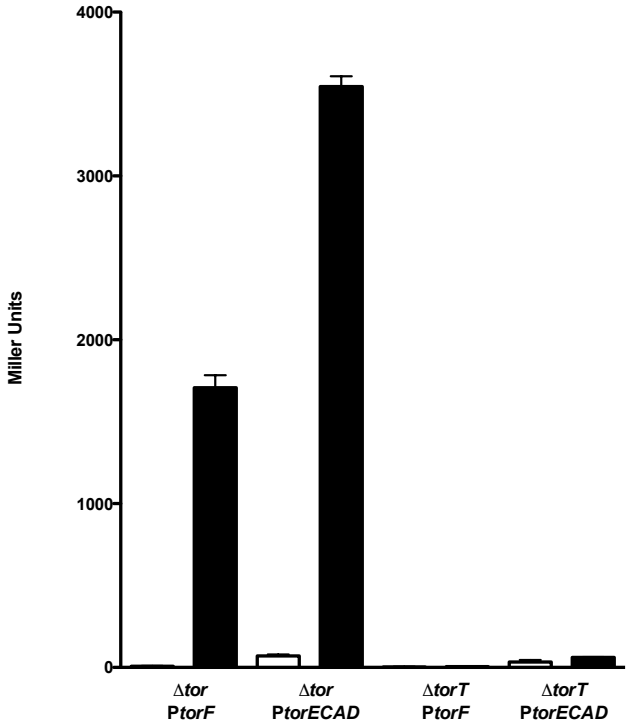

Figure 5. Miller assay data for strains grown aerobically in LB in the presence (black) and absence (white) of 1 mM TMAO. The genotype and promoter driving *lacZ* expression are listed.

Deletion of *torF* does not alter growth on TMAO or DMSO

torF expression has been shown to be highly up-regulated in the presence of TMAO and DMSO, however its function is unknown (12, 21). Deletion of *torF* did not result in a growth defect when there were stoichiometric equivalents of lactate and TMAO or DMSO (figure 6). A small difference in survival between *ΔtorF* and WT was seen in the TMAO limited condition with Δ*torF* maintaining a higher OD in stationary phase compared to WT (figure 7). Over-expression of *torF* by introducing a plasmid with *torF* into WT did not enhance the observed difference under acceptor limiting conditions (figure 8).

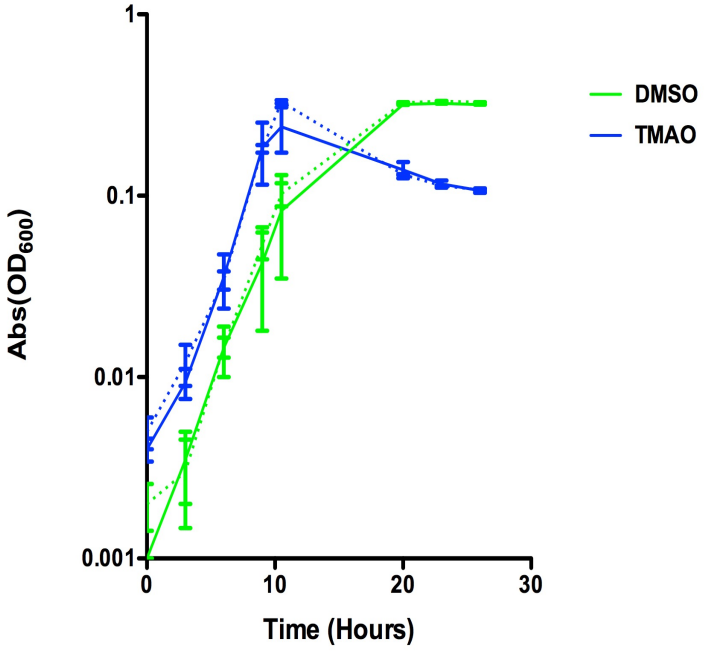

Figure 6. Anaerobic growth of WT (solid lines) and *ΔtorF* (dashed lines) in SBM with 20 mM lactate and 40 mM TMAO or DMSO.

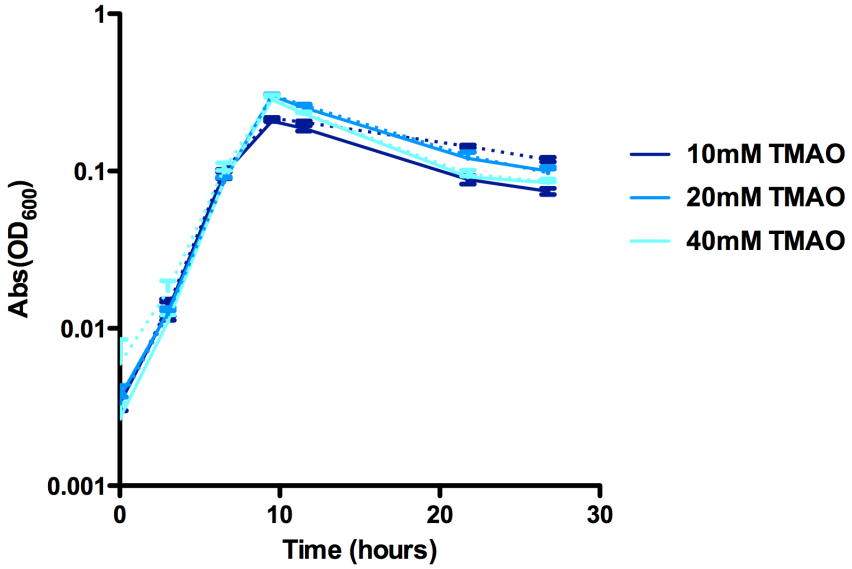

Figure 7. Anaerobic growth of WT (solid lines) and *ΔtorF* (dashed lines) in SBM with 20 mM lactate and varying concentrations of TMAO.

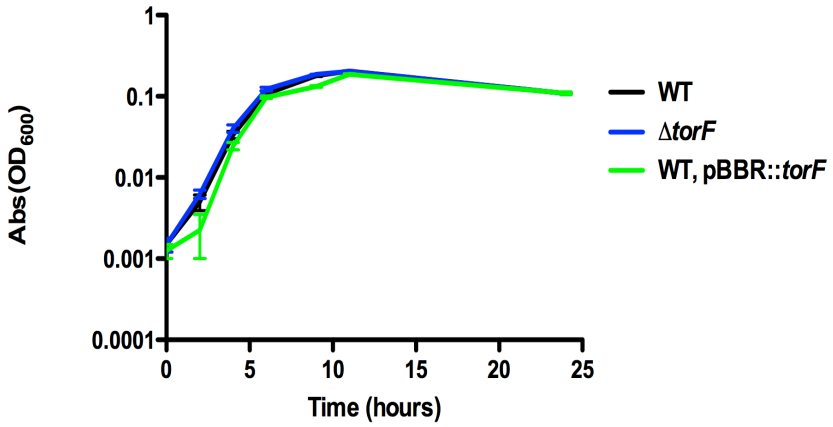

Figure 8. Anaerobic growth in SBM with 20 mM lactate and 40 mM TMAO.

