May 2020

Mechanisms and Regulation of Aerobic Respiration in Shewanella Oneidensis MR-1

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MECHANISMS AND REGULATION OF AEROBIC RESPIRATION IN *SHEWANELLA ONEIDENSIS* MR-1

by

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A Dissertation Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

in Biological Sciences

at

The University of Wisconsin-Milwaukee

May 2020

ABSTRACT

MECHANISMS AND REGULATION OF AEROBIC RESPIRATION IN *SHEWANELLA ONEIDENSIS* MR-1

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Under the Supervision of Dr. Daâd Saffarini

Shewanella oneidensis MR-1 expresses three terminal oxidase complexes predicted to participate in aerobic respiration: an *aa*$_3$-type cytochrome *c* oxidase, a *cbb*$_3$-type cytochrome *c* oxidase, and a *bd*-type quinol oxidase. An intermediate *bc*1 complex is used for transfer of electrons to the *aa*$_3$-type and *cbb*$_3$-type cytochrome *c* oxidase complexes. Use of the terminal aerobic oxidases in *S. oneidensis* is atypical. The *aa*$_3$-type cytochrome *c* oxidase is the expected primary oxidase complex in oxygen-replete conditions; however, it has no observed function in aerobic growth in *S. oneidensis*. The *cbb*$_3$-type cytochrome *c* oxidase and *bd*-type quinol oxidase are the primary oxidases used for aerobic respiration in this organism, regardless of oxygen tension. The regulatory systems CRP, a cyclic AMP (cAMP) binding protein, and the ArcAB two-component system regulate anaerobic respiration and appear to play a role in aerobic growth if *S. oneidensis*. Furthermore, the *S. oneidensis* the cyclic AMP phosphodiesterase, *cpdA* (SO_3901) also appears to affect aerobic growth, but its exact role in this process is not known. .

This work analyzes aerobic growth of mutants deficient in terminal oxidase complexes and regulatory proteins when exposed to different sole carbon sources in basal medium. A

deletion mutant of both the *bc*1 complex and the *bd*-type quinol oxidase exhibited a severe growth deficiency under all conditions tested. Mutants deficient in CRP and ArcA demonstrated slight aerobic growth deficiencies. The *cpdA* mutant demonstrated a severe growth deficiency. Carbon source does not drastically affect the growth phenotype of mutants tested. In this work, aerobic growth of two CRP-like proteins (SO_2550 and SO_2551) was also analyzed. These proteins contain the cyclic nucleotide binding domain as well as the helix-turn-helix motif of CRP-family proteins. Deletion of genes that encode these proteins results in an aerobic growth deficiency. We also analyzed promoter expression of the two major aerobic terminal oxidase complexes in these regulatory protein deletion mutants. To assess promoter expression over time, a fluorescent timer system was developed using a mutant mCherry that fluoresces blue after translation and matures to red. This system allows analysis of growth and promoter expression simultaneously. Promoter expression did not drastically change during the growth cycle of *S. oneidensis*, confirming that growth deficiencies of regulatory system mutants are not due to lack of expression of terminal oxidase complexes.

Recovery of the growth deficiency of the *cpdA* deletion mutant was also analyzed in this work. Addition of casamino acids to a basal medium restored aerobic growth in this mutant. Growth of a *cpdA* deletion mutant was also restored by the inactivation of SO_3550 (an anti-sigma factor). The SO_3550 cognate sigma factor encoded by SO_3551 is an ECF, or extracytoplasmic function sigma factor. ECF sigma factors are typically used in stress response in bacteria, suggesting that the action of this sigma factor is able to remedy the growth deficiency of the *cpdA* mutant.

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ACKNOWLEDGEMENTS

First, I would like to extend my gratitude to my advisor, Dr. Daâd Saffarini. Her guidance and support were fundamental in my success as a graduate student. She encouraged my independence, but was always available to help troubleshoot experiments and provide a fresh perspective on my research and analysis. I have become a substantially stronger writer in my time here as well, largely due to her editing and advice.

I would also like to thank my committee: Dr. Mark McBride, Dr. Sergei Kuchin, Dr. Sonia Bardy, and Dr. Steven Forst. They provided invaluable suggestions for additional experiments and new methods of testing. Their recommendations also aided my presentation abilities, as they encouraged detailed yet concise explanations of my work. My lab mate Rini Banerjee deserves a special thank you for her help with ideas for experimental design and always knowing where everything is in the lab.

Lastly, I would like to thank my family and friends, in particular my fiancée Kelly Grant. The impact her emotional support has had on my success these past three years cannot be overstated. My pug Bowser also deserves a very special thank you for insisting on two-hour naps in my lap most days, which helped me finish writing. I'm looking forward to the adventures ahead, and I'm so grateful for everyone who has been on this journey with me. Thank you.

CHAPTER 1

INTRODUCTION

Cellular respiration is a series of redox reactions by which organisms create an ion gradient and generate ATP via an electron transport chain. This process is fundamental for energy generation in many organisms. Aerobic respiration (using O_2 as the terminal electron acceptor) is the type of respiration employed by eukaryotic organisms. When oxygen is absent, microorganisms can generate energy by fermentation or anaerobic respiration. Many bacteria and archaea are able to respire anaerobically using a variety of different compounds as terminal electron acceptors (1). As such, anaerobic respiration is significant in the biogeochemical cycling of many elements, including nitrogen, sulfur, and metals. Organisms in the *Shewanella* genus are facultative anaerobes adept at respiring different electron acceptors and are consequently used as a model to study respiration.

Shewanella Genus

The genus *Shewanella* is comprised of gram-negative, rod-shaped, non-spore-forming bacteria that are catalase and cytochrome *c* oxidase positive. They are motile by means of a single polar flagellum and exhibit electron acceptor taxis, or movement towards electron acceptors such as oxygen and nitrate (2). Most species are unable to ferment glucose (2). The genus is well known for its respiratory versatility, including the ability to use radionuclides, elemental sulfur, and metal oxides as terminal electron acceptors (3). They have also been found to reduce azo dyes (4). A unique feature of *Shewanellae* is that they are facultative anaerobes – many other metal and sulfur reducers are obligately anaerobic. Therefore, *Shewanellae* commonly inhabit redox interfaces of aquatic systems, both freshwater and marine (3). However,

these bacteria are not limited to aquatic environments. The first reported organism in this genus, "*Achromobacter putrefaciens*" (now classified as *Shewanella putrefaciens*), was first isolated from rancid butter in the 1930s (5, 6). *Shewanella* species are mostly non-pathogenic, with the exception of *S. alga* and *S. haliotis;* however, they are involved in the putrefaction of fish – the reduction of TMAO by *S. putrefaciens* gives rotting fish its smell (2, 7, 8). The genome sequences of more than 20 *Shewanella* species have been sequenced and analyzed. Commonly studied species are *S. loihica* PV-4, *S. denitrificans,* and *S. oneidensis* MR-1.

***Shewanella oneidensis* MR-1**

Shewanella oneidensis MR-1 is the *Shewanella* species used to study respiration. It was isolated from Oneida Lake in New York due to its ability to reduce manganese (MR – manganese reducing), and designated *Alteromonas putrefaciens* (9). It was reclassified as part of the *Shewanella* genus based on 5S rRNA sequencing, and the sequences of 16S rRNA and *gyrB* (DNA gyrase subunit B) determined it is a distinct species (2, 6). Respiration is its sole source of energy production, and it thrives in redox stratified environments. *S. oneidensis* can use 14 different electron acceptors, including oxygen, dimethyl sulfoxide (DMSO), trimethylamine oxide (TMAO), fumarate, nitrate, nitrite, sulfite, and oxidized metals (including iron (III), manganese (IV), uranium (IV), and chromium (IV)) (10).

The ability to use metals as terminal electron acceptors has spurred research into electricity generation by *S. oneidensis*. The reduction of iron (III) and manganese (IV) occur extracellularly, with the terminal reductase complexes presenting on the outer surface of the outer membrane of the cell. The bacteria associate with the surface of the metal or electrode, allowing for a mediator-free electron transfer and electricity production (3, 11, 12). *Shewanella*

species are also able to produce long olefinic hydrocarbons, likely as an adaptation to cold temperatures. These hydrocarbons may be used as biofuels or in other chemical applications (13, 14). *S. oneidensis* is also able to reduce graphene oxide to graphene (a 2D graphitic material with remarkable strength and conductive properties) using the Mtr respiratory pathway that is used for reduction of other metal oxides. This microbially-reduced graphene demonstrates excellent conductivity and the production process avoids the toxic chemicals used in current large-scale graphene oxide reduction methods (15-18).

Respiration in *S. oneidensis*

The genome of *S. oneidensis* encodes 42 *c*-type cytochromes, which are essential for its respiratory versatility (10). *c*-type cytochromes are electron transfer proteins characterized by covalently bound heme (19, 20). Anaerobic respiration has been the primary focus of research in this organism, and many of the cytochromes and pathways have been well studied. As mentioned previously, metal reduction occurs on the outer membrane of *S. oneidensis*; DMSO reduction occurs on the outer membrane as well (3). The tetraheme *c* cytochrome CymA functions as an intermediate in these pathways, transporting electrons from the menaquinone pool to the outer membrane complexes for extracellular substrate reduction. Menaquinone serves as an electron carrier in anaerobic respiration. The menaquinone pool refers to total menaquinone, both oxidized and reduced. CymA is a multifunctional intermediate that also transfers electrons to the periplasmic fumarate and nitrate reductases (21). *S. oneidensis* has several atypical *c*-type cytochromes including SirA, the sulfite reductase, an octaheme with atypical heme binding sites (22). There appears to be functional redundancy in cytochrome expression and usage and atypical regulation of respiration when compared to other model organisms.

Aerobic respiration is less well studied in *S. oneidensis*. It employs three terminal oxidase complexes encoded by the *cox*, *cco*, and *cyd* genes as well as an intermediate oxidase complex encoded by the *pet* genes. The categories of terminal oxidases can be divided into heme-copper oxidases and *bd*-type quinol oxidases (23). Heme-copper oxidases can be further divided into A, B, or C types. Type A includes mitochondrial cytochrome *c* oxidase (aa_3), *c*-type cytochromes from *Paracoccus denitrificans*, the *cox* (aa_3-type *c* cytochrome) from *S. oneidensis*, as well as non-*c*-type cytochromes, such as the bo_3 terminal oxidase in *Escherichia coli* (20, 24, 25). Type B consists of cytochromes such as the ba_3 oxygen reductase of *Thermus thermophilus* and the quinol oxidase of the archaeon *Acdianus ambivalens* (24, 26). Type C comprises exclusively cbb_3-type cytochrome *c* oxidases, including the cbb_3-type encoded by the *cco* genes in *S. oneidensis* (27). The intermediate *bc*1 (*pet*) complex is a ubiquinol:cytochrome *c* oxidoreductase that is a common intermediate in many organisms, including *S. oneidensis*. It oxidizes quinol (aerobic respiration electron carrier) and transfers those electrons to the terminal heme-copper oxidases. The *cyd* genes in *S. oneidensis* code for the *bd*-type quinol oxidase. This oxidase transfers electrons directly from the quinone pool to molecular oxygen, thereby bypassing the *bc*1 complex (20). These oxidases and their functions will be discussed in more detail in Chapter 3.

Regulation of Respiration

S. oneidensis respiration is controlled by three primary regulatory systems: EtrA, ArcAB, and CRP. EtrA is analogous to *E. coli* FNR (**f**umarate and **n**itrate **r**eductase). It contains an Fe-S cluster that is activated under anaerobic conditions, allowing for transcription of genes involved in anaerobic respiration (28, 29). This protein is inactive in the presence of oxygen and does not

affect aerobic respiration in *S. oneidensis*. *E. coli* ArcAB (**a**noxic **r**espiratory **c**ontrol) is a two-component signal transduction system, wherein ArcB is the sensor kinase and ArcA is the response regulator. ArcB senses the redox state of the menaquinone pool as well as total amount of ubiquinone in the cell. Under low-oxygen and anaerobic conditions, ArcB is active. A phosphorelay is initiated and ArcA becomes phosphorylated and regulates transcription of a variety of metabolic genes. Menaquinone oxidizes rapidly in the presence of oxygen, and ArcB becomes inactive (30, 31). *S. oneidensis* has an orthologous ArcA; however, ArcB is split into two separate proteins, ArcB1 and HptA (32). ArcA mutants are deficient in DMSO respiration, but not fumarate respiration, suggesting that additional regulators of anaerobic respiration are present in this organism (33, 34).

CRP is a **c**yclic-AMP **r**eceptor **p**rotein and a global regulator that has been studied at length in *E. coli*. It is recognized for its role in carbon metabolism, but is also involved in iron uptake, heat shock response, flagellar synthesis, and aerotaxis (35). CRP in *S. oneidensis* does not have a role in carbon metabolism as the bacterium does not use a wide array of carbon sources. CRP does, however, regulate respiration in *Shewanella*. CRP deletion mutants are deficient in anaerobic respiration on a variety of substrates, including DMSO, fumarate, and iron and manganese oxides (36). Cyclic-AMP (cAMP) is synthesized by adenylate cyclases and degraded by phosphodiesterases. The genome of *S. oneidensis* codes for three adenylate cyclases, CyaA, CyaB, and CyaC. It has one known phosphodiesterase, SO_3901 (CpdA). These respond to environmental stimuli to regulate the cAMP concentration inside the cell and influence CRP activity (37, 38).

In addition to global regulatory mechanisms, bacteria can employ more specific transcriptional control via alternate sigma factors. Sigma factors lend specificity to RNA

polymerase by binding to RNAP and creating the holoenzyme, thereby targeting the enzyme to particular promoter sequences and aiding in transcription initiation. The housekeeping sigma factor, RpoD, is involved in cell growth under ideal conditions for the organism (39, 40). Alternate sigma factors react to cellular stress and will initiate transcription of genes for adaptation to that specific stress. Anti-sigma factors bind to and inactivate their cognate sigma, contributing an additional level of regulation (40). The genome of *S. oneidensis* encodes ten sigma factors, five of which are suggested to have extracytoplasmic function (ECF). These sigma factors are not well characterized; however, ECF sigma factors often aid in stress responses of bacteria (41). Regulatory mechanisms in *S. oneidensis*, including CRP, ArcA, and sigma factors will be further discussed in Chapters 4 and 5.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Generation of Mutants

The bacterial strains used in these experiments are outlined in Table 2.1. Generation of deletion mutants was carried out using established methods (22). Using the primers listed in Table 2.2 and Phusion polymerase (New England Biolabs), 1kb fragments flanking the gene(s) of interest were amplified. The primers were designed to add a restriction site that replaces the deleted gene(s). For each deletion, the fragments were amplified, digested with the appropriate restriction enzyme, and then ligated together. The resulting 2kb fragments were inserted into the SmaI site of the suicide vector pER21 (Table 2.3). The plasmids were used to transform *E. coli* strain EC100D+, and recombinant colonies were screened on Luria-Bertani (LB) medium supplemented with gentamicin (25 μg/mL) (42). Insertions in the SmaI site of pER21 disrupt the alpha fragment of the *lac*Z gene; therefore, transformants were plated on media supplemented with isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for white/blue screening. Plasmids containing the fragments of interest were identified by PCR, then used to transform *E. coli* β2155 followed by conjugation with the desired strain of *S. oneidensis*. The first recombinants were plated on gentamicin, and secondary selection to obtain the deletions was carried out using 5% sucrose plates. The plasmid pER21 contains the *sac*B gene (encoding levansucrase) that causes sucrose toxicity in gram-negative bacteria. Sucrose resistant colonies were screened for deletion of the genes of interest by PCR.