Conclusion

TMAO respiration via the DMSO reductase has not been well documented in *S. oneidensis*. Given the similarity of DMSO and TMAO, and the ability of some organisms to use the same reductase to respire both compounds, it is not surprising that the *S. oneidensis* DMSO reductase is active towards TMAO. The data presented in this chapter demonstrated the capability

of *S. oneidensis* to use two distinct pathways to respire TMAO. However, deletion of the DMSO reductase did not result in a growth defect, indicating TorA is likely the primary reductase.

The necessity of TorT for TMAO sensing and respiration has been documented in *E. coli* and *V. parahaemolyticus,* although not as thoroughly in *S. oneidensis*. The residues necessary for TMAO binding are highly conserved across species, including in *S. oneidensis*. Although the leucine residue reported by Baraquet et al. is not conserved in the *S. oneidensis* TorT protein, the data presented in this thesis and the widespread conservation of other key residues suggest TorT has maintained its TMAO binding ability in *S. oneidensis* (15).

In addition to TorT, studies addressing the role of TorF in *S. oneidensis* are lacking. Although *torF* is highly induced in the presence of TMAO, its role in TMAO respiration is unknown. Under standard TMAO growth conditions (20 mM lactate and 40 mM TMAO) deleting *torF* does not appear to have an effect. *torF* expression was reported to increase in the presence of DMSO. However, miller assays with one of the P*torF::lacZ* strains grown in the presence of 40 mM DMSO do not support this observation (data not shown). Additionally, growth of a *torF* mutant is not impaired on DMSO. In TMAO limiting conditions, the *torF* mutant did not die as quickly as WT, the reason for which is unknown. Additional studies looking at *torF* expression under a range of conditions, or studies looking for phenotypes of a *torF* mutant under various conditions would be helpful for understanding its function.

Chapter 3
Using the Tor Pathway to Regulate Expression of a Gene of Interest

Introduction

The ability to modulate gene expression is important not only for large-scale biotechnological applications, but also for understanding the physiology of an organism. Advances in synthetic biology have led to a variety of tools that can be used to regulate gene expression at the transcriptional and translational level. Using these tools in *S. oneidensis* could help improve its performance in biotechnological applications by enabling manipulation of fluxes through desired pathways. While many non-native promoters, such as the *lac* and arabinose promoters, are functional in *S. oneidensis,* a native inducible promoter system used to drive expression of a gene of interest has yet to be demonstrated (25, 26).

While manipulating gene expression is important for biotech applications, it is critical for cells to modulate gene expression in their environment in response to fluctuating conditions. As a result, cells have native inducible promoter systems that respond to various stimuli (27). The Tor system described in the previous chapter is an example of a native inducible system that could be used to control expression of a gene of interest with TMAO.

The *torECAD* operon and *torF* gene are induced in the presence of TMAO when activated TorR binds to specific sites within their promoter regions. The *S. oneidensis* TorR protein is highly specific to the hexameric repeats, and is unable to bind when a mutation is introduced (12). Within the *S. oneidensis* genome, the repeats are only found upstream of *torECAD*, *torF,* and *torR* (figure 9). These attributes are ideal for use in biotech applications involving *S. oneidensis.*

Expression driven by the three promoters in response to TMAO is influenced by the location of the repeats. For example, the TorR binding site in the *torR* promoter lies within the -10 promoter element and results in negative autoregulation (12). The binding sites are upstream of the -10 and -35 regions in the *torECAD* and *torF* promoters, and TorR binding induces gene expression, although to different degrees. Initial studies using real time PCR to compare expression found *torF* is induced 60-fold in the presence of TMAO compared to a 20-fold induction of *torECAD* (25).

Figure 9. Promoter region of *torF* (A), *torECAD* (B), and *torR* (C). TorR binding sites are boxed and hexameric repeats are bold. The putative -10 element is underlined, transcriptional start site is blue, and the start codon is red.

DMSO has also been shown to influence expression of *torF* and *torECAD.* Microarray data demonstrated an 8-fold expression increase for *torF* and increased expression, ranging from 0.67 to 6.4-fold, for the *torECAD* genes in the presence of DMSO (21). In this experiment, the most highly induced gene in the DMSO respiration pathway was only upregulated 2.8-fold in the presence of DMSO. The microarray data suggest the tor promoters may be responsive to DMSO.

The utility of the Tor system for inducing a gene of interest is discussed in this chapter. Miller assays were used to characterize the *torECAD* and *torF* promoters. Several conditions were tested to examine responses in different mediums with varying TMAO concentrations in the presence and absence of oxygen.

Materials and Methods

Reagents

Phusion High- Fidelity DNA polymerase, enzymes, Antarctic phosphotase, and T4 ligase were purchased from New England Biolabs (Ipswich, MA). Reactions were set up according to the manufacturer's protocol. Gel extraction and plasmid mini-prep kits were purchased from Qiagen (Valencia, CA). The Invitrogen PureLink PCR clean up kit was used (Carlsbad, CA).

Primers and gene blocks were ordered from Integrated DNA Technologies via the University of Minnesota Genomics Center (UMGC). Sanger sequencing was carried out at the UMGC facility.

Culturing conditions

Strains used in this study are listed in table 2. All *E. coli* strains were grown at 37°C on Luria-Bertania (LB) media and supplemented with kanamycin or DAP when appropriate. *S. oneidensis* strains were grown at 30°C on either LB or SBM. 20 mM lactate served as the carbon source in SBM and fumarate was added to a final concentration of 40 mM when cells were grown anaerobically. Vitamins, minerals and CAA were added to SBM. Glycerol stocks stored at -80°C were used to streak LB agar plates and obtain isolated colonies. Medium for overnight liquid cultures was inoculated with a single colony and grown overnight, shaking at 225 rpm, at the appropriate temperature.