Complementation of Mutants

The mutants were complemented by introduction of the appropriate wild-type gene and its promoter cloned into pJBC1. If the *lac* promoter was used in place of the gene's native promoter (as used for *E. coli crp* mutant complementation in Chapter 4), primers were designed with an NdeI cut site at the start of the gene(s) of interest and a BamHI site at the other end of the gene(s). The genes were amplified using Phusion polymerase (New England Biolabs), cut with NdeI and BamHI, and ligated into NdeI/BamHI cut pJBC1.

Table 2.1. List of strains used in this study.

Strain	**Description**	**Source**
E9	*Escherichia coli* Dh5α	*E. coli* Genetic Stock Center
E254	*Escherichia coli* EC100D+	Epicenter Technologies
E380	*Escherichia coli* β2155	(43)
E976	*Escherichia coli* WM3064	(44)
E1180	*Escherichia coli* ΔCRP	*E. coli* Genetic Stock Center
SR1	*Shewanella oneidensis* MR-1	(2)
SR 694	ΔCRP	(37)
SR 722	ΔSO_3901	(38)
SR1507	ΔSO_2364-2357 (complete deletion of *cco* operon)	(38)
SR 1623	Δ*pet*Δ*cyd*	This work
SR 1637	ΔSO_608-610 (deletion of *petABC*)	This work
SR 1648	ΔSO_4606-4609 (complete deletion of *cox* operon)	This work
SR 1649	Δ*cco*Δ*cox*	This work
SR 1650	Δ*cyd*Δ*cox*	This work
SR 1694	Δ*cya*B	This work
SR 1699	*Shewanella oneidensis* with insertion in SO_3550	This work
SR 1700	ΔSO_3901 with insertion in SO_3550	This work
SR 1701	ΔCRP with insertion in SO_3550	This work
SR 1716	Δ*cyd* (deletion of *cydAB*)	This work
SR 1717	ΔSO_2551	This work
SR 1718	ΔSO_2550-2551	This work

Table 2.2. List of primers used in this study.

Primer Name	**Sequence**	**Comment**

2357F	GATCGGATCCCCCAAGCGAATGAATT	*cco* deletion
2357R	CCACACGCTGATCAATCAC	*cco* deletion
2364F	GCAGATGTGTTCGATGCCCTTTC	*cco* deletion
2364R	GATCGGATCCGCTTCCACTTATT	*cco* deletion
2550F	CATCATAGTTCAGCTAAAACAGCG	ΔSO_2550-2551 deletion
2550fNDE	GATCCATATGTCTCAAAACGCCCTAG	ΔSO_2550 and ΔSO_2550-2551 complementation
2550R	GATCGGATCCGTGTGAATTAGCTGCTTATTCG	ΔSO_2550-2551 deletion
2550rBAM	GATCCGATCCGAAGAGATGCTGTTAG	ΔSO_2550 and ΔSO_2550-2551 complementation
2551F	GCTTAAGCGTAGCGAGATTAATGTTG	ΔSO_2551 deletion
2551F1	GATCGGATCCGCAAATACCATAGCAACTTCAGTC	ΔSO_2551 and ΔSO_2550-2551 deletion
2551fNDE	GATCCATATGGATTGAAAGTAGTGTC	ΔSO_2551 complementation
2551R	GATCGGATCCGGTTAACAACTACAGC	ΔSO_2551 deletion
2551R1	CCATTGCTTTAGTTGCCGTAGC	ΔSO_2551 and ΔSO_2550-2551 deletion
2551rBAM	GATCGGATCCGGCAGGTTTATACAGG	ΔSO_2550-2551 and ΔSO_2551 complementation
3550-3552F	GAGTCGGCTCTAACAAACCTCGC	SO_3550-2 complementation
3550-3552R	CGCTTCGTTAAGAGCGTGCG	SO_3550-2 complementation
3550insF	GCATCATCCGCAGCAACTTTCG	SO_3550 insertion
3550insR	GGCGTTACACCAAGAAGTCTCAG	SO_3550 insertion
615	TCGGGTATCGCTCTTGAAGGG	Himar sequencing
aa3compF	CCTCAAATGACAATCTCGCGC	*aa3* mutant complement
aa3compR	CTGGGCCTTATTGGTCTCAATCCC	*aa3* mutant complement
aa3proF	GATCAAGCTTCTCCGTATTTAGCCTTGAAAGCC	*aa3* promoter
aa3proR	GATCGGATCCATGGCACAACACTTCTCCTCTGCC	*aa3* promoter
ccoccompR	GACTCGGGTCAGCAGATCGTG	*cco* mutant complement
ccocompF	GTTAAACCAATTCATTCGCTTGGG	*cco* mutant complement

ccocompintF	GGCATCAGCCGTGATTGTGG	*cco* mutant complement
ccocompintR	GCTATTTACTCAGCATGGTGG	*cco* mutant complement
ccoPF	CGACGGGATCCATCATAATGCTTCCA	*cco* promoter
ccoPR	GTACGGTACCCGGTGAGAGGCTAATT	*cco* promoter
cyabF5	GCTATGCGCTTATCATGATGTTG	*cya*B deletion
cyabF6	GATCGAATTCCGCTTGCTGGGGATGC	*cya*B deletion
cyabR5	GATCGAATTCCAATTTTACAGCCTAG	*cya*B deletion
cyabR6	CTGTATTGCTCGAGCTCTGC	*cya*B deletion
cydABcompF	CCATATAAATACCGTCCATCTGG	*cyd*AB mutant complement
cydABcompR	CCGTAGCAAAACCCATCTAAACC	*cyd*AB mutant complement
CydABF1	CATGCTCAAGAGGGTGCC	*cyd*AB deletion
CydABF2	GATCGGTACCGGTTCCAATTGCTACG	*cyd*AB deletion
CydABR1	GATCGGTACCCCAAATCGCTAGATCC	*cyd*AB deletion
CydABR2	CTCGATTTGAAATATGCCG	*cyd*AB deletion
cydAPF	GATCAAGCTTCTGCAGCATTTCTAAT	*cyd*AB promoter
cydAPR	GATCGGATCCATCGGTGACTCCTTAC	*cyd*AB promoter
JBC 1/2F	CCCAGTCACGACGTTGTAAAACG	Plasmid promoter
JBC 1/2R	AGCGGATAACAATTTCACACAGG	Plasmid promoter
mCherryF2	CGATAAGGATCCGTCGAGATCTATGG	Fluorescent timer
mCherryR2	GATCTCTAGACACTCGGCATGGGGTC	Fluorescent timer
petABCcompF	CAGCATGTGACAAAGTTAACGC	*pet*ABC mutant complement
petABCcompR	GGATACTACATTTTACCCGTTATTGG	*pet*ABC mutant complement
SO4606-9F2	ATCGAAGCTTCTCGACCTAAGTAACG	*aa*$_3$ deletion
SO4606-9R1	ATCGAAGCTTCACCTCAACTCTAGC	*aa*$_3$ deletion
SO4606-9R2	CCGCCGTGATACCAATGC	*aa*$_3$ deletion
SO4606F1	CCGAAAATGAAGACTACTCACC	*aa*$_3$ deletion
SO608-10F1	GGAGGGAGTAATTCATGGG	*pet*ABC deletion
SO608-10F2	GATCGGTACCGCAAACGGGTAAAATG	*pet*ABC deletion
SO608-10R1	GATCGGTACCGCAGACGGTCAGATAG	*pet*ABC deletion
SO610R2	GCTTAGGCGGCTCGTCCTTG	*pet*ABC deletion

Table 2.3. List of plasmids used in this study.

Plasmid	**Description**	**Source**
pER21	Suicide vector	(22)
pMC10	lacZ reporter	(32)
pJBC1	Complementation vector	(45)
RB1	mini*HIMAR* Transposon	(46)

pKMB1	Fluorescence vector	This work
pUC119	High copy number plasmid	(47)

Transposon Mutagenesis

Transposon mutants were generated using the pMini*himar* RB1. The plasmid was transferred into *S. oneidensis* via conjugation using *E. coli* WM3064 as the donor strain (46). Mutants were selected on basal medium agar supplemented with 30mM lactate, 0.01% casamino acids, and 25μg/mL kanamycin. Chromosomal DNA was isolated from the mutants and digested with BamHI. The DNA was re-ligated and transformed into *E. coli* strain EC100. RB1 contains an origin of replication but lacks a BamHI restriction site; therefore, successful transformation indicates that the colonies contain the transposon plasmid and adjacent chromosomal DNA fragments. The chromosomal fragments on these plasmids were sequenced (Eurofins Scientific) using the primer 615 (Table 2.2). The resulting sequences were analyzed, and mutants of interest were selected for further study.

Growth Conditions and Assays

Overnight cultures of *E. coli* and *S. oneidensis* were primarily grown in LB medium. Basal medium (BM) (36) supplemented with 0.01% casamino acids and 30mM lactate, pyruvate, N-acetyl glucosamine (NAG), or 20mM acetate as the carbon source was used for aerobic growth assays. The strains used are listed in Table 2.1. Optical density was measured using the Tecan Infinite® m200 PRO plate reader. Twenty-four well plates were used for the assay. The wells contained 600μL of supplemented basal medium inoculated with overnight cultures at a 1/100 dilution. Wild-type *S. oneidensis* MR-1 was used as a control for all experiments. The experiments were carried out in the plate reader per manufacturer's instructions. The plate reader

was calibrated by measuring culture optical density (OD) of 23 culture dilutions in a Thermo Spectronic Genesys 20 spectrophotometer, pipetting into the 24 well plate, and measuring in the Tecan plate reader. The results were plotted, and a standard equation was determined. Data plotted in the figures in this document are the means and standard deviations of the growth assays. All cultures were assayed in triplicate.

Promoter Expression

To assess promoter activity of the terminal oxidase complexes, the plasmid pMC10, which carries a promoterless *lacZ*, was used as described previously (32). The promoters of interest (*cco*, *cyd*, *cox)* were amplified by PCR, digested with BamHI and NdeI, and inserted into the pMC10 plasmid. Measurement of β-galactosidase activity allows us to infer promoter activity under different growth conditions. β-galactosidase activity was determined as described by Miller, using ortho-Nitrophenyl-β-galactoside (ONPG) as a lactose analog that yields a yellow compound when digested by β-galactosidase. The intensity of the yellow color is reported in Miller units and graphed as percentage of wild-type activity (48). Supplemented basal medium was inoculated with overnight cultures and incubated for 3-4 hours at 30°C with shaking. The β-galactosidase activity was measured in these cultures as described previously (32, 48).

The limitations of these assays are that they only capture a snapshot of the activity of the promoter. Once a β-galactosidase assay is performed, the cells are dead and cannot be tested further. To test promoter expression over time, a plasmid was developed using a fluorescent timer protein. Monomeric fluorescent timers change color over time at specific rates. *lacZ* in pMC10 was replaced with Fast-FT mCherry from pBAD/His-Fast-FT. This fluorescent timer fluoresces blue at 15 minutes after translation and matures to red in seven hours at 37°C.

Excitation/emission wavelengths for blue and red are 403/466nm and 583/606nm, respectively (49). The *cco*, *cox*, and *cyd* promotors were cloned upstream of mCherry in the resultant plasmid pKMB1. The plasmids were transferred into *E. coli* β2155 by electroporation and then into *S. oneidensis* wild type and mutant strains by conjugation. Growth curves were performed using the Tecan Infinite® m200 PRO plate reader as described above measuring OD600 as well as fluorescence emission. The overnight cultures for these assays were grown anaerobically in LB medium supplemented with 30mM lactate and 10mM fumarate. These growth conditions were chosen to minimize the activity of the aerobic oxidase promoters in the starter cultures.

CHAPTER 3

FUNCTION OF AEROBIC OXIDASE COMPLEXES IN *SHEWANELLA ONEIDENSIS*

Introduction

The reduction of oxygen to water has the highest redox potential of any respiratory reaction, making aerobic respiration the most favorable form of energy generation (50, 51). As mentioned above, *S. oneidensis* is unique among metal reducing organisms in that it is a facultative anaerobe. *S. oneidensis* employs many common proteins in its usage of oxygen; however, evidence suggests that their expression is atypical.

The genome of *S. oneidensis* encodes three terminal aerobic oxidases (*aa*$_3$- and *cbb*$_3$-types and a *bd*-type) as well as the intermediate *bc*1 type oxidase for the utilization of oxygen (10, 52). The primary terminal aerobic oxidase in many organisms is the Type A HCO or *aa*$_3$-type cytochrome *c*. It has low affinity for oxygen and is therefore used under high oxygen tensions (53). In *S. oneidensis*, the *cox* operon codes for this complex. *coxB*, *coxA*, and *coxC* (SO_4606, 4607, and 4609) encode the structural subunits of the *aa*$_3$-type cytochrome *c*, of which CoxA and CoxB (Subunits I and II) are the catalytic subunits. Both subunits contain heme binding motifs. Subunit II transfers electrons to the heme-copper binuclear site of Subunit I, where the reduction of O_2 to H_2O takes place (19, 20, 25, 27). Subunit III does not bind heme, nor does it pump protons; it may contribute to assembly or stability, but it is not necessary for reduction of O_2 (19, 54).

The *cbb*$_3$-type cytochrome *c* oxidase is a high-affinity *c* cytochrome. It is often the primary *c* cytochrome used under microaerobic conditions (55). It is encoded by the *cco* operon, and the structural subunits are encoded by *ccoNQOP* (SO_2364-2361) (27). CcoO and CcoP are

c-type cytochromes that are membrane-bound and serve as intermediates in the transfer of electrons from the *bc*1 complex to the catalytic subunit CcoN. CcoN contains 12 transmembrane helices and the heme-copper binuclear site necessary for reduction of O_2. CcoQ has no described function in *S. oneidensis* (55, 56). It is a small membrane-bound polypeptide that, in other organisms, is suggested to stabilize CcoP (57).

During aerobic respiration, the intermediate *bc*1 complex transfers electrons from the quinone pool to the aa_3 and cbb_3 terminal oxidase complexes. In *S. oneidensis*, this complex is encoded by *petABC* (SO_608-610) (27). *petA* encodes the Reiske iron-sulfur protein, *petB* encodes a *b*-type cytochrome containing two hemes *b*, and *pet*C encodes a c_1-type cytochrome. Electron transfer occurs from the quinol pool to the Fe-S center of the Reiske protein, to the hemes *b*, then from the *c* cytochrome to the terminal oxidase complex (20, 58-60).

S. oneidensis has an additional high-affinity terminal oxidase complex proposed to function in low oxygen concentration, the *bd* complex. This complex is not part of the heme-copper oxidase family of oxygen reductases, but rather is part of a distinct class of quinol oxidases. The *bd* complex directly oxidizes quinol and reduces O_2, creating proton motive force via membrane charge separation (61). *S. oneidensis cydAB* (SO3286-5) encodes this complex (27). Subunit I (CydA) contains two hemes *b*, and Subunit II (CydB) contains one heme *d.* The complex is membrane bound, and it reduces O_2 at the junction between the two subunits (23). This process generates less energy than the two-step process of the heme-copper oxidase pathway (20). This chapter explores the relationship between these cytochrome complexes and growth rate on four different carbon sources.

Results

As mentioned in the Materials and Methods section, mutants were generated that lacked the structural components of the oxidases and the genes involved in their maturation (Table 2.1). These mutants included single and double mutants that lacked one or more of the predicted oxidase operons. However, attempts to generate a mutant that lacked both the *cbb*3-type cytochrome *c* oxidase and the *bd*-type quinol oxidase were not successful. Previous reports suggested that loss of both oxidase complexes may be lethal (25, 62). Attempts to isolate this double mutant under anaerobic conditions were also not successful. Complementation of mutants was successful with the exception of Δ*cco* operon mutants. The deletion comprises approximately 7kb and its large size was prohibitive in insertion of the fragment into the complementation plasmid pJBC1.

Aerobic Growth with Different Carbon Sources

Wild-type and mutant strains were tested for aerobic growth with lactate, pyruvate, acetate, and N-acetyl glucosamine as sole carbon sources. N-acetyl glucosamine supported aerobic growth of each mutant except for the Δ*pet*Δ*cyd* double mutant (data not shown). This mutant was deficient in aerobic growth with N-acetyl glucosamine, lactate, and pyruvate (data not shown and Figures 3.1 and 3.3).

Mutants deficient in the *bd*-type quinol oxidase (Δ*cyd*), the aa_3-type cytochrome *c* oxidase (Δ*cox*), or both grew similarly to the wild type in the presence of lactate as the sole carbon source. The *bc*1 complex deletion (Δ*pet*) grew at the same rate as wild-type *S. oneidensis* as well. Mutants lacking the cbb_3 oxidase (Δ*cco* and Δ*cco*Δ*cox*) had a slight growth deficiency, but reached wild-type culture densities at approximately six hours (Figure 3.1). A mutant lacking

the *bc*1 complex and the *bd*-type quinol oxidase (Δ*pet*Δ*cyd*) had a severe growth deficiency and did not reach wild-type culture density in 18 hours. Complementation of this mutant with either *petABC* or *cydAB* restored aerobic growth similar to wild-type density (Figure 3.2)

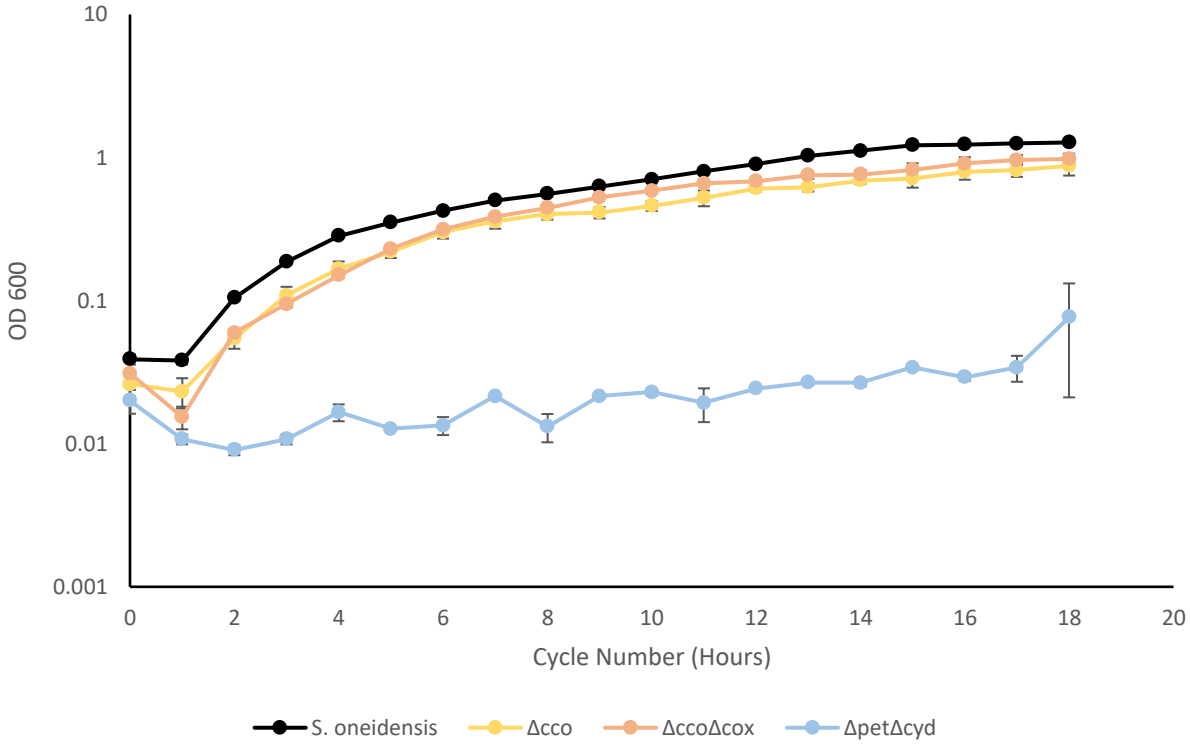


Figure 3.1 Aerobic growth of wild-type *S. oneidensis* and deletion mutants in basal medium supplemented with 30mM lactate and 0.01% casamino acids.

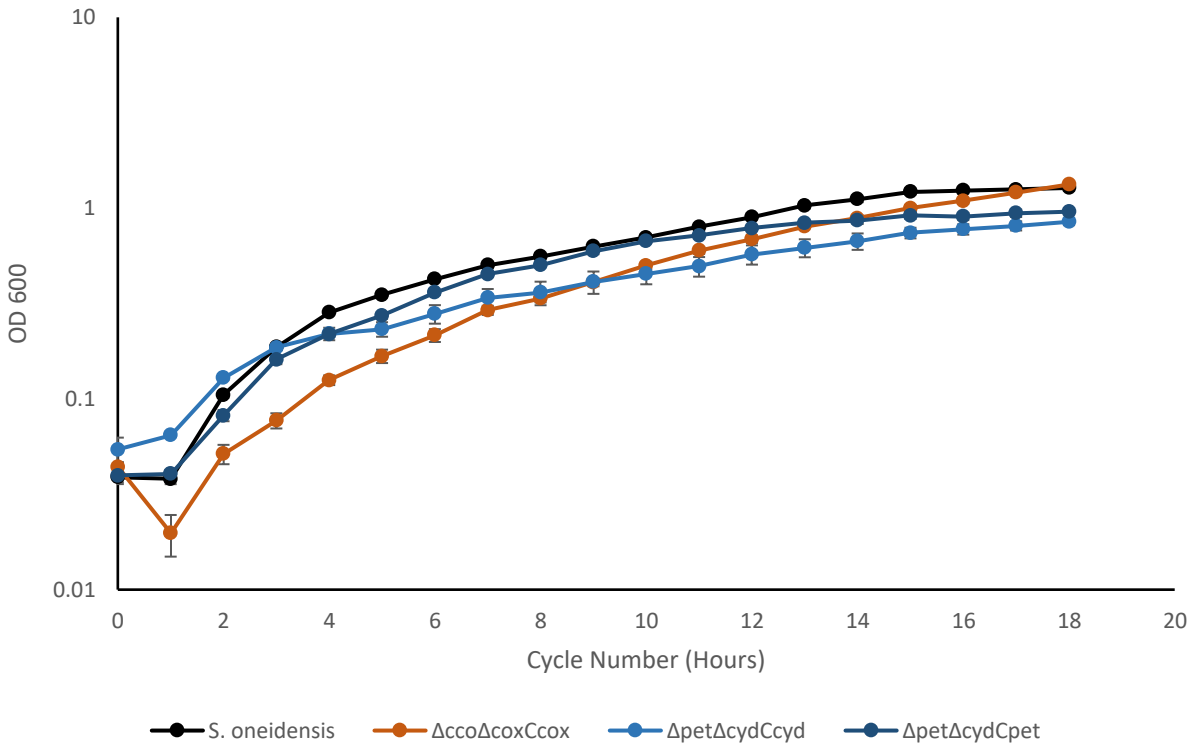


Figure 3.2 Aerobic growth of wild-type *S. oneidensis* and complemented mutants in basal medium supplemented with 30mM lactate and 0.01% casamino acids.

Similar results were obtained when the oxidase mutants were grown in the presence of pyruvate. Loss of the *bc*1 complex, the *bd*-type oxidase, or the *aa*3-type oxidase did not appear to affect growth compared to the wild type. Deletion of the *cco* genes did not cause a major growth deficiency but deletion of both the *cco* and *cox* operons resulted in a growth deficiency and mutant cultures did not reach wild-type culture density after 18 hours. The Δ*pet*Δ*cyd* mutant had a severe growth deficiency in pyruvate (Figure 3.3). Complementation of the mutants restored their ability to grow with pyruvate similar to the wild type (Figure 3.4).

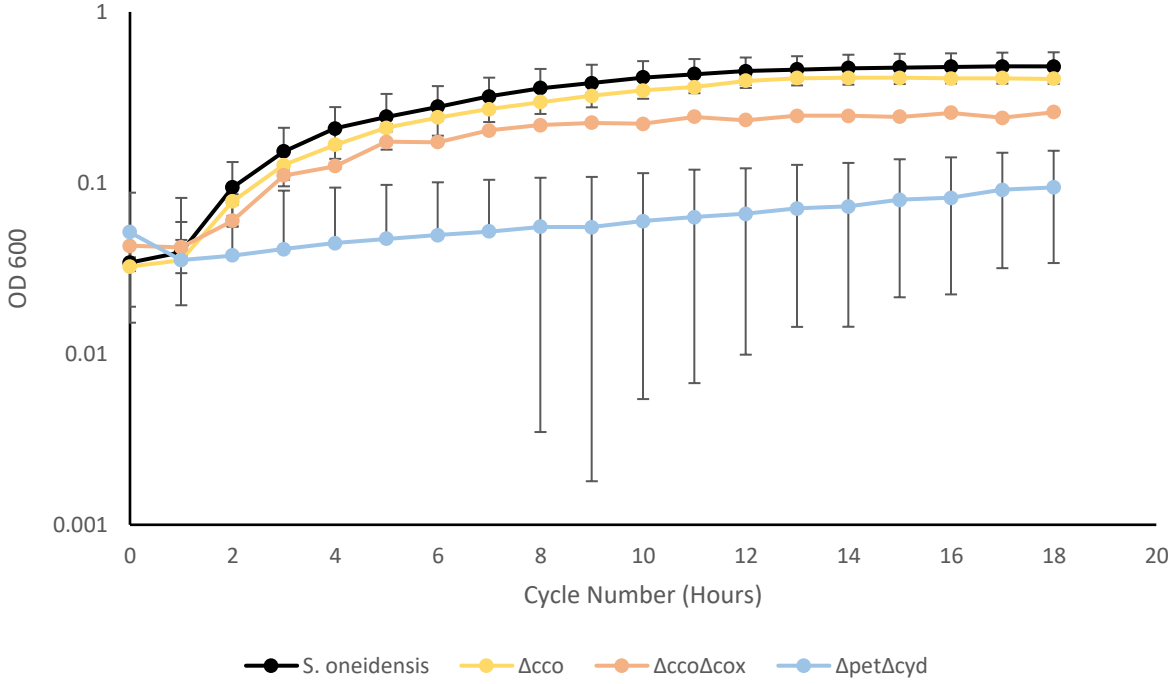


Figure 3.3 Aerobic growth of wild-type *S. oneidensis* and deletion mutations in basal medium supplemented with 30mM pyruvate and 0.01% casamino acids.

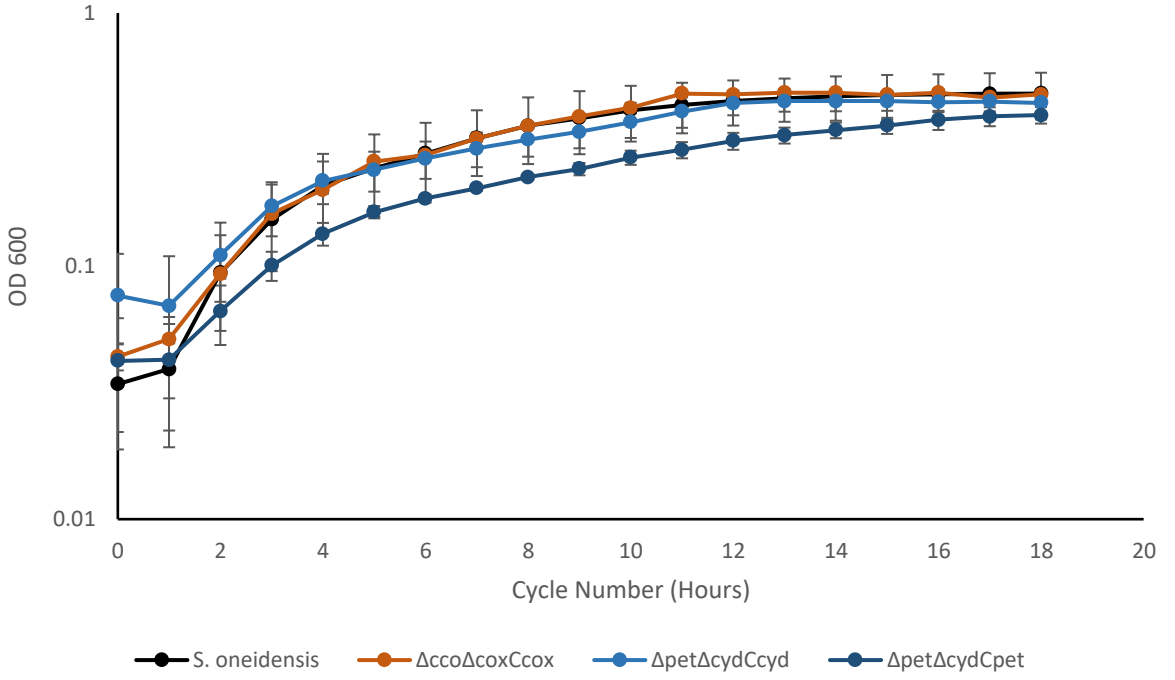


Figure 3.4 Aerobic growth of wild-type *S. oneidensis* and complemented mutants in basal medium supplemented with 30mM pyruvate and 0.01% casamino acids.

The overall growth rate of wild-type *S. oneidensis* cultures grown in basal medium supplemented with 20mM acetate and 0.01% casamino acids is slower than with other carbon sources. Therefore, growth with acetate was monitored over a period of 48 hours. Mutants deficient in the *bd*-type quinol oxidase, the *aa*$_3$-type cytochrome *c* oxidase, or both grew at the same rate as wild-type *S. oneidensis* (Figure 3.5). A growth deficiency was observed in Δ*pet*Δ*cyd* mutant (Figure 3.5). Surprisingly, this deficiency was not as severe as when other carbon sources were used (Figures 3.1 and 3.3). The deletions that include the *cbb*$_3$-type cytochrome *c* oxidase (Δ*cco* and Δ*cco*Δ*cox*) had a more pronounced deficiency using acetate than they do with other carbon sources. They did not reach the same culture density as the wild-type cultures and they reached stationary phase approximately 10 hours after the wild-type cultures. In addition, acetate was the only carbon source tested in which the *bc*1 complex deletion mutant demonstrated a growth deficiency. Complementation of mutants restored their ability to grow similar to the wild type (Figure 3.6)

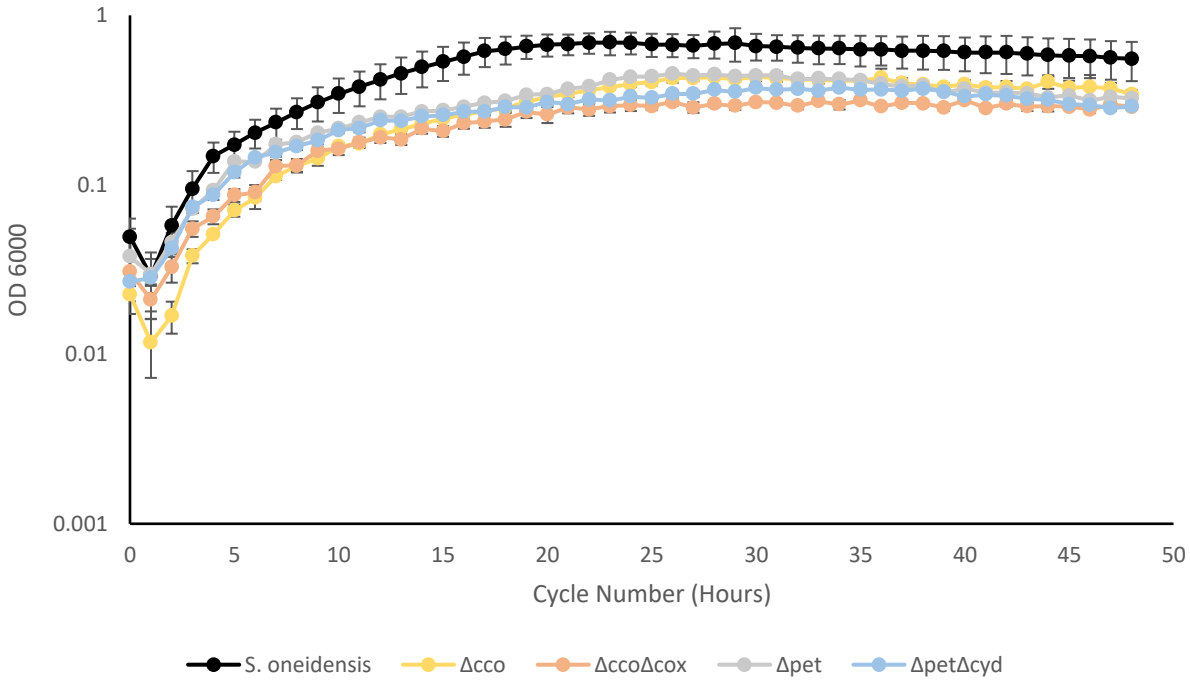


Figure 3.5 Aerobic growth of wild-type *S. oneidensis* and deletion mutants in basal medium supplemented with 20mM acetate and 0.01% casamino acids.