Table 2. Strains and plasmids used in this study

***E. coli* Strains**	**Genotype/Characteristics**	**Reference**
UQ950	DH5α λ(*pir*) cloning host; F^-Δ(*argF-lac*)169 Φ80d*lacZ58*ΔM15 *glnV*44(AS) *rfbD1 gyrA96*(Nal^R) *recA1 endA1 spoT1 thi-1 hsdR17 deoR* λpir^+	(23)
WM3064	Donor strain for conjugation; *thrB1*004 *pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD)567 ΔdapA*1341::[*erm pir*(wt)]	23)
***S. oneidensis* Strains**	**Genotype/Characteristics**	**Reference**
JG274	WT MR-1	(24)
JG3332	WT with P*torF::lacZ* in *glmS* site	This Study
JG3333	WT with P*torECAD::lacZ* in *glmS* site	This Study
JG3334	Δ*torF* with P*torF::lacZ* in *glmS* site	This Study
JG3335	Δ*torECADF* with P*torECAD::lacZ* in *glmS* site	This Study
JG3363	Δ*tor*Δ*dms* with P*torF::lacZ* in *glmS* site	This Study
JG3364	Δ*tor*Δ*dms* with P*torECAD::lacZ* in *glmS* site	This Study
Plasmids	**Genotype/Characteristics**	**Source**
pEB007	Used to insert promoter-*lacZ* fusions into the genome at the *glmS* site	Evan Brutinel

Background strain construction

Using the *tor* promoters to induce expression means that the inducer, TMAO, will be depleted as it is respired to TMA. This attribute may be useful if only a transient induction in needed. In other applications, it may be ideal for the inducer to be present at a stable concentration. For this to occur, TMAO respiration needs to be prevented. To abolish TMAO respiration, *torECAD* and *torF* were deleted. As discussed in chapter 2, this strain uses the DMSO reductase to respire TMAO, so a Δ*dms*Δ*tor* strain was also made. The promoter-*lacZ* fusions were inserted into the WT, Δ*torECADF* (Δ*tor*), and Δ*dms*Δ*tor* strains following the procedure outlined in chapter 2.

Miller assays

After the promoter fusions were inserted into the genome, their strengths and induction conditions were determined using miller assays. Each promoter was tested aerobically and anaerobically in LB and SBM. Overnight LB cultures, were spun down and resuspended to an OD of 1.0 in the desired medium. Assays carried out in SBM were washed two times prior to resuspension. 10 ml cultures of medium were inoculated with 75 µl of the resuspension. TMAO was added to the cells to the desired concentration. The cultures were grown at 30°C while shaking until they reached an OD of about 0.1. The rest of the assay was carried out as outlined in chapter 2.

Results and Discussion

The TMAO concentration needed for induction varies between media

The promoter-*lacZ* fusions were assayed in SBM and LB over a range of TMAO concentrations. For both promoters in each background, the concentration at which induction occurred was lower when cells were grown in LB (figure 10A, 10B). Initial induction occurred around 30 µM and reached full activation around 100 µM. Promoters for cells grown in SBM were initially activated around 100 µM with maximal induction occurring around 700 µM.

LB is nutrient rich and enables faster cell growth compared to SBM. The differences between the mediums might influence cellular transcription and translation rates. It is possible that cells grown in LB have higher levels of the proteins needed for induction. Having more TorT present could enable TMAO detection at lower concentrations. Although the promoters are induced at lower TMAO concentrations in LB, expression is slightly higher in SBM once the promoters are induced.

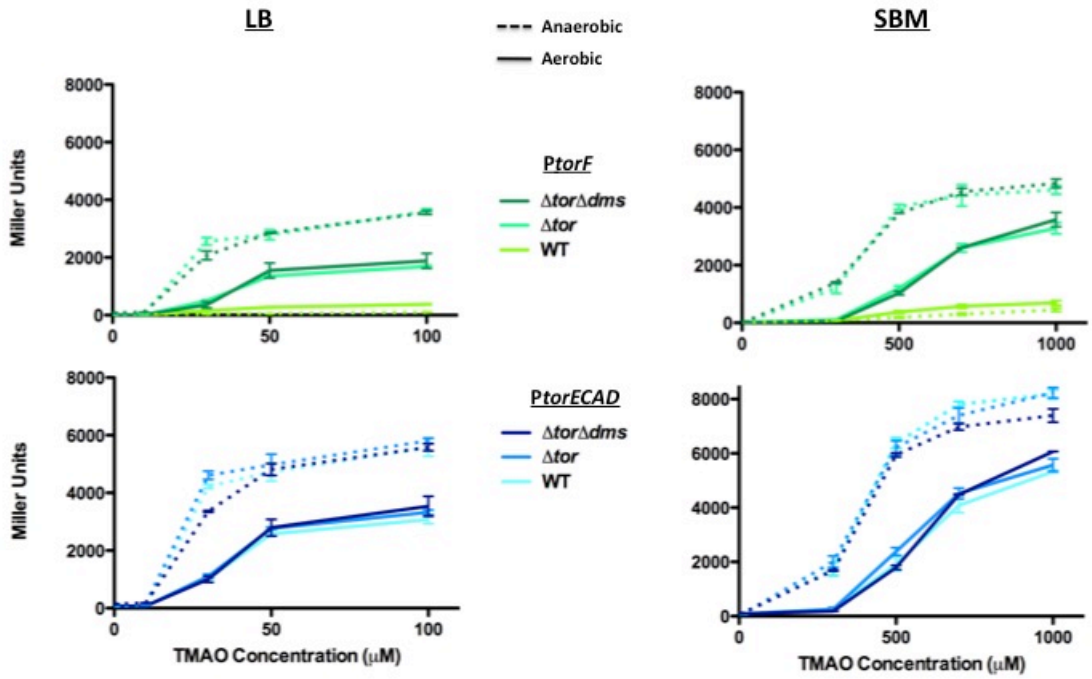

Figure 10. Miller assays performed for strains with the P*torF::lacZ* fusion and P*torECAD::lacZ* fusion.

Oxygen has an effect on the *tor* promoters

Both promoters are highly expressed aerobically and anaerobically with higher levels observed anaerobically, except for the *torF* promoter in the WT background. A similar result was observed for expression of the *E. coli torCAD* operon (16). At low cell densities, expression of *torCAD* is lower aerobically than anaerobically. Miller assays over a range of cell densities demonstrate a positive correlation between cell density and Miller units, and maximum expression was nearly the same aerobically and anaerobically (16). The Miller assays performed for this thesis were done at the same optical density. Performing Miller assays over a range of optical densities may produce a result similar to what is observed for *E. coli.*

Aerobic expression of *torECAD* presents the idea of TMAO reduction in the presence of oxygen, which occurs in *E. coli* (16). Reducing TMAO in the presence of oxygen is energetically unfavorable, but may confer a growth advantage. TMA production increases the pH and may help the cells cope with media acidification due to acetate production. In *E. coli,* TMAO activates expression of genes (*tnaLAB)* that help it survive in alkaline environments and potentially outcompete other bacteria (28).

Similar studies looking at whether *S. oneidensis* respires TMAO in the presence of oxygen need to be performed. *torECAD* transcription occurs in the presence of oxygen, but it is unknown whether functional protein is produced. If reduction does occur in the presence of oxygen, there may be different advantages for *S. oneidensis*. When *S. oneidensis* is grown on TMAO without enough buffer, the cells die faster than when the medium is properly buffered. Unlike *E. coli,* up-regulation of alkaline stress response genes has not been reported. TMA production may help counteract acidification, but further investigation is needed.

torECAD drives higher expression levels regardless of TMAO concentration

In all conditions tested, expression driven by *torECAD* was higher in the presence and absence of TMAO (Table 3). Without TMAO, the background expression of P*torECAD* is significantly higher than that of P*torF,* which is essentially off. When fully induced, P*torECAD* results in a higher absolute expression than P*torF.* Due to higher background level expression, P*torECAD* has a lower fold induction than P*torF.*

Table 3. Maximum fold inductions based on Miller assay data

LB Medium		
Genotype, Promoter driving *lacZ* expression	**Aerobic (± SD)**	**Anaerobic (± SD)**
WT, P*torF*	90.9 ± 0.944	183.0 ± 2.46
Δ*tor,* P*torF*	233.9 ± 0.82	364.1 ± 1.61
Δ*tor*Δ*dms*, P*torF*	143.7 ± 0.48	45.2 ± 0.16
WT, P*torECAD*	47.0 ± 0.27	90.0 ± 0.48
Δ*tor*, P*torECAD*	47.2 ± 0.29	91.9 ± 0.13
Δ*tor*Δ*dms*,P*torECAD*	50.2 ± 0.25	38.3 ± 0.03
SBM Medium		
Genotype, Promoter driving *lacZ* expression	**Aerobic (± SD)**	**Anaerobic (± SD)**
WT, P*torF*	50.5 ± 0.42	62.2 ± 0.78
Δ*tor,* P*torF*	139.7 ± 0.77	331.2 ± 0.52
Δ*tor*Δ*dms*, P*torF*	312.7 ± 0.46	109.8 ± 0.15
WT, P*torECAD*	48.0 ± 0.25	77.5 ± 0.12
Δ*tor*, P*torECAD*	62.7 ± 0.66	104.5 ± 0.60
Δ*tor*Δ*dms*,P*torECAD*	89.0 ± 0.03	67.4 ± 0.22

P*torF* behaves differently in different strains

The TMAO concentration at which induction occurs is the same for both promoters, regardless of genetic background. However, there is a difference in the level of induction for P*torF* that seems to be strain dependent. For all tested conditions, the fold induction for P*torF* in the WT background was significantly lower than the Δ*tor* and Δ*tor*Δ*dms* backgrounds. This was not observed for P*torECAD*, which behaved the same in both backgrounds. Despite sequence verification of the promoter region and remaking the strain, the difference was still observed. The fold induction of the WT strain with the *torF* promoter is still significant, but is much lower than the other two *torF* promoter strains. The data suggests there is an additional form of regulation for the *torF* promoter.

Conclusion

This work has illustrated the ability to use a native *S. oneidensis* pathway to reliably induce gene expression under aerobic and anaerobic conditions. The *torECAD* and *torF* promoters can be used to drive expression of a gene of interest, as demonstrated by the miller assays. For both promoters, expression is less in the presence of oxygen. The promoters are activated by the same concentration of TMAO, which is lower when cells are grown in rich medium versus minimal. Expression driven by P*torECAD* is higher in the presence and absence of TMAO compared to P*torF* expression, and is the same for different background strains in the conditions tested. Conversely, expression driven by P*torF* was lower in the WT background compared to the *Δtor* and Δ*tor*Δ*dms* backgrounds in all conditions tested.

Chapter 4
Engineering Extracellular Electron Transport to be TMAO-Inducible

Introduction

Shewanella species are well known for their ability to respire a vast number of compounds, many of which are extracellular. Some of the electron acceptors they use are of particular interest to the biotechnology industry. For example, *Shewanella* species may be suitable for bioremediation of certain heavy metals, such as Uranium and Technetium, which are immobilized in their reduced forms (1). Additionally, *Shewanella* species can interface with an electrode, which presents opportunities for its use in electrosynthesis and microbial fuel cells.

Respiration of insoluble extracellular acceptors necessitates a mode of transferring intracellular electrons to the exterior. Transposon mutagenesis was crucial for identifying the proteins involved in this process in *Shewanella* species (29,30). Characterization of mutants unable to respire extracellular metals, such as iron oxide and manganese, has led to our current model of EET.

Electrons generated from carbon oxidation enter the quinone pool and can reduce menaquinone or ubiquinone. CymA, an inner membrane *c*-type cytrochrome, oxidizes manaquinone and transfers the electrons to different respiratory pathways (31). When an extracellular metal or an electrode is the acceptor, electrons from CymA are transferred to MtrA, a *c*-type cytochrome periplasmic electron carrier, and then to the outer membrane *c*-type cytochrome, MtrC (30). MtrB, an outer membrane beta barrel protein stabilizes MtrA and MtrC (32). The Mtr proteins form a conduit that, with CymA, forms what is known as the Mtr pathway (figure 11).