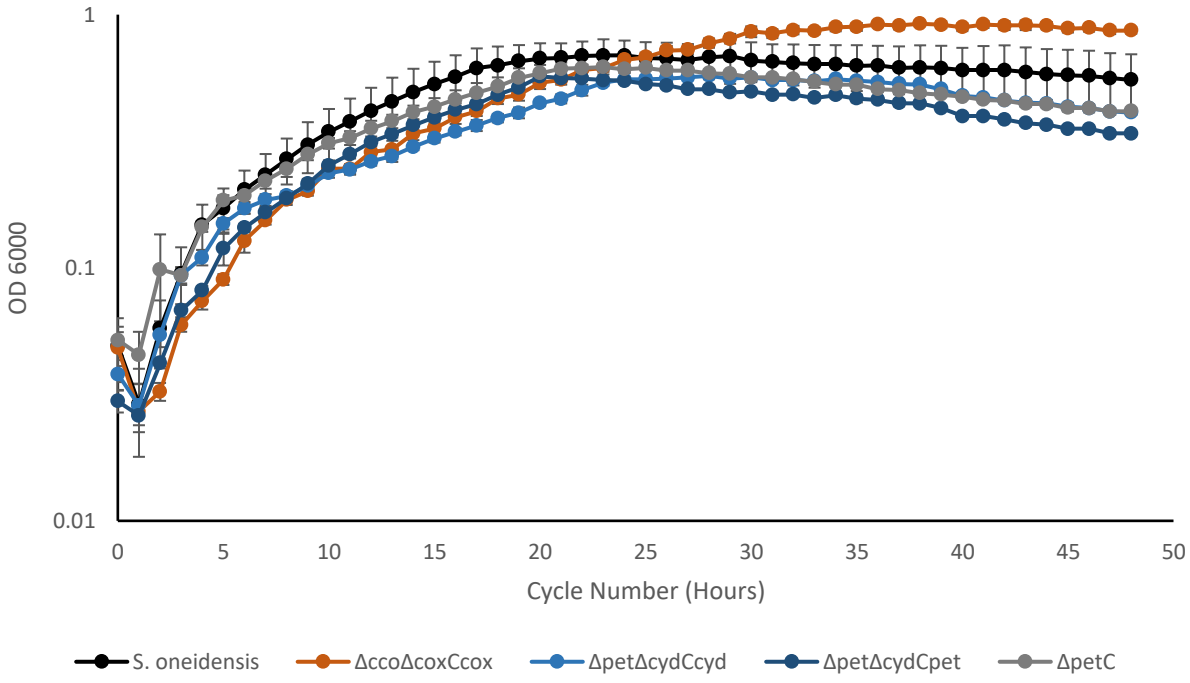


Figure 3.6 Aerobic growth of wild-type *S. oneidensis* and complemented mutants in basal medium supplemented with 20mM acetate and 0.01% casamino acids.

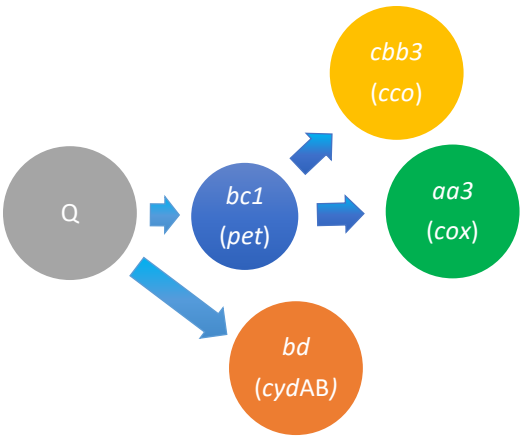


Figure 3.7 Diagram of the path of electrons from the quinone pool (Q) to the terminal aerobic oxidase complexes.

Discussion

As mentioned above, the *cbb*$_3$-type cytochrome *c* oxidase and the *bd*-type quinol oxidase both have a high affinity for O_2 and therefore function under microaerobic conditions (23, 55). The low affinity *aa*$_3$-type cytochrome *c* oxidase is the major oxidase in other organisms and functions under oxygen replete conditions (53). The results demonstrate that deletions of the *aa*$_3$-type and *bd*-type oxidases have no effect on the growth of *S. oneidensis* under the tested conditions, indicating that neither of these complexes are necessary for aerobic growth. Promoter expression experiments for the *aa*$_3$-type oxidase (data not shown) demonstrate little to no activity of this promoter under any conditions tested. This lack of function for the *aa*$_3$-type cytochrome *c* oxidase tested is supported by other works, suggesting it has a yet unknown function – possibly under nutrient starvation conditions (25, 62, 63). The *cbb*$_3$-type cytochrome *c* oxidase deletions have a slight effect on the growth rate of *S. oneidensis*. As this is the only single terminal oxidase deletion to affect growth rate under the conditions tested, it is likely the primary aerobic oxidase in *S. oneidensis*. Combined with the lack of detection of the *aa*$_3$-type oxidase, it is possible that

the *bd*-type terminal oxidase is functioning in the Δ*cco* mutants to compensate for the loss of the *cbb*$_3$-type oxidase. The inability to obtain a deletion of both the *cbb*$_3$-type and *bd*-type oxidases supports this hypothesis. These are the major oxidases in this organism; therefore, one must be present for aerobic respiration to occur. Both of these oxidases commonly function under microaerobic conditions, but it appears that they function in oxygen-replete environments in *S. oneidensis*. These terminal oxidases playing a less common role in *S. oneidensis* is supported by biochemical analyses and by analyses of other organisms with atypical cytochrome functions (25, 64).

The exclusive mutant with a severe growth deficiency is the deletion of both the *bc*1 complex and *bd*-type terminal oxidase. Figure 3.7 indicates the hypothesized flow of electrons from the quinone pool to the terminal aerobic oxidases for *S. oneidensis*. As mentioned above, the *bc*1 complex is an intermediate electron transfer protein between the quinone pool and the *cbb*$_3$- and *aa*$_3$-type terminal oxidases (Figure 3.7). The *bd*-type terminal oxidase bypasses this system and accepts electrons directly from the quinone pool. The inability of the Δ*cco*Δ*cyd* mutant to be obtained aerobically suggests that the Δ*pet*Δ*cyd* mutant would also not survive under aerobic conditions as the same pathway is being knocked out in both mutants. The *cbb*$_3$-type cytochrome is unable to accept electrons from the quinone pool; therefore, it is likely this mutant survives by fermenting amino acids or small peptides present in LB or basal media (42, 56, 65).

Carbon source choice has a small effect on the growth phenotype. *S. oneidensis* does not use a wide variety of carbon sources and is unable to ferment glucose due to lack of the enzyme 6-phosphofructokinase (66). The main carbon source used is lactate, a three-carbon molecule that is converted to pyruvate by the enzyme lactate dehydrogenase. *S. oneidensis* has a different class

of lactate dehydrogenase enzymes than *E. coli*. Even though the genome of *S. oneidensis* does code for *ldh*A, the lactate dehydrogenase for D-lactate, this is a fermentative enzyme and there is no evidence that *S. oneidensis* uses sugar fermentation as a method of energy generation. The lactate dehydrogenase enzymes in *S. oneidensis* are Dld-II and LldEFG for oxidation of D- and L-lactate, respectively (66-68). *S. oneidensis* can metabolize pyruvate into lactate, formate, acetate, CO_2, and H_2. Conversion of pyruvate to acetate via acetyl-CoA is the primary energy generation pathway for this carbon source. Also, pyruvate fermentation can enhance survivability of *S. oneidensis* under anaerobic conditions (68, 69). *S. oneidensis* degrades chitin and thus use its monomer, N-acetyl glucosamine, as a sole carbon source (66, 70). Acetate may be produced by the degradation of lactate and pyruvate. *S. oneidensis* is then able to use it as a carbon source under aerobic conditions. When both lactate and acetate are present, they are used simultaneously, i.e. there is no diauxic growth, implying lack of strict regulation of carbon source usage (71). Usage of acetate is mediated by the glyoxylate shunt, and upregulation of its enzymes can be seen when *S. oneidensis* is growing on acetate as a sole carbon source (72, 73).

The results suggest a lack of regulation in carbon source usage with regard to aerobic respiration, as the results do not change drastically based on carbon source chosen. As stated above, the only severe growth deficiency is observed when the *bd*-type quinol oxidase and the *bc*1 complex are deleted simultaneously. The deficiency is less severe when acetate is the carbon source, especially in comparison to the phenotypes of the other deletion mutants, Δ*cco*, Δ*cco*Δ*cox*, and Δ*pet*. Overall growth rate is slower while using this carbon source, likely due to the necessity of ATP consumption to convert acetate into acetyl-CoA (71). Kinetic models of *S. oneidensis* suggest that the organism often uses futile cycles, or cycles that are suboptimal in terms of biomass production as they do not result in a predicted amount of energy based on

mathematical models. This is unsurprising because *S. oneidensis* occupies suboxic and nutrient-poor environments in nature. Usage of futile cycles may confer an advantage in these environments, offering *S. oneidensis* a higher metabolic rate versus yield, allowing them to outcompete for a niche even if biomass is sacrificed (72, 74). *S. oneidensis* does not have a metabolic framework for thriving in a nutrient-rich, highly oxygenated culture, meaning it lacks strict regulation of carbon source utilization and does not use the *cbb*$_3$ and *aa*$_3$ terminal oxidase complexes as predicted based on oxygen tension (74). This is the likely explanation for the observed phenotypes; *S. oneidensis* thrives almost as a scavenger in the natural environment, particularly with regard to oxygen. Therefore, analyzing a streamlined aerobic process in a simplified environment results in a more complicated answer than metabolic models based strictly on presence of genes for specific oxidase complexes would predict.

CHAPTER 4

REGULATION OF AEROBIC RESPIRATION IN *SHEWANELLA ONEIDENSIS*

Introduction

Bacteria that can respire several electron acceptors employ regulatory networks and exhibit redox taxis (75) to maximize energy generation. *Shewanella* species employ these energy taxis systems and regulate cellular processes, including carbon metabolism, flagellar synthesis, and nutrient import based on electron acceptor redox potential. They also preferentially congregate toward the electron acceptor from their native habitat (76-78). As mentioned above, one of the regulatory systems involved in electron acceptor usage is EtrA, the *E. coli* FNR homolog in *S. oneidensis*. The EtrA of *S. oneidensis* is able to complement *E. coli* FNR (29). EtrA directly senses oxygen – aerobic conditions oxidize and inactivate the Fe-S center of EtrA. Therefore, it is only used to regulate anaerobic respiration, specifically the use of DMSO as a terminal electron acceptor (28, 29, 79).

The ArcAB two-component system is an additional redox-sensing system in *S. oneidensis*. Under microaerobic and anaerobic conditions, the kinase ArcB responds to reduced oxygen conditions and activates the response regulator ArcA by phosphorylation leading to activation or repression of genes involved in specific metabolic processes (30, 31, 33). The Arc system in *S. oneidensis* is atypical, where ArcB domains are encoded by two genes *arc*B1 and *hpt*A. ArcB1 (also referred to as ArcS) contains the sensor domain and HptA contains the phosphotransfer domain (32, 33). *S. oneidensis* ArcA is highly similar to the *E. coli* ArcA, but there is little functional overlap. The *bd*-type quinol oxidase is repressed by ArcA under anaerobic conditions in *S. oneidensis*, which is the opposite of *E. coli* (23, 33, 80). The *cbb*$_3$-type

oxidase (*cco*) is negatively regulated by ArcA under anaerobic conditions. The fumarate reductase is not regulated by ArcA, and DMSO reduction is partially regulated (32, 33, 80). Deletion mutants are not universally deficient in respiration, though, which suggests other regulatory mechanisms for both aerobic and anaerobic respiration, possibly through other activators.

The global regulator CRP is primarily involved in carbon metabolism in *E. coli* and can activate a variety of metabolic processes as mentioned in Chapter 1. There is a considerable lack of overlap between CRP regulation in *E. coli* and *S. oneidensis*. The major function of CRP in *S. oneidensis* is regulation of respiration, which is surprising considering CRP does not have redox-sensing capabilities (36, 81). Deletion mutants of CRP are deficient in anaerobic respiration with many substrates, including DMSO, fumarate, and iron and manganese oxides (36).

Activation of CRP requires cAMP, which is produced by adenylate cyclases. cAMP levels are further regulated by the activity of phosphodiesterases (35). *E. coli* possesses one adenylate cyclase that is activated when glucose levels are low (82). *S. oneidensis* synthesizes three adenylate cyclases, encoded by *cyaA*, *B*, and *C* and the one phosphodiesterase is encoded by SO_3901 (*cpd*A) (27). Like CRP, adenylate cyclases are not redox-sensing; therefore, the mechanism of activation is not directly based on presence of oxygen. CyaA and CyaC are the adenylate cyclases that are active under anaerobic conditions. CyaA is cytoplasmic and CyaC is membrane bound and deletions of both of them are phenotypically comparable to a CRP deletion (37), and increased cAMP levels induce fumarate and iron reductases. According to previous microarray analysis performed in this lab, the *bd*-type quinol oxidase (*cyd*) is upregulated and the *aa*$_3$-type cytochrome *c* (*cox*) is downregulated in ΔCRP mutants under O_2-limited and anaerobic

conditions, respectively (37, 83). CRP and CyaB are discussed further below, and SO_3901 (*cpdA*) will be discussed in this chapter as well as in Chapter 5 in more detail.

In addition to CRP, the *S. oneidensis* genome encodes two CRP-like proteins, SO_2550 and SO_2551. They are labeled as such due to the presence of a helix-turn-helix motif, or DNA-binding domain, and cyclic nucleotide binding domain. The intergenic region is 125bp, suggesting they are co-transcribed (27). This chapter will explore the effect these regulatory systems have on aerobic respiration and assess if deficiencies in aerobic growth are as a result of differences in expression of terminal aerobic oxidase complexes.