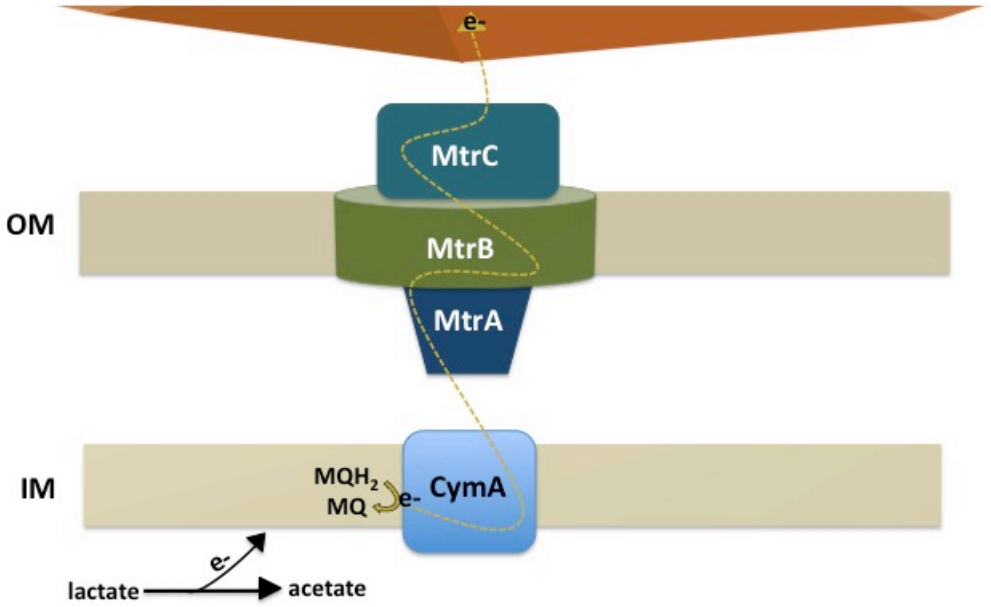

Figure 11. The Mtr pathway. The dashed line represents electron travel from the menaquionne pool to an extracellular substrate. Blue proteins correspond to multiheme cytochromes.

From the Mtr pathway, electrons either directly reduce the acceptor, or electrons are transferred to a shuttle. When a soluble acceptor is present, such as ferric citrate, MtrC transfers electrons directly (30). When the cell is not in direct contact with the substrate it is respiring, MtrC reduces flavins that shuttle electrons to the acceptor (33). Flavin shuttles travel to and are oxidized by the insoluble acceptor, a process that is important for efficient electrode respiration (34).

Understanding regulation of the Mtr genes is important for optimizing applications that exploit them. Prior to *mtrC* discovery, *mtrA* and *mtrB* were thought to form a two-gene operon due to the identification of putative terminators upstream of *mtrA* and downstream of *mtrB* (29). When *mtrC* was discovered, 5' Rapid Amplification of cDNA Ends (5' RACE) was used to investigate whether the genes constituted an operon. The results, which were confirmed by a separate study, show that the promoter upstream of *mtrC* drives expression of all three genes (29, 35).

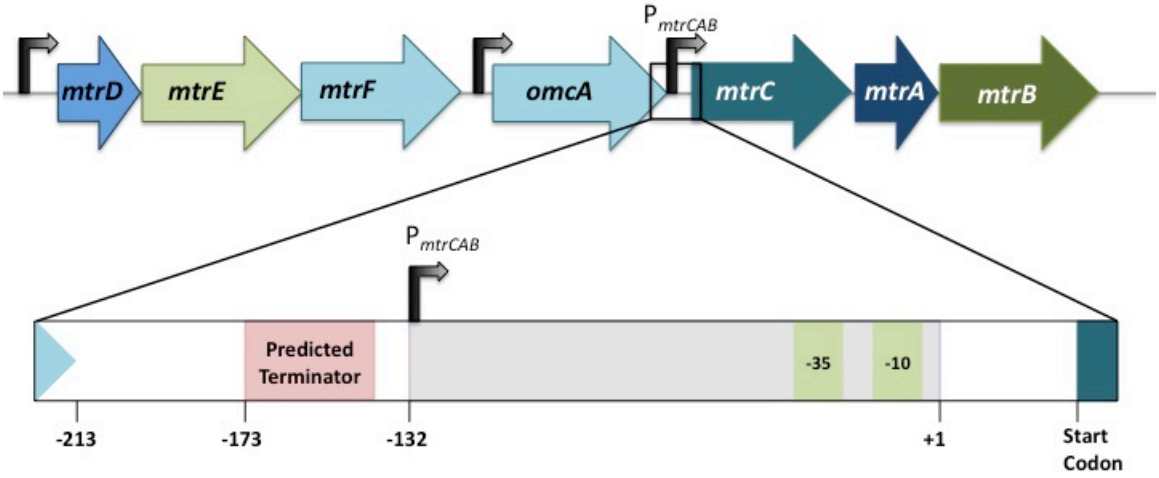

Figure 12. *mtrCAB* genomic context and annotation of intergenic region upstream of *mtrCAB*. Numbers within the intergenic region indicate position relative to the transcriptional start site (+1). The shaded region was replaced with the *torF* promoter.

Expression of *mtrCAB* is influenced by several factors. Cyclic AMP receptor protein (CRP) activates expression of *mtrCAB* and is necessary for iron and manganese reduction (35,36). *mtrCAB* may also be negatively regulated. The region 101 to 203 base pairs upstream of the transcriptional start site is thought to be associated with transcriptional repression, as higher expression is observed when this region is deleted (35).

Donor to acceptor ratios and growth phase may also influence expression driven by P*mtrCAB* (35, 37). Higher *mtrC* expression was observed in acceptor limiting conditions during stationary phase compared to mid-log phase. An opposite trend was observed in carbon limiting conditions (35). Interestingly, cells in stationary phase had higher expression aerobically than anaerobically on fumarate (35). The key promoter elements are diagrammed in figure 12.

The ability to precisely control and maximize gene expression is ideal for enhancing biotechnological applications involving *S. oneidensis.* In the past, this has been achieved using a plasmid-based system (38). While this is a valid approach in the laboratory, it is not ideal for large-scale applications where plasmid stability is a significant concern. Integrating genes and regulatory elements into the genome addresses this issue. This chapter demonstrates the successful replacement of the native *mtrCAB* promoter with the *torF* promoter, on the genome. The replacement enables reliable induction of EET upon the addition of TMAO. This work demonstrates the ability to synthetically regulate gene expression without relying on a plasmid.

Materials and methods

Reagents

DNA polymerase (Phusion High-Fidelity), enzymes, Antarctic phosphotase, and T4 ligase were purchased from New England Biolabs (Ipswich, MA). Reactions were set up according to the manufacturer's protocol. Qiagen Gel extraction and plasmid mini-prep kits were used (Valencia, CA). The Invitrogen PureLink PCR clean up kit was used (Carlsbad, CA). Primers were ordered from Integrated DNA Technologies via the University of Minnesota Genomics Center (UMGC). Sanger sequencing was carried out at the UMGC facility.

Culturing conditions

Strains used in this study are listed in table 4. All *E. coli* strains were grown at 37°C on Luria-Bertania (LB) medium in the presence of 50 μg/mL kanamycin (Km). *E. coli* WM3064 was also supplemented with diaminopimelic acid (DAP). *S. oneidensis* strains were grown at 30°C on either LB or *Shewanella* Basal Medium (SBM). Lactate was added to SBM medium for a final concentration of 20 mM. When grown anaerobically, fumarate was added to a final concentration of 40 mM. SBM is supplemented with 5ml/L vitamins and minerals and has a final concentration of 0.05% CAA. Glycerol stocks stored at -80°C were used to streak LB agar plates and obtain isolated colonies. Medium for overnight liquid cultures was inoculated with a single colony and grown overnight, shaking at 225 rpm, at the appropriate temperature.