Results

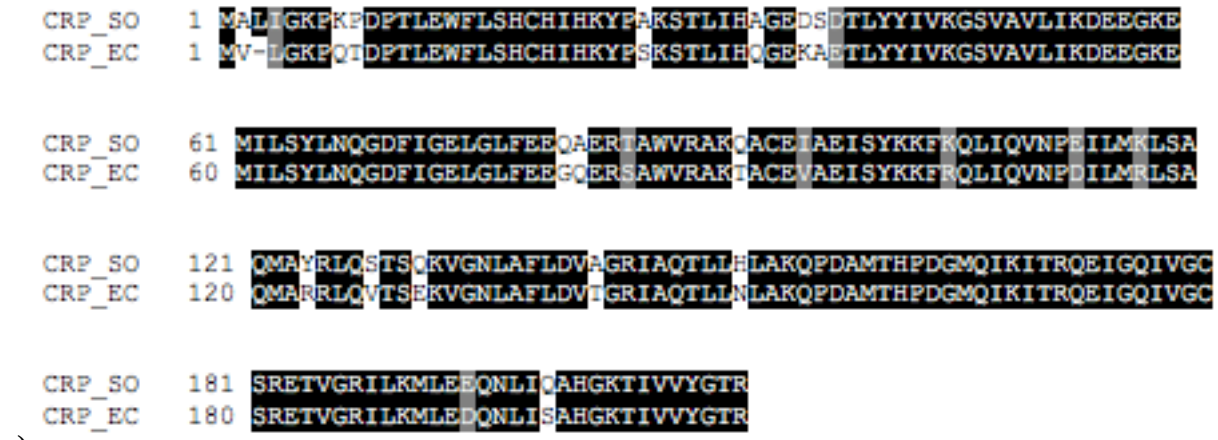
Analysis of *arc*A, *crp,* and SO_3901 Mutants and Complemented Strains

The *arc*A, *crp,* and SO_3901 deletion mutants and complemented strains were created by previous members of this lab. In addition, mutants were generated that lack genes predicted to encode cAMP-receptor proteins (ΔSO_2550-2551 and ΔSO_2551). The ΔSO_2551 mutant was complemented with SO_2551 and the ΔSO_2550-2551 mutant was complemented with SO_2550-2551 and SO_2551 to investigate the phenotype of SO_2550, of which a deletion has yet to be completed. SO_2550 and SO_2551 were of interest because of their similarity to CRP and because of the involvement of cAMP in aerobic and anaerobic respiration in *S. oneidensis*.

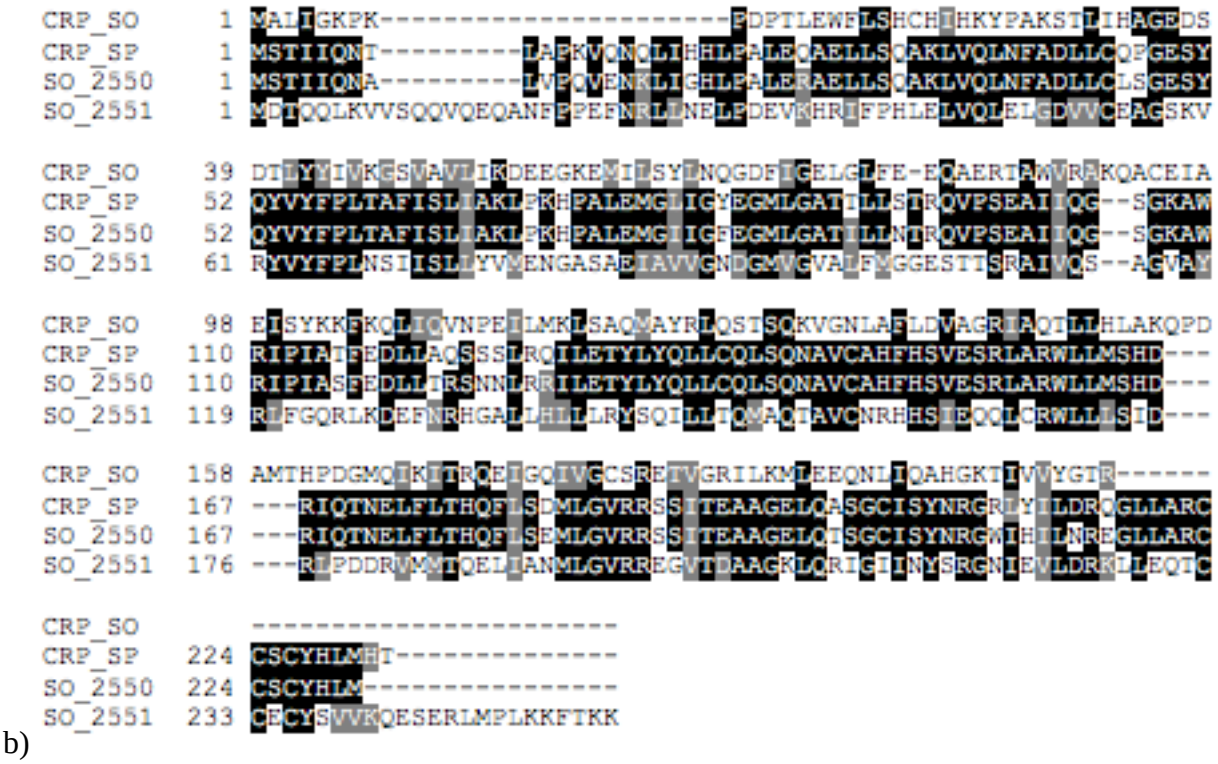
Analysis of Protein Sequence

There is little identity overlap of amino acids between the *S. oneidensis* CRP and SO_2550 and SO_2551 (Figure 4.1a); however, there is a substantial degree of identity between

a suggested CRP in *S. putrefaciens* and SO_2550. The helix-turn-helix domains as well as the cyclic nucleotide binding domains are indicated in Figure 4.1c and d.

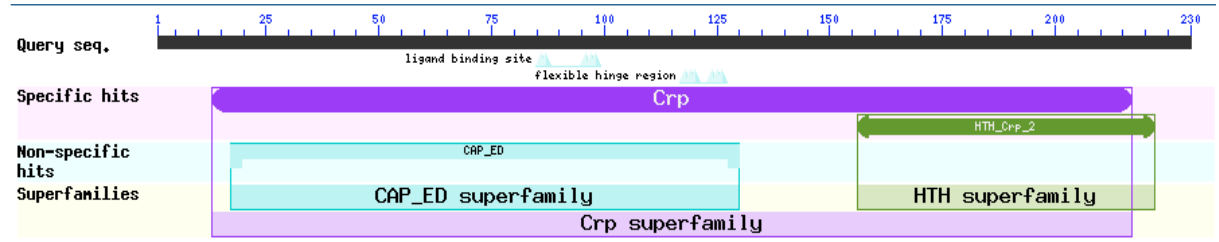


a)



b)

c)



d)

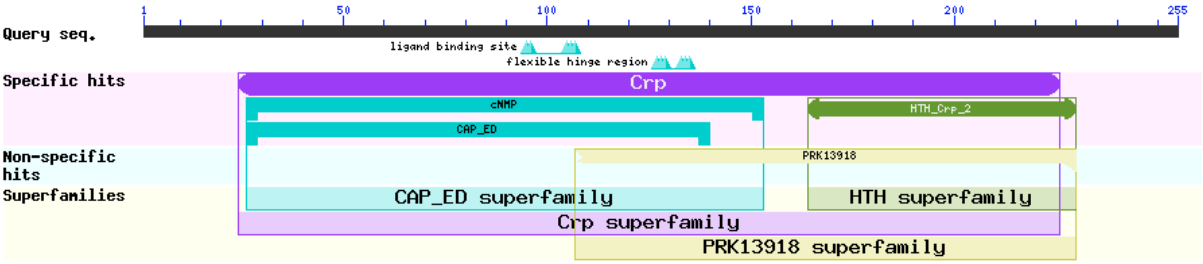


Figure 4.1 a) Sequence alignment the CRP of *S. oneidensis* (CRP_SO) and the CRP from *E. coli* (CRP_EC) (84) b) Sequence alignment of SO_2550 and SO_2551 compared with CRP in *S. oneidensis* (CRP_SO) and a CRP-like protein from *Shewanella putrefaciens* (CRP_SP) (84) c) CRP-like regions of SO_2550 d) CRP-like regions of SO_2551 (85).

To assess the ability of the SO_2550 and/or SO_2551 to complement an *E. coli* CRP deletion mutant, complementation plasmids were created with the gene(s) of interest cloned downstream of the *lac* promoter as described in Chapter 2. Plasmids were created containing SO_2550, SO_2551, and SO_2550-2551. The plasmids were transferred into an *E. coli crp* mutant (Table 2.1) and plated on LB plates containing X-gal. Gene expression was induced with IPTG. Breakdown of X-gal in the transformed cells would indicate complementation of *crp* deletion in this *E. coli* strain. None of the transformed colonies were able to breakdown X-gal, suggesting that neither gene complemented the loss of *crp* in *E. coli*.

Aerobic Growth with Different Carbon Sources

To determine the role of known and putative regulators on aerobic respiration, mutants deficient in *crp*, *cpdA* (SO_3901), and SO_2550-2551 were tested for aerobic growth in the

presence of different carbon and energy sources. A description of the mutants and growth conditions is provided in Chapter 2. In the presence of lactate, the ΔSO_3901 (Δ*cpd*A), Δ*crp* and ΔSO_2550-2551 mutants exhibit growth deficiencies to varying degrees (Figure 4.2). Deletion of *cpdA* caused the most severe growth deficiency. Deletion of *crp* negatively affected the growth rate and the mutant reached less than half the culture density of wild-type *S. oneidensis.* The ΔSO_2550-2551 mutant grew slightly better than the Δ*crp* mutant; however, it still only reached half of the final culture density of wild type. The wild-type growth phenotype of the SO_2550-2551 was restored by the introduction of SO_2551 (Figure 4.3). This suggests the phenotype of the SO_2550-2551 mutant is as a result of deletion of both genes, not just of SO_2550. The ΔSO_2551 single deletion mutant grew at the same rate as wild-type *S. oneidensis*, further illustrating that the growth deficiency is the result of the deletion of both SO_2550 and SO_2551. Deletion of *arc*A affected growth early in the experiment; however, the mutant reached the same culture density as wild type by 18 hours of growth.

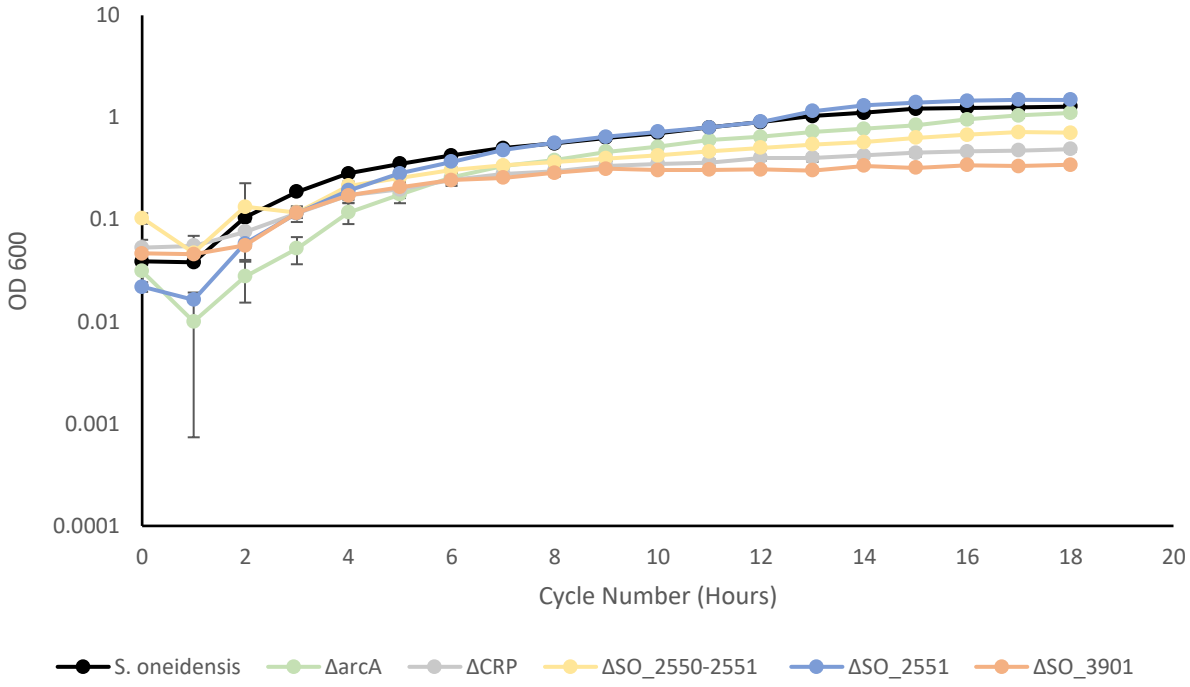


Figure 4.2 Aerobic growth of wild-type *S.* oneidensis and deletion mutants in basal medium supplemented with 30mM lactate and 0.01% casamino acids.

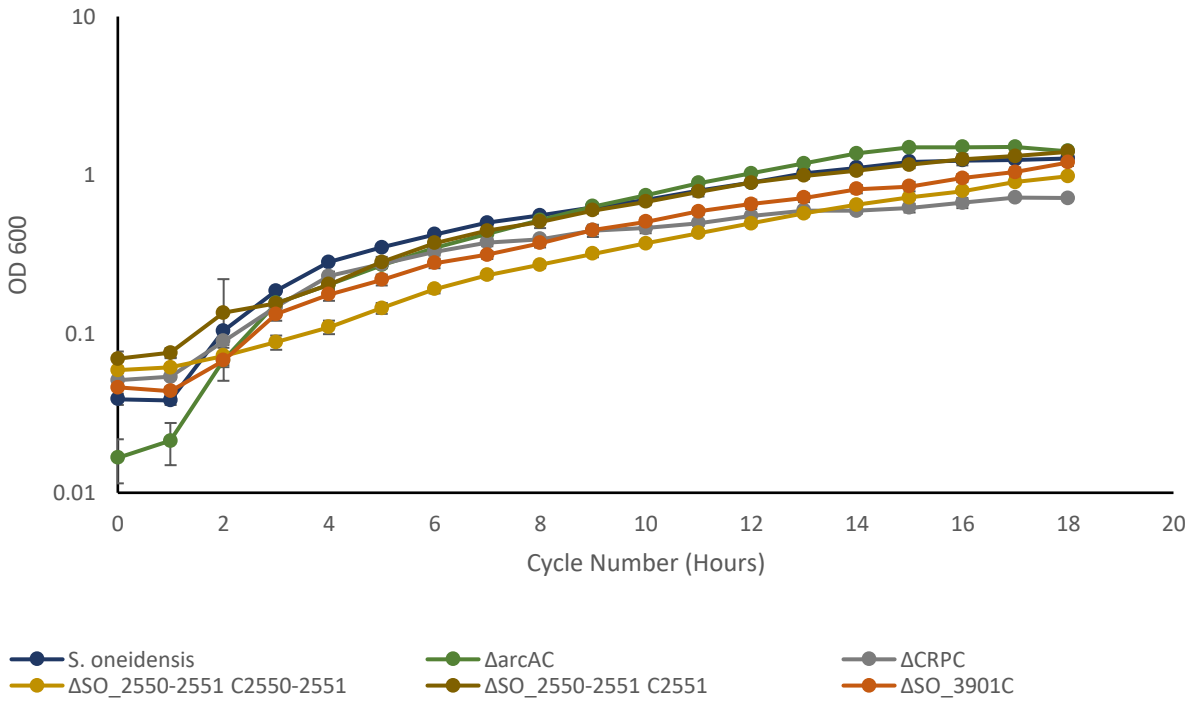


Figure 4.3 Aerobic growth of wild-type *S. oneidensis* and complemented mutants in basal medium supplemented with 30mM lactate and 0.01% casamino acids.

When grown with pyruvate, Δ*crp* and ΔSO_2551 grew similar to the wild type (Figure 4.4). This was surprising because we expected Δ*crp* to exhibit the same growth deficiency observed in the presence of lactate (Figure 4.2). The Δ*arc*A, ΔSO_3901, and ΔSO_2550-2551 mutants exhibited severe growth deficiencies compared to the wild type. Interestingly, the Δ*arc*A mutant appeared to have a long lag phase, but reached a similar cell density as the wild type after 18 hours of incubation. The ΔSO_3901 (Δ*cpd*A) and ΔSO_2550-2551 mutants have a similarly severe growth deficiency under these conditions, reaching less than half of the final density of wild type (Figure 4.4). Complementation of Δ*cpd*A, Δ*arc*A, and ΔSO_2550-2551 mutants restored aerobic growth with pyruvate similar to the wild type (Figure 4.5).