Table 4. Strains and plasmids used in this study

***E. coli* Strains**	**Genotype/Characteristic**	**Reference**
UQ950	DH5α λ(*pir*) cloning host; F^-Δ(*argF-lac*)169 Φ80d*lacZ58*ΔM15 *glnV*44(AS) *rfbD1 gyrA96*(Nal^R) *recA1 endA1 spoT1 thi-1 hsdR17 deoR λpir*$^+$	(23)
WM3064	Donor strain for conjugation; *thrB1*004 *pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD)567 ΔdapA*1341::[*erm pir*(wt)]	(23)
***S. oneidensis* strains**	**Genotype/Characteristic**	**Reference**
JG274	WT MR-1	(24)
JG3278	Δ*torECADF*	This Study
JG3544	Δ*torECADF*Δ*dmsEFABGH (*Δ*tor*Δ*dms*)	This Study
JG3459	WT with P*torF::mtrCAB*	This Study
JG3460	Δ*torECADF* with P*torF:mtrCAB*	This Study
JG3631	Δ*torECADF*Δ*dmsEFABGH (*Δ*tor*Δ*dms*) with P*torF::mtrCAB*	This Study

JG 3571	WT with pBBR1MCS2::*torF*	This Study
Plasmids	**Genotype/Characteristic**	**Reference**
pBBR1MCS2	Broad-host-range cloning vector, Km^r	(39)
pSMV3	Deletion vector, Km^r, *sacB*	(23)

Promoter Fusion Construction

The *torF* promoter was chosen to replace the native *mtrCAB* promoter because it is virtually off in the absence of TMAO and is highly induced in its presence. The same promoter region that was used in the previous sections was used to replace 132 base pairs upstream of and including the *mtrCAB* transcriptional start site. The replacement was achieved by ligating P*torF* between fragments (~1000 bp) corresponding to the regions upstream and downstream of the *PmtrCAB* segment to be replaced. The fusion was ligated into pSMV3 and inserted into the *S. oneidensis* genome via homologous recombination as described in Chapter 2. The fusion was integrated into the following backgrounds: WT, Δ*tor*, and Δ*dms*Δ*tor*. The strains were sequenced using primers upstream and downstream of the replaced promoter

Quantitative reverse-transcriptase PCR

mtrC expression levels in the presence and absence of TMAO were quantified in WT, and the promoter replacement strains. The strains were struck on LB plates from glycerol stocks. Liquid SBM cultures were inoculated with a single colony and grown aerobically overnight while shaking at 30°C. This was done in duplicate.

The following morning, cells were spun down, washed, and re-suspended to and OD of 1.0 in SBM. 50 µl of each overnight culture were transferred to SBM with and without 1mM TMAO. Fumarate was added as the electron acceptor, and the cells were grown anaerobically while shaking until they reached an OD of 0.1. When the cells reached and OD of 0.1, they were normalized to an OD of 1.0 in 0.5 mL of RNAprotect reagent (QIAGEN Sciences, Germantown, MD) and the pellets were stored at -80°C.

mRNA was extracted from the pellets using the RNeasy mini kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol, with the on column DNase digestion step. cDNA was generated using the primers listed in Table S1. Reactions containing 9 µL RNase-free H_2O, 1µL oligo mix (2 pmol/µL of each primer), 1 µL dNTP mix, and 2 µL of 50 ng/µL RNA were heated at 65°C for 5 minutes and then placed on ice for 1 minute. The following were added to the mixtures: 4 µL of 5x First strand buffer (Invitrogen, Carlsbad, CA), 1 µL DTT (100 mM), 1 µL RNaseOUT, and 1 µL of Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). A negative control without reverse transcriptase was also prepared. Samples were incubated at 50°C for 1 hour and then inactivated at 70°C for 15 minutes. 2 µL cDNA (2 ng/µL) was added to 10 µL SYBR green (Life Technologies, Carlsbad, CA), 1 µL of primer, and 6µL ddH_20. PCR reactions were performed on a Biorad CFX connect machine. Data was analyzed using the delta delta Ct method.

Iron Reduction Quantification

FerroZine assays were performed to quantify iron reduction for the promoter fusion strains (40). WT was included as a positive control and Δ*mtrC* served as a negative control. LB plates were struck from -80°C freezer stocks and LB liquid medium was inoculated with a colony. This was performed in triplicate. The LB cultures grew throughout the day while shaking. Fresh LB medium with or without 1mM TMAO was inoculated from the day culture and grown overnight. The following morning, cells were spun, washed in SBM and resuspended to an OD of 1.0.

The assay was performed on a 96 well plate containing SBM with 20 mM lactate, 10 mL/L vitamins and minerals, and 5 mM of an iron source (either ferric citrate or iron oxide). For each strain, iron reduction was assayed in the absence and presence of 1mM TMAO. The plates were inoculated to an OD of 0.1 and incubated at room temperature in an air-tight anaerobic chamber made anaerobic by degassing for 15 minutes with nitrogen gas.

To measure the iron concentration, 5M HCL was added to the cells to stop the reaction. An aliquot was transferred to 0.5 M HCL. An aliquot of that solution was mixed with ferroZine reagent and allowed to react for 15 minutes, at which point the absorbance at 562 nm was determined. A standard curve with $FeSO_4$ was used to calculate the Fe_{III} concentration.

Results and Discussion

P*torF* is driving *mtrCAB* expression in the promoter replacement strains

Quantitative PCR was used to determine the success of the promoter replacement and the degree to which *mtrC* is induced in the presence of TMAO (Table 5). Unlike Miller Assays, which use protein levels as a proxy for promoter strength, qPCR measures transcript levels. Although qPCR and Miller assay data cannot be directly compared, the general trends observed for the qPCR data were expected to be similar to the Miller Assay data. However, there are several factors that could lead to discrepancies. In the promoter replacement strain, the genomic context and ribosomal binding site are different than the promoter-*lacZ* fusion. Additionally, unknown regulatory elements might influence *mtrCAB* expression, and mRNA stability could lead to differences between the qPCR and Miller Assay data.

qPCR was only performed for cells grown anaerobically in SBM. The data demonstrate P*torF* is driving *mtrCAB* expression for both replacement strains. As expected, *mtrC* was highly induced in the Δ*tor* strain (Table 5). Based on the Miller assay data, the fold expression change was expected to be lower in the WT background, but it was much lower than expected. A higher fold expression change was observed for WT with the native promoter compared to WT with the replaced promoter. It should be noted that this experiment was only performed once in Biological duplicate, and the standard deviations for WT with the native promoter and the Δ*tor* strain were very high. This experiment needs to be repeated before any confident conclusions can be drawn. Despite this, the data demonstrates *mtrC* transcription is occurring for both replacement strains.

Table 5. Fold expression change of *mtrC* in the presence of TMAO

Strain	Fold Expression Change (± SD)
WT	3.6 ± 3.2
WT, P*torF::mtrCAB*	1.3 ± 0.09
Δ*tor*, P*torF::mtrCAB*	1658.8 ± 895.4

EET in the P*torF::mtrCAB* strains is enhanced in the presence of TMAO

Optimal respiration of a metal or an electrode requires functional MtrA, MtrB and MtrC, although some reduction occurs in the absence of *mtrCAB* due to the presence of paralogs. When MtrC, MtrA, and MtrB are present, they form the primary conduit through which electrons are transferred (25). The qPCR data demonstrate transcription is being driven by *PtorF* in the promoter replacement strains. To test whether functional protein is being produced and EET is

occurring, Iron reduction assays using iron citrate or iron oxide as a terminal electron acceptor were used (Figure 13, 14).

TMAO induced ferric citrate and iron oxide reduction for both promoter replacement strains (figure 13, 14). In the absence of TMAO, iron reduction was lower than observed for the *mtrC* mutant. The defect was stronger when iron oxide served as the electron acceptor, possibly because Mtr homologs are more able to compensate when ferric citrate is the terminal electron acceptor. When MtrC and MtrA mutants are grown on ferric citrate, homologs are able to facilitate electron transport, although at a slower rate. When grown on iron oxide, an MtrC mutant can still reduce Fe(III), but MtrA homologs are unable to compensate (25). Lack of *mtrCAB* expression in the replacement strains in the absence of TMAO, and the inability of homologs to restore iron oxide reduction likely explain the more pronounced iron reduction defect when grown on iron oxide.

The extent of iron oxide reduction differed between the WT and Δ*tor* backgrounds, while a difference was not observed for iron citrate reduction. Expression variances might explain the difference. Miller assay and qPCR data suggest expression driven by P*torF* is higher in the Δ*tor* strain than the WT background. Higher expression may not enhance ferric citrate reduction, but may enhance iron oxide reduction. Ferric citrate is soluble and reduction occurs faster, as demonstrated by the different time scales for iron citrate and iron oxide reduction assays. Higher *mtrCAB* expression might not have benefited cells grown on iron citrate in the tested condition. It is possible that using fewer cells would produce a phenotype similar to that observed with iron oxide. Another explanation is that the WT fusion cells are reducing TMAO rather than iron oxide. If that were the case, a larger defect for WT with the native promoter, in the presence of TMAO, would be expected.

The FerroZine data demonstrate the ability to induce EET for both promoter replacement strains, despite the low fold expression change of *mtrC* determined by qPCR. Iron reduction for both promoter replacement strains in the presence of TMAO was similar to WT reduction levels. This suggests that higher *mtrCAB* transcription levels do not necessarily increase EET. If that were the case, the Δ*tor* strain would have a higher reduction rate. It is possible that higher transcription does not actually result in more functional protein, which could be assessed using cytochrome stains. Another possibility is that electron transport via the Mtr conduit is not the rate-limiting step. Although reduction rates did not exceed WT levels, the data demonstrate the ability to successfully induce EET with TMAO.

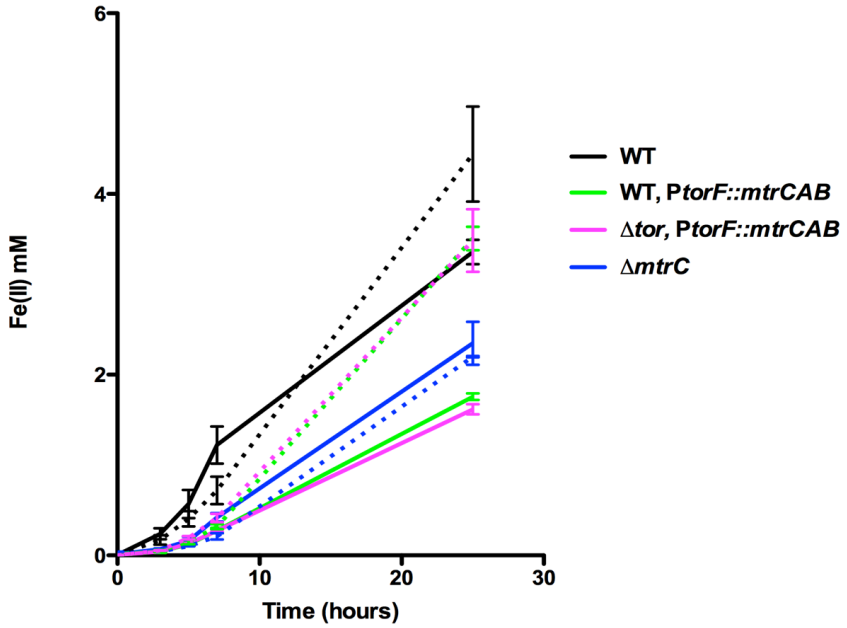

Figure 13. Iron reduction assay using 5 mM ferric citrate in the absence of TMAO (solid lines) and presence of 1 mM TMAO (dashed lines).

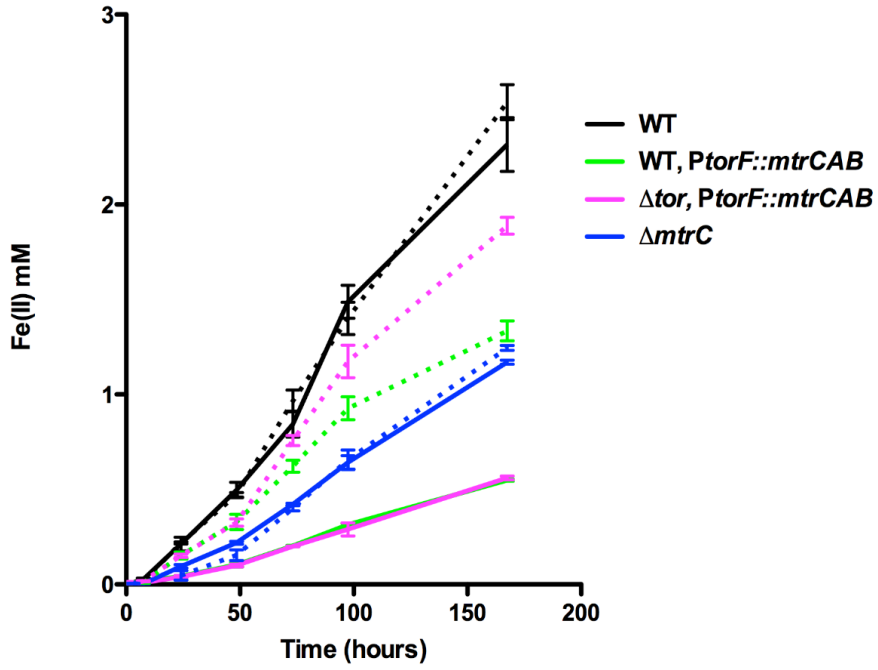

Figure 14. Iron reduction assay using 5 mM iron oxide in the absence of TMAO (solid lines) and presence of 1 mM TMAO (dashed lines).