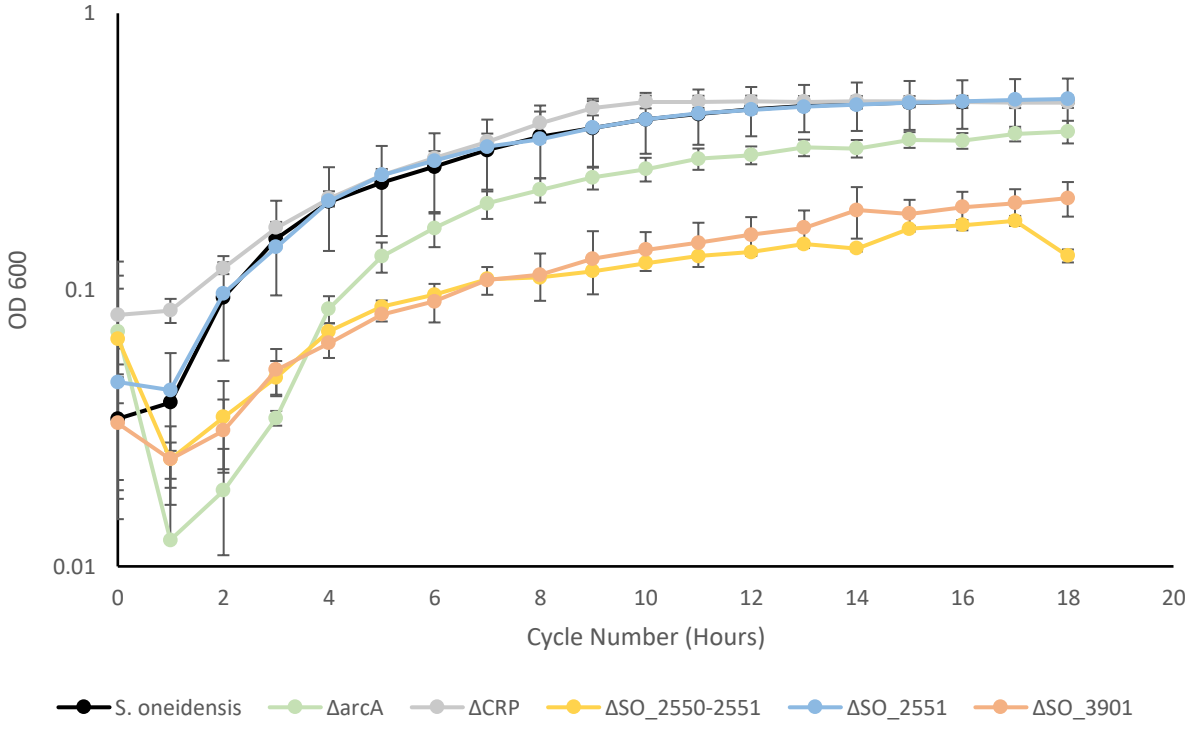


Figure 4.4 Aerobic growth of wild-type *S. oneidensis* and deletion mutants in basal medium supplemented with 30mM pyruvate and 0.01% casamino acids.

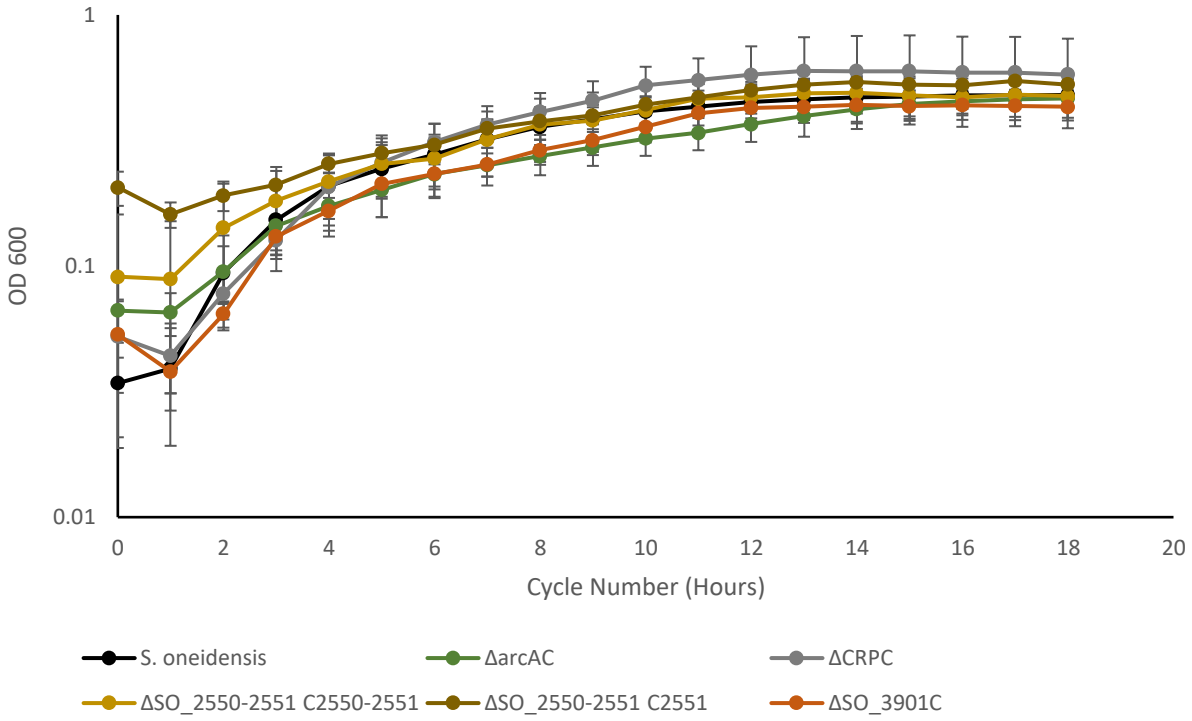


Figure 4.5 Aerobic growth of wild-type *S. oneidensis* and complemented mutants in basal medium supplemented with 30mM pyruvate and 0.01% casamino acids.

When grown with acetate, Δ*crp* and ΔSO_2551 mutants grew at the same rate as wild-type *S. oneidensis*. The Δ*arc*A mutant exhibited a long lag phase before growth resumed to wild type levels. Similar results were obtained with ΔSO_2550-2551 mutant (Figure 4.6). The deletion of SO_3901 (*cpd*A) had the most severe growth deficiency, similar to its phenotype with other carbon sources. Complementation of the mutants with the respective plasmids restored their ability to grow with acetate similar to the wild-type (Figure 4.7).

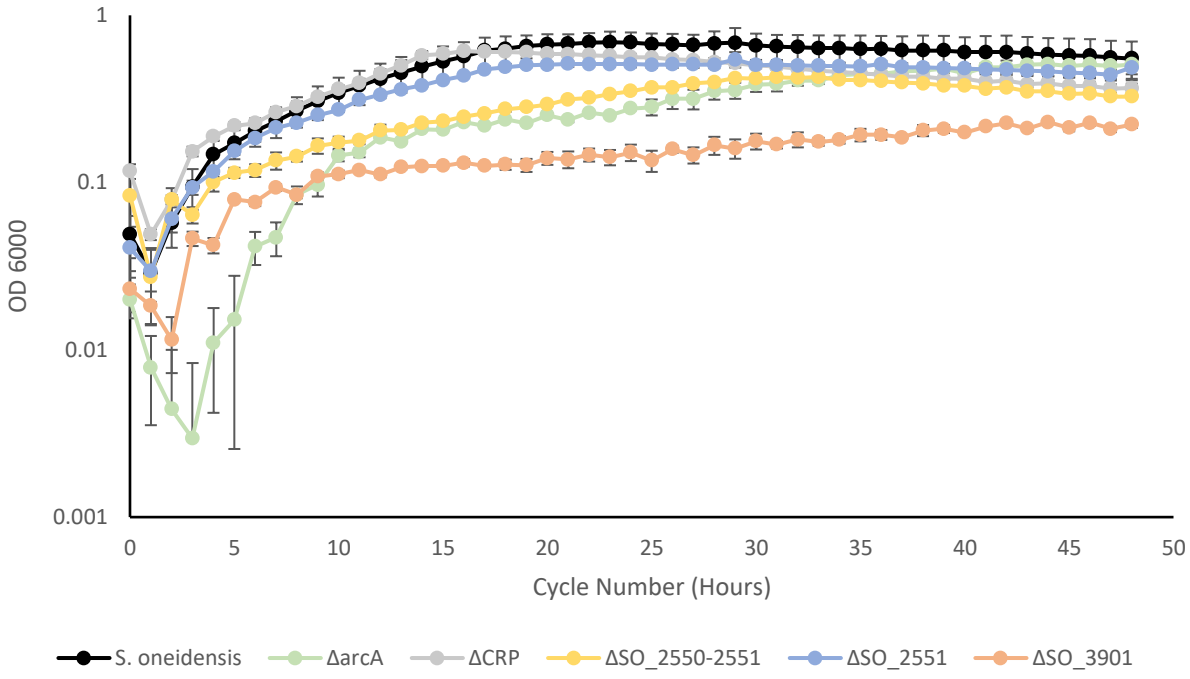


Figure 4.6 Aerobic growth of wild-type *S. oneidensis* and deletion mutants in basal medium supplemented with 20mM acetate and 0.01% casamino acids.

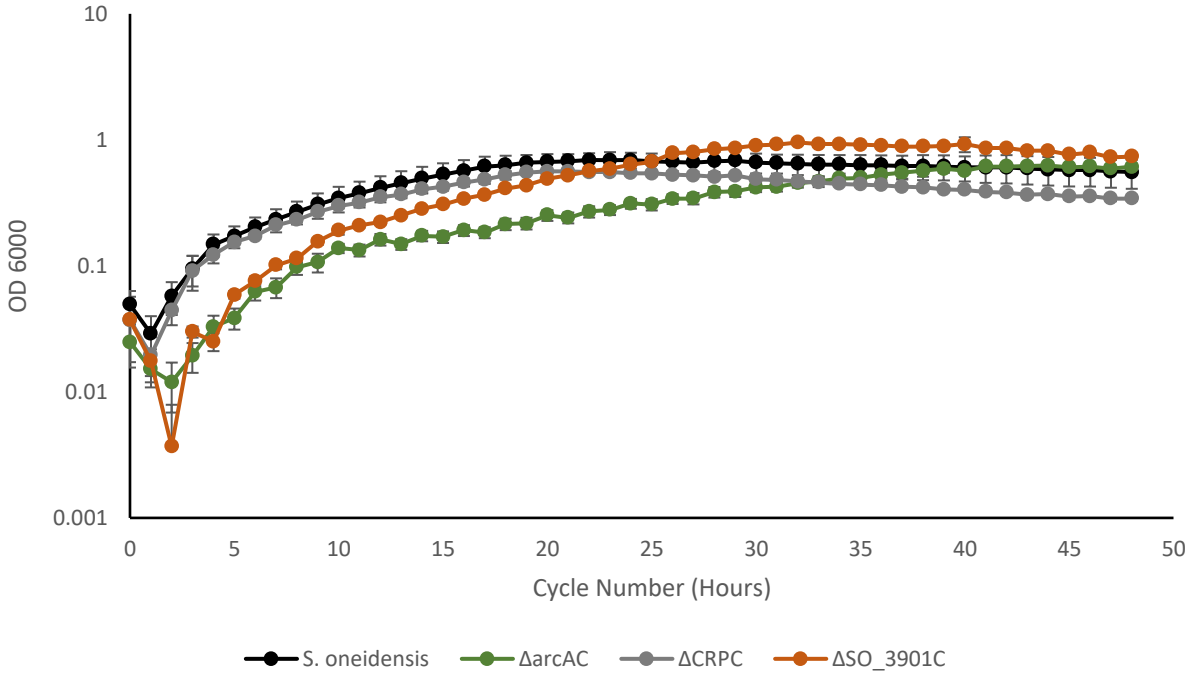
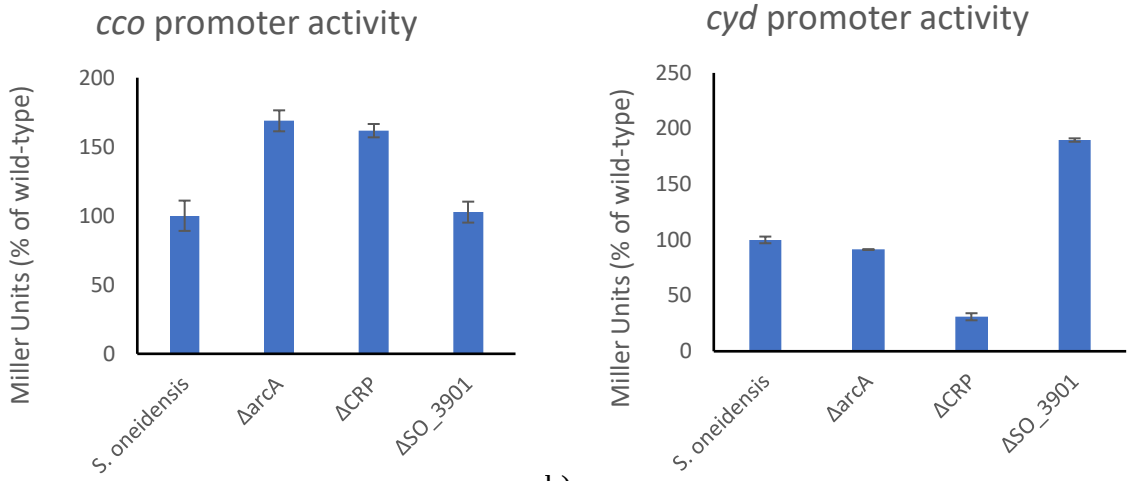


Figure 4.7 Aerobic growth of wild-type *S. oneidensis* and complemented mutants in basal medium supplemented with 20mM acetate and 0.01% casamino acids.