Conclusion

The native *mtrCAB* promoter was successfully replaced with P*torF,* enabling the regulation of extracellular electron transport with TMAO. Quantitative PCR data demonstrated the ability of the *torF* promoter to drive expression of *mtrC,* although to a lesser degree in the WT background. FerroZine assays corroborated the qPCR data, as both promoter replacement strains were able to reduce ferric citrate and iron oxide. Iron reduction was significantly lower in the absence of TMAO, demonstrating the ability to control EET with a TMAO inducible promoter. The results demonstrate a method to regulate gene expression without relying on plasmid, making it useful for large-scale biotechnological applications.

Chapter 5
Conclusions and Future Directions

The data in this thesis demonstrate the ability to use components of the Tor pathway to induce expression of a gene of interest. P*torECAD* and P*torF* are both highly induced in the presence of TMAO, which fostered the idea of using them to modulate gene expression. The promoters were characterized and found to respond to a range of TMAO concentrations aerobically and anaerobically. After demonstrating the ability to induce *lacZ* expression with both promoters, the native *mtrCAB* promoter was replaced with P*torF*. Rather than relying on a plasmid, P*mtrCAB* was replaced with P*torF* on the genome. Without TMAO, iron reduction was less than an *mtrC* mutant. TMAO addition increased EET and iron reduction was similar, although slightly less, to WT levels.

In this work, only the *mtrCAB* promoter was replaced, but replacement of additional promoters may help optimize processes involving *S. oneidensis*. For example, to successfully maximize and induce EET, promoters in the flavin production and export pathway could also be targeted alone or in combination with Mtr pathway promoters. Replacing multiple promoters in the EET pathway might be necessary for producing a system in which EET only occurs in the presence of TMAO.

Additionally, the Tor promoters could be modified to change expression levels. The location of the TorR binding site may influence transcription and could be moved within the Tor promoter and potentially alter expression. It may also be possible to make a non-Tor promoter inducible with TMAO by introducing TorR binding sites. Engineering new TMAO inducible promoters with varying strengths would be useful for tailoring expression levels for a given application.

Metal reduction assays, such as the FerroZine assay, are useful for analyzing manipulations to genes involved in EET. However, many of the applications pertaining to *Shewanella* rely on its ability to interface with an electrode. It is crucial to understand the effects of genetic modifications in the context of an electrode. Although induction of iron reduction has been demonstrated, the ability of the promoter replacement strains to interface with an electrode and to induce current production with TMAO needs to be verified. Maximizing the ability of *S. oneidensis* to interface with an electrode will likely require several approaches. The work presented in this thesis offers new tools and a method that could aid in the endeavor to develop useful processes involving *Shewanella* species.

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Supplementary Information

Table S1. Primers used in this work

Primer	Sequence	Restriction site
TorD-F	CGTTATTACCGCAGTTAAAGGC	SpeI
TorD-R	GCGGTTGAACGAAAGCG	ApaI
TorE-F	GCATGGTGGGCTTTGTC	ApaI
TorE-R	GCCAATACCAATCCCAGTACC	SacI
torF UP-F	GCTGCGCTTAGTGATAATGCC	BamHI
torF UP-R	CATCATCTCAAAGTGATTGGGAATGG	ApaI
torF DN-F	TAATCTTTTTTGCTTGCACGTTCA	ApaI
torF DN-R	ATCCATCTATGCCAGAGCGAG	SpeI
torT UP-F	TGCTGATTAATGTGCGCTACAG	SpeI
torT UP-R	TGTGAGCAAAGCTTGCCAAAAT	NheI
torT DN-F	CTATCAAGTACGGCCTCCATCTTTA	NheI
torT DN-R	TTGAGGAGACTGTAAAAGCTCGG	SacI
mtrC UP-F	GAGTGCATACACTAAATCGAG	BamHI
mtrC UP-R	TAGTGAGGTGGAAATAAAAAGAGAGAGG	NheI
mtrC DN-F	GGGAATTCTATTTCCAGCATCCACTTAA	NheI
mtrC DN-R	CGATGTCTACGTGACAGCTAGAACA	NotI
mtrC screen-F	GATGGAAGCACACGGTAACTAAGTC	NA
mtrC screen-R	CTACCATCATTGCCGTTATTACCATC	NA
CymA UP-F	GACAACATTGAGCGTTTCAGTGC	BamHI
CymA UP-R	ACTTCAACTCCAGCTAATACATTCATC	NheI
CymA DN-F	CATAAAATAGTCTTATAAAAACTGATTTTGAC	NheI
CymA DN-R	AAAATTATCAGTGATGCTTGTACC	NotI
PtorF-F	ATTTCTATAAATGACACTGC	NheI
PtorF-R	ATCGTAAAAATAAGTTGTTAATG	SphI
PtorECAD-F	TATCAAACTGCCGAGTGCGATC	NheI
PtorECAD-R	TGACATCACTATAAAGGGGAGATGT	SphI
SO recA RT	AGCCGCTAAGCCCATGTGTGAGT	NA
SO envZ RT	GAGGCGGATACGAGTCAGCGGC	NA
SO mtrC RT	TTTGTGAATTTCGAACTCAGCAGTG	NA
SO recA sense	TGTGTTCACAGCCCGATAC	NA
SO recA antisense	CGCTACCGAGTCAACGATAAT	NA
SO envZ sense	CGAGTTTCCCGACCCTTTAC	NA
SO envZ antisense	GATCTTGCTCTAGCTGCTTCAT	NA
SO mtrC sense	TGTGCAGACCCTGCATTT	NA
SO mtrC antisense	GGCGAGTACTTGGTGCTTTA	NA