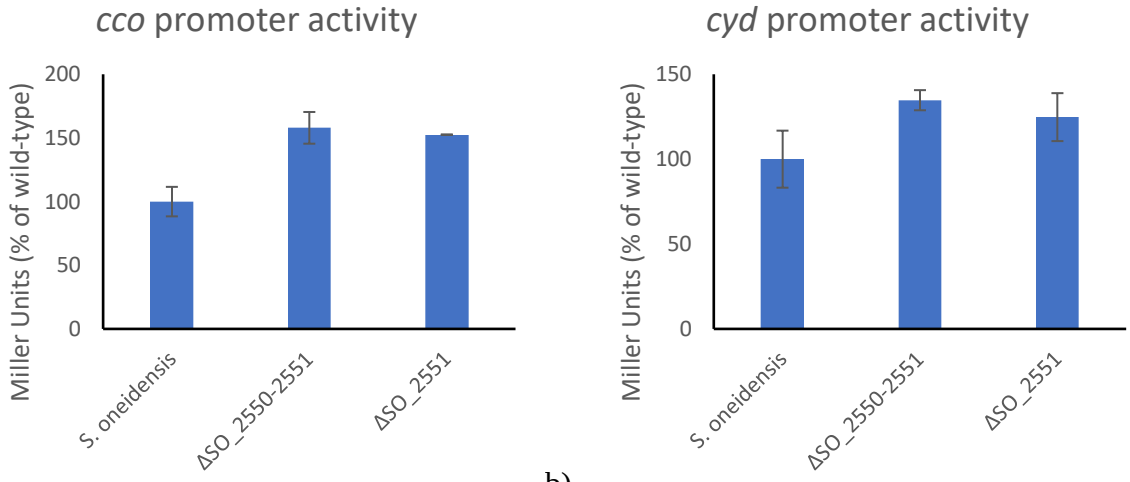
Effect of CRP, ArcA, SO_2550-1, SO_2551, and CpdA on the Expression of *cco* and *cyd* Promoters

To determine the effect of regulatory proteins on the expression of the oxidase genes, the *cco* and *cyd* promoters were inserted into pMC10 upstream of the *lac*Z gene as previously described (32). Stationary phase cultures were used to inoculate basal medium supplemented with 30mM lactate and 0.01% casamino acids and incubated aerobically at 30°C with shaking. The β-galactosidase assays were performed approximately three hours after inoculation. Figures 4.8a and 4.9a display the *cco* promoter activities in deletion mutants and wild-type *S. oneidensis*. The ΔSO_3901 (Δ*cpd*A) mutant expressed the *cco* promoter at the same level as wild type. All other mutants had increased *cco* promoter expression at three hours aerobic growth. The increased expression ranged from approximately 1.5x-1.7x the level of wild type. Figures 4.8b and 4.9b display the *cyd* promoter activities of these same cultures. The *cyd* promoter activity varied more than the *cco* promoter activity. The Δ*arc*A mutant expressed the *cyd* promoter at approximately the same level as wild-type *S. oneidensis*. The Δ*crp* mutant appeared to negatively regulate *cyd* promoter expression, with β-galactosidase levels at approximately 0.3x that of wild type. The ΔSO_2550-2551, ΔSO_2551, and ΔSO_3901 mutants all exhibited increased expression of the *cyd* promoter, with the ΔSO_3901 mutant expressing β-galactosidase at approximately 1.9x wild-type levels.



a) b)

Figure 4.8 Promoter expression assays for *cco* (a) and *cyd* (b) promoters. Basal medium supplemented with 30mM lactate and 0.01% casamino acids. Assay performed after 3 hours aerobic growth at 30°C with shaking.



a) b)

Figure 4.9 Promoter expression assays for *cco* (a) and *cyd* (b) promoters. Basal medium supplemented with 30mM lactate and 0.01% casamino acids. Assay performed after 3 hours aerobic growth at 30°C with shaking.

Fluorescence Growth Assays

The FastmCherry gene was used to replace *lacZ* in pMC10 plasmid (32) and generate pKMB1 as described in Chapter 2. Newly synthesized Fast mCherry expresses a blue fluorescent

protein with an emission of 466 nm, and can be used to indicate recent transcriptional activity (49).

The growth assay of the *cco* promoter expression of FastmCherry in pKMB1 (Figure 4.10) is ultimately reflective of the expression of β-galactosidase displayed in Figure 4.8a and 4.9a – all mutants displayed more expression than wild type at three hours. The expression of the *cco* promoter decreased with culture growth. *S. oneidensis*, ΔCRP, and ΔSO_2551 stabilized at similar points at 14 hours. The expression of *cco* in the Δ*arc*A and ΔSO_2550-2551 mutants stabilized at similar points at 14 hours as well, although the points were lower than the remaining cultures. The *cco* promoter expression of the ΔSO-3901 (Δ*cpd*A) mutant was expressed at a higher level than wild-type *S. oneidensis* for the entirety of the assay. The ratio of fluorescence to culture density decreased for the first 8 hours, but instead of the ratio stabilizing as it did in the other cultures, it slowly increased for the remaining readings.

The *cyd* promoter expression of FastmCherry in pKMB1 (Figure 4.11) is also reflective of the *cyd* promoter expression of β-galactosidase demonstrated in Figure 4.8b and 4.9b. The *cyd* expression for *S. oneidensis* and the ΔSO_2551 mutant is similar at all points tested. The ratios of the fluorescence to culture density for Δ*arc*A, ΔSO_2550-2551, and ΔSO_3901 decreased for the duration of the assay, with Δ*arc*A and ΔSO_2550-2551 ratios stabilizing at similar points at approximately 14 hours. The ratio for the ΔSO_3901 mutant behaved similarly to the *cco* expression, that is, the ratio stabilized at approximately six hours and slowly increased for the remainder of the assay. The ratio of fluorescence to culture density was relatively constant (and low) for the Δ*crp* mutant through the duration of the assay.

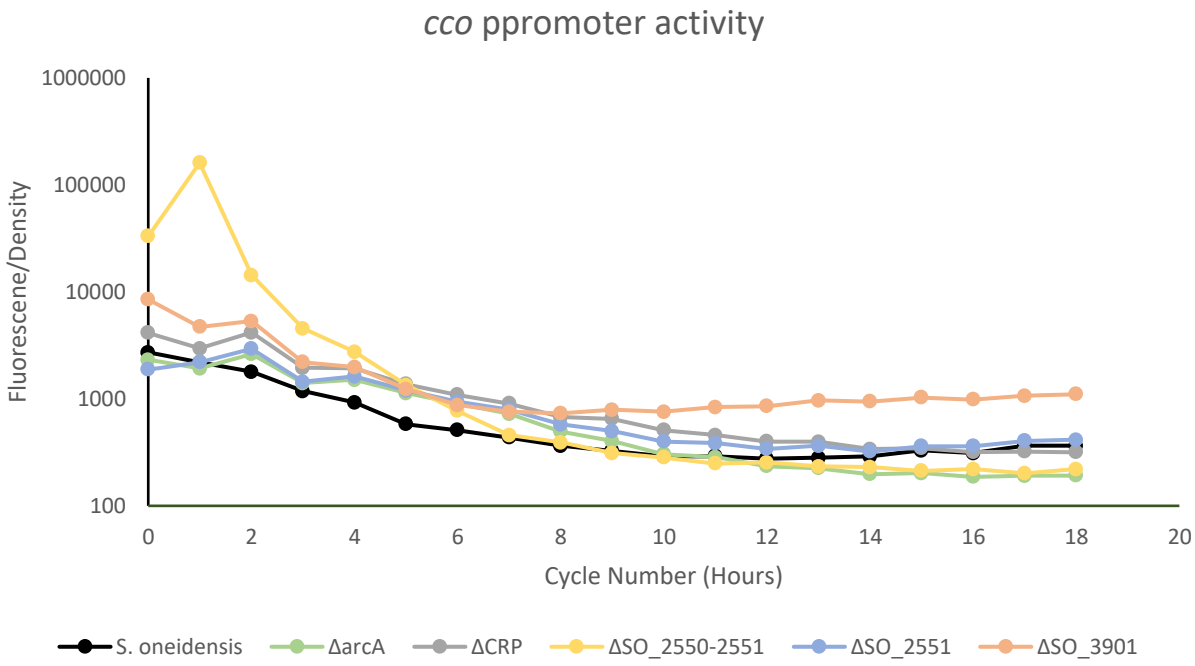


Figure 4.10 Aerobic growth in basal medium supplemented with 30mM lactate and 0.01% casamino acids. Growth and fluorescence were measured simultaneously and are plotted as a ratio of fluorescence to culture density.

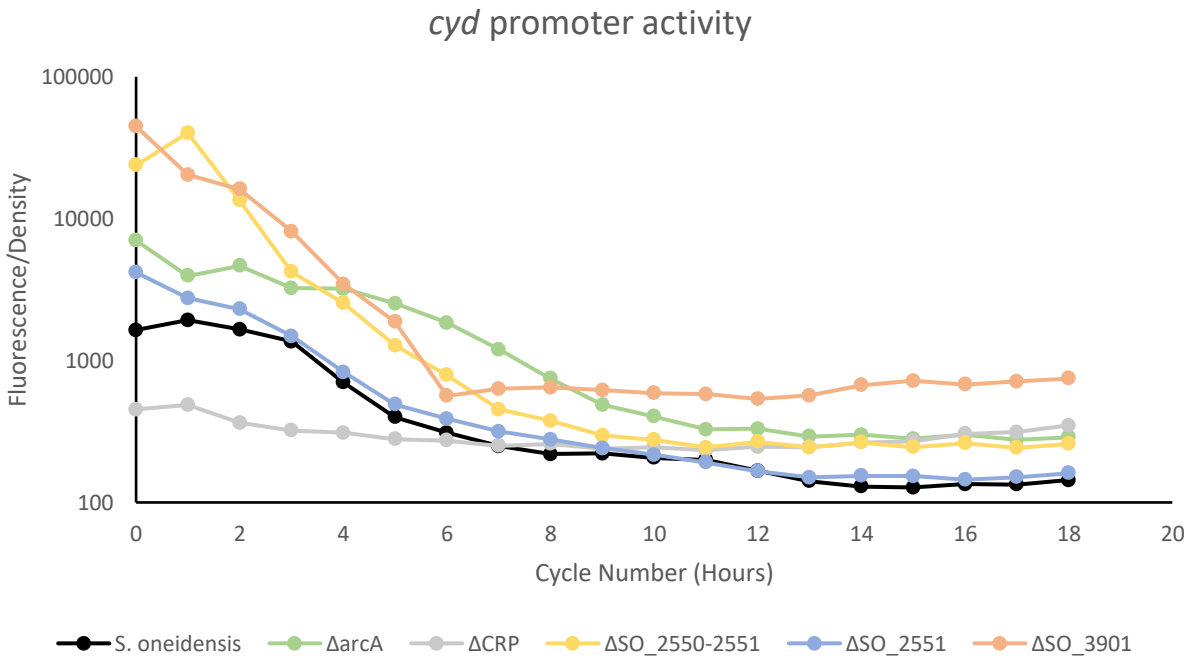


Figure 4.11 Aerobic growth in basal medium supplemented with 30mM lactate and 0.01% casamino acids. Growth and fluorescence were measured simultaneously and are plotted as a ratio of fluorescence to culture density.

Discussion

S. oneidensis employs several well-studied systems to regulate its wide range of electron acceptor usage. As discussed above, CRP and the Arc system appear to be the major players in the regulation of aerobic respiration as well as anaerobic respiration (32, 36, 80). ArcA plays a major role in the regulation of anaerobic DMSO reduction, and to a lesser extent in aerobic respiration in rich media (32, 33). In this work, the ability of Δ*arcA* to grow aerobically in defined media supplemented with different carbon and energy sources was tested. The major deficiency noted in these growth assays was the unusually long lag phase. The growth rate markedly increased after this lag phase, and the cultures reach almost the same density as wild-type *S. oneidensis*. These results were consistent regardless of the carbon source used. The likely explanation for this may be that ArcA mutants have stationary phase defects. In *E. coli*, ArcA is required for downregulation of the tricarboxylic acid (TCA) cycle during carbon starvation. Deletion of *arcA* in *E. coli* leads to an increased rate of respiration and causes decreased stationary phase survival due to buildup of reactive oxygen species (86, 87). Despite the structural similarity between the ArcA proteins of *S. oneidensis* and *E. coli,* it is unclear how extensive the overlap of ArcA regulons is between the two organisms. Some regulatory functions in *S. oneidensis* are the opposite of *E. coli*, e.g. the *cyd* operon is repressed by ArcA under anaerobic conditions in *S. oneidensis* (32, 33). Bioinformatic and microarray analyses of an ArcA mutant in *S. oneidensis* suggest that there is little overlap with the regulatory effects of ArcA compared with *E. coli* (80). However, recent studies suggest that *S. oneidensis* exhibits electrochemical gating of the TCA cycle in a redox-dependent manner, implicating ArcA in that regulation (88). Also, the ArcA regulon of *E. coli* is larger than previously proposed (80) and ArcA binding sites appear to have plasticity, thereby expanding the regulatory network and

allowing for regulation of one promoter by multiple proteins. ArcA is suggested to provide an advantage, then, in natural environments with a variety of available carbon sources and electron acceptors (89). Taking these studies into account, we can conclude that ArcA affects *S. oneidensis* aerobic metabolism, if not through TCA gating, possibly through carbon metabolism.

CRP is a cAMP binding regulatory protein that acts as the major regulator of anaerobic respiration. Its role in regulating anaerobic respiration is unusual because it lacks a redox-sensing domain. In other organisms, CRP regulates carbon metabolism (36, 81) . Under aerobic conditions, growth of Δ*crp* appears to depend on the available carbon source. Growth with pyruvate (Figure 4.4) or acetate (Figure 4.6) were not markedly different from the growth rate of wild-type *S. oneidensis*, although the culture density did begin to decrease after approximately 20 hours when acetate was used as the sole carbon source. The culture density slightly decreased in wild-type *S. oneidensis* under these conditions, albeit at a slower rate. Growth of Δ*crp* was most drastically affected when lactate was the carbon source used (Figure 4.2). Overall rate of growth was slower, and did not reach the stationary cell density of wild-type cultures. *S. oneidensis* preferentially uses D-lactate when presented with a racemic mixture, and CRP has been demonstrated to regulate expression of the D-lactate permease and dehydrogenase, which suggests that the CRP protein has an unexpected role in both carbon metabolism and respiration in this organism (90, 91).

The genes SO_2550 and SO_2551 are suggested to belong to the CRP family of transcriptional regulators based on sequence analysis (85). Growth assays of the single deletion ΔSO_2551 did not reveal any growth deficiencies regardless of carbon sources tested. The SO_2550-2551 double deletion mutant was deficient in aerobic growth under all conditions tested. Growth in lactate (Figure 4.2) was decreased when compared with wild-type *S.*

oneidensis, and cultures exhibited an extremely long lag phase (30 hours) before growth resumed (Figure 4.6). The most marked deficiency was demonstrated when pyruvate was used as the sole carbon source (Figure 4.4).

SO_2550 and SO_2551 are not similar in sequence to the CRP of *S. oneidensis*; however, SO_2551 is very similar to a CRP-family protein from *S. putrefaciens* (Figure 4.1). SO_2550 has a putative cAMP-binding domain, and SO_2551 contains a predicted helix-turn-helix motif (DNA-binding domain). Growth assays of complemented ΔSO_2550-2551 suggest that observed aerobic growth deficiencies are due to loss of both SO_2550 and SO_2551, possibly as a result of redundant function of the proteins (Figures 4.3 and 4.5). The complementation assay mentioned above indicates that neither of these proteins (either separately or together) are able to complement an *E. coli crp* mutant. The *S. oneidensis* genome is predicted to encode four CRP family genes, and variant CRPs are prevalent in other organisms; therefore, it is possible these proteins could have a similar regulatory function to the CRP of *E. coli* without being able to directly complement it (66, 82). The *S. putrefaciens* CRP is not discussed in the literature; therefore, comparison of function is not possible at this time. Complementation of a *S. putrefaciens* CRP mutant with SO_2550-2551 would be valuable in future work. As these specific proteins have yet to be reported in the literature, speculative analysis would suggest that this combined deletion plays a role in aerobic respiration based on carbon source usage.

The cAMP phosphodiesterase SO_3901(CpdA) is, to date, the only classified 3',5'-cAMP phosphodiesterase encoded by *S. oneidensis* genome. Loss of CpdA has no effect on anaerobic growth or aerobic growth in rich media. Aerobic growth, however, is impaired in minimal media supplemented with acetate or lactate (Figures 4.2 and 4.6). In the presence of pyruvate, growth of the *cpdA* mutant was very slow compared to the wild type (Figure 4.4).

Previous work by A. Banerjee showed increased cAMP levels in this mutant, similar to the anaerobic levels of cAMP in wild-type *S. oneidensis* (38). It is possible, therefore, that the *cpdA* mutant behaves as if it is growing under anaerobic conditions (36). However, acetate is only used by *S. oneidensis* under aerobic conditions, so actions of anaerobiosis cannot completely explain the observed phenotype (92). Complementation of the ΔSO_3901 (Δ*cpd*A) phenotype is discussed in detail in Chapter 5.

It has been suggested that there is decreased production of aerobic cytochromes *c* in this mutant and that is the cause of the poor aerobic growth phenotype (93); however, promoter expression experiments do not support this assumption. The promoter expression assays in Figures 4.8 and 4.9 show relative expression of the *cco* or *cyd* promoter following 3 hours of incubation. The mutant strains had higher *cco* promoter activity than wild-type *S. oneidensis* and all mutant strains except Δ*crp* had higher *cyd* promoter activity. These results indicate that early in the growth cycle of these cultures, this assertion of decreased production of aerobic oxidases is not supported.

Promoter expression is commonly demonstrated by using a β-galactosidase assay. As mentioned above, this assay only captures one point in the growth cycle of a culture. While this provides valuable information, it does not answer the question of how promoter expression changes over time. Even though the static promoter expression assays do not support the hypothesis that decreased promoter expression is the cause of the growth deficiencies, it could be possible that the *S. oneidensis* terminal oxidase complexes may be produced at reduced levels at varying points during the growth cycle, thereby explaining the growth deficiencies of the regulatory complex mutants. The plasmid pKMB1 was developed for this study to address that question. As demonstrated in Figures 4.10 and 4.11, expression of the variant mCherry from

these promoters reflects the expression of β-galactosidase, indicating successful application of this method. All of the strains tested showed high levels of promoter expression relative to culture density in the beginning of the assay that decreased and then stabilized over time. The *cyd* expression of the Δ*crp* mutant is the only result that shows low expression over the course of the experiment, consistent with the β-galactosidase results and consistent with previous work published by this lab (37). All other mutants show elevated *cco* and *cyd* expression relative to wild-type *S. oneidensis*. However, there was no variation in expression based on time of the assay, negating the hypothesis that the promoter expression in the mutants was decreased at some points in the growth cycle. This assay may prove valuable, though, for other protein complexes that are temporally regulated.

Carbon metabolism and oxidase expression in *S. oneidensis* (as explained in detail in Chapter 4) does not appear to be strictly regulated. No terminal oxidase on its own is indispensable for aerobic growth, and none of the mutants tested are unable to grow on any of the carbon sources used. Expression of a variety of terminal reductases is observed when *S. oneidensis* is under anaerobic conditions; therefore, it is possible to infer that expression of aerobic oxidase complexes is similar (i.e. both expressed) when oxygen is present (94).

CHAPTER 5
ANALYSIS OF A SUPPRESSOR MUTATION OF ΔSO_3901

Introduction

Cyclic AMP levels are tightly regulated in the bacterial cell. As mentioned in the previous chapter, the *S. oneidensis* MR-1 genome encodes three adenylate cyclases (*cya* genes) and one known cAMP phosphodiesterase. This class III 3',5'-cAMP phosphodiesterase SO_3901 (*cpd*A) lowers the intracellular cAMP concentration, affecting CRP activity (95). In other organisms, cAMP levels are implicated in the regulation of a variety of cellular processes, including carbon metabolism, iron uptake, and virulence (96-98). The *cpd*A (SO_3901) deletion mutant generated in this lab is deficient in aerobic but not anaerobic respiration (38). This chapter will evaluate if additional nutrients can ameliorate the *cpdA* deletion deficiencies. In addition, to assess if this mutant can return to a wild-type growth phenotype via a compensatory mutation, transposon mutants were generated in a *cpdA* deletion background, and a disruption in an anti-sigma factor (SO_3550) was found to rescue the phenotype of the ΔSO_3901 mutant.

Bacterial sigma factors are used in concert with RNA polymerase (RNAP) to bind specific sequences of DNA to create the open complex and activate transcription. Without a sigma factor, RNAP is only capable of nonspecific binding at DNA ends or nicks. The most extensively studied sigma factor is σ_{70}, encoded by *rpo*D. In *E. coli,* this sigma factor is involved in "housekeeping" functions and the bulk of transcription during growth in nutrient-rich environments (39, 40). The anti-sigma factor encoded by SO_3550 is in an operon with SO_3551, a sigma factor (σ_{24}) described as "ECF-like," meaning extracytoplasmic function (27, 41). These ECF sigma factors, including RpoE (σ_{24}), respond to membrane and extracellular signals, and are often employed during stress responses in bacteria. They are tightly regulated by

a cognate anti-sigma factor, which impedes binding to the specific promoter sequence (40). The *S. oneidensis* genome encodes ten sigma factors, five of which are described as σ_{24} or ECF sigma factors. Three of these sigma factors: RpoE, RpoE2, and SO_3096, have been described in the literature and are involved in diverse stress responses, such as: temperature, minimal nutrient supply, oxidative conditions, and heavy metals (41, 94). The function of SO_3551 has not been identified. This chapter will discuss the effect the anti-sigma factor SO_3550 has on *S. oneidensis* growth.

Results

Aerobic Growth of Δ*cpd*A Can Be Restored by the Addition of Casamino Acids

The Δ*cpdA* (ΔSO_3901) mutant is able to grow aerobically in LB. However, it failed to grow in basal medium supplemented with different carbon sources (Chapter 4). To determine if the loss of CpdA affects amino acid biosynthesis, and thus contributes to loss of growth in amino acid-poor media, growth assays were completed with different concentrations of casamino acids (Figure 5.1) The results indicated that addition of excess casamino acids overcomes the growth deficiency caused by the deletion of *cpd*A (SO_3901). The basal medium we typically use for growth of *S. oneidensis* is supplemented with 0.01% casamino acids. These growth assays were performed with 0.05%, 0.1%, 0.2%, and 0.5% concentrations. Both wild-type *S. oneidensis* and Δ*cpdA* exhibited an accelerated growth rate with increasing casamino acid concentration. At 0.5% casamino acids, the ΔSO_3901 mutant reached the same culture density as the wild type in the same conditions, albeit at a slightly slower rate. This is the only casamino acids concentration tested that almost completely overcame the growth deficiency of the mutant.

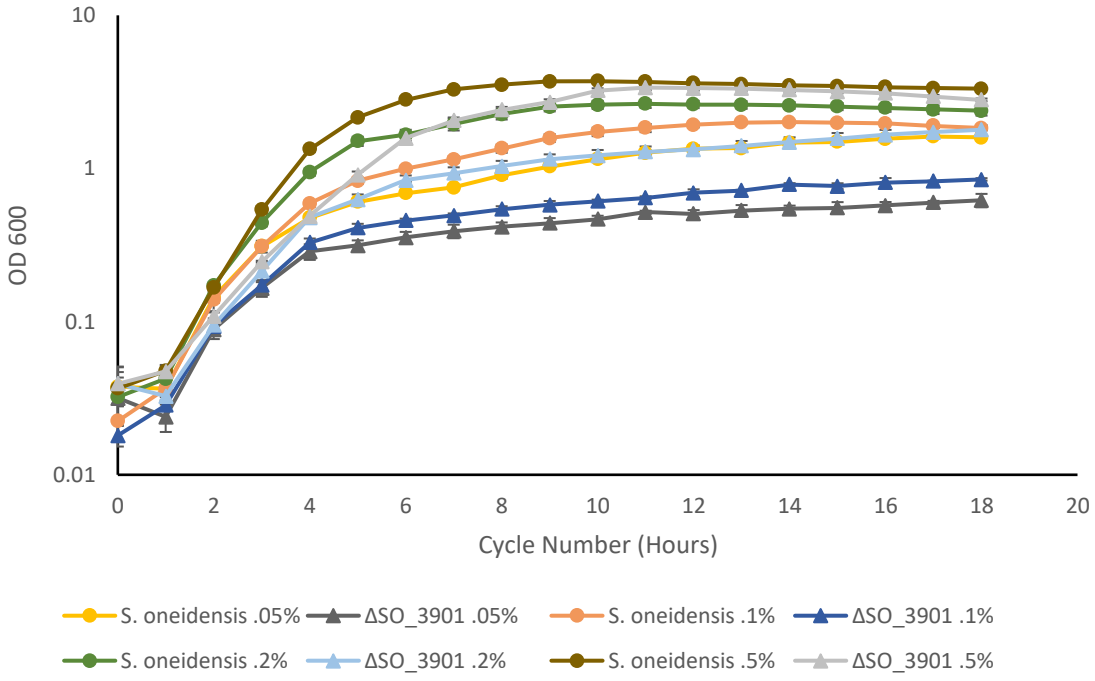


Figure 5.1 Aerobic growth of wild-type *S. oneidensis* and ΔSO_3901 mutant in basal medium supplemented with 30mM lactate and varying concentrations of casamino acids (0.05%, 0.1%, 0.2%, and 0.5%).

Identification of a Δ*cpd*A Suppressor Mutation

To begin to understand the role of CpdA in aerobic respiration, we generated transposon mutants in a Δ*cpdA* background, and selected the resulting mutants on basal medium agar supplemented with lactate. Because Δ*cpdA* failed to grow under these conditions, mutants that grew were expected to have transposon insertions in genes that may compensate for the original mutation. One of the mutants generated had an insertion in the anti-sigma factor SO_3550. A gentamicin resistant insertional mutant of SO_3550 was also generated in Δ*crp*, Δ*cpdA*, and wild-type *S. oneidensis* backgrounds. A complemented strain of SO_3550-3552 in pJBC1 is currently being prepared for analysis.

The gene SO_3550 is located on the minus strand of *S. oneidensis* genome directly downstream of SO_3551. SO_3550 does not appear to contain identified conserved domains;

however, it is labelled as a putative anti-sigma factor. SO_3551 is predicted to be a sigma factor and contains a conserved DNA-binding region at the C-terminus (Figure 5.3).

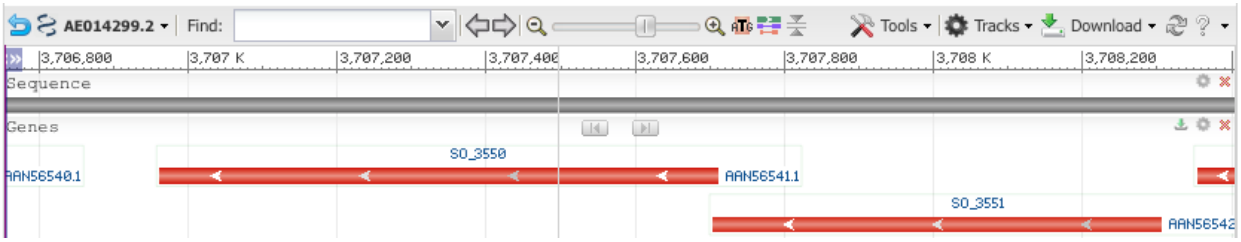


Figure 5.2 Map of SO_3550 and SO_3551 in *S. oneidensis* genome (85).

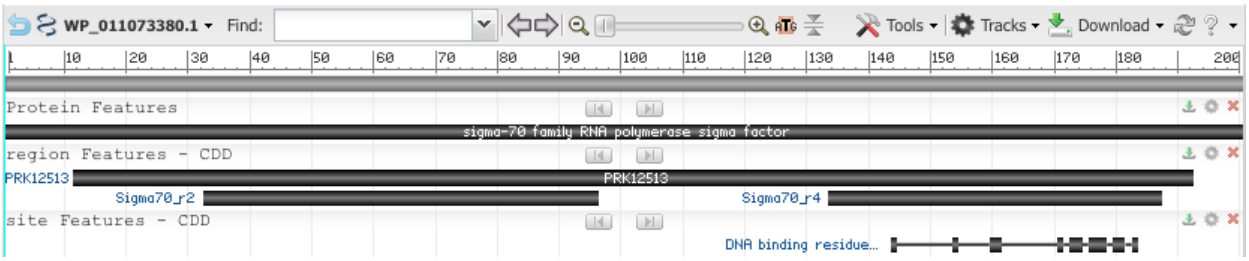


Figure 5.3 Map of sigma factor SO_3551 highlighting DNA-binding region (85).

Aerobic growth of wild-type *S. oneidensis*, Δ *cpd*A, and the *cpd*A/SO_3550 double mutant is shown in Figure 5.4. As mentioned above, Δ*cpd*A barely grows aerobically in basal medium supplemented with lactate. We attribute the initial growth observed to be due to the presence of casamino acids. This growth deficiency was not observed in the mutant that lacks CpdA and SO_3550 functions. Complementation of the double mutant with *cpdA* on a plasmid did not appear to affect growth suggesting that a mutation in SO_3550 only may not have any visible effects on aerobic growth.

To further test the role of SO_3550, the suicide vector pER21 was used to create an insertional mutation in that gene. Insertional mutants were selected on gentamycin agar plates,

and then tested for growth as described in Chapter 2. Figure 5.5 illustrates that the insertional mutation did not negatively affect the growth of *S. oneidensis*. An insertional mutation in SO_3550 was also generated in a Δ*crp* mutant and the resulting mutant grew similar to the wild type. The SO_3550 insertional mutation was also generated in Δ*cpdA* to confirm the results obtained with the transposon mutagenesis experiment described above and in Figure 5.5. Insertional inactivation of SO_3550 using pER21 in a Δ*cpdA* backgrounds restored growth to the Δ*cpdA* mutant further supporting its role in suppressing Δ*cpdA* phenotype.

Anaerobic growth experiments were carried out in Basal Medium supplemented with 30mM lactate, 0.01% casamino acids, and either 10mM DMSO or 10mM fumarate as the electron acceptor. The insertion in SO_3550 did not affect growth of wild-type *S. oneidensis*, Δ*cpdA*, or Δ*crp*. In addition, *lac*Z assays were performed on these samples, and the addition of SO_3550 did not affect promoter activity of the *dms* or *fcc* operons (data not shown).

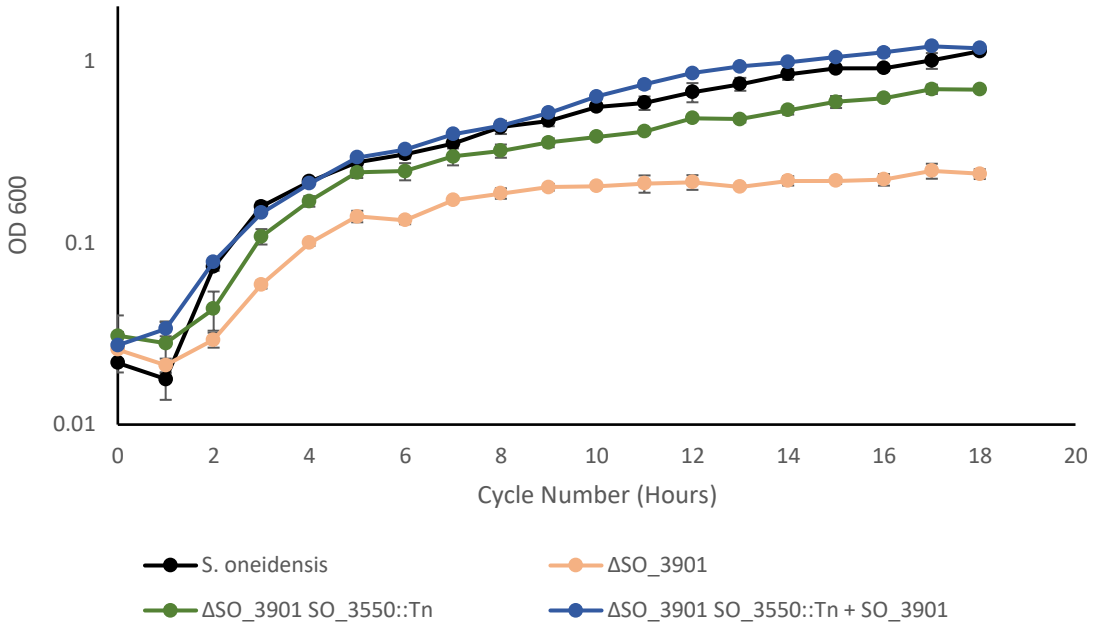


Figure 5.4 Aerobic growth of wild-type *S. oneidensis*, ΔSO_3901, and ΔSO_3901/SO_3550 in basal medium supplemented with 30mM lactate and 0.01% casamino acids.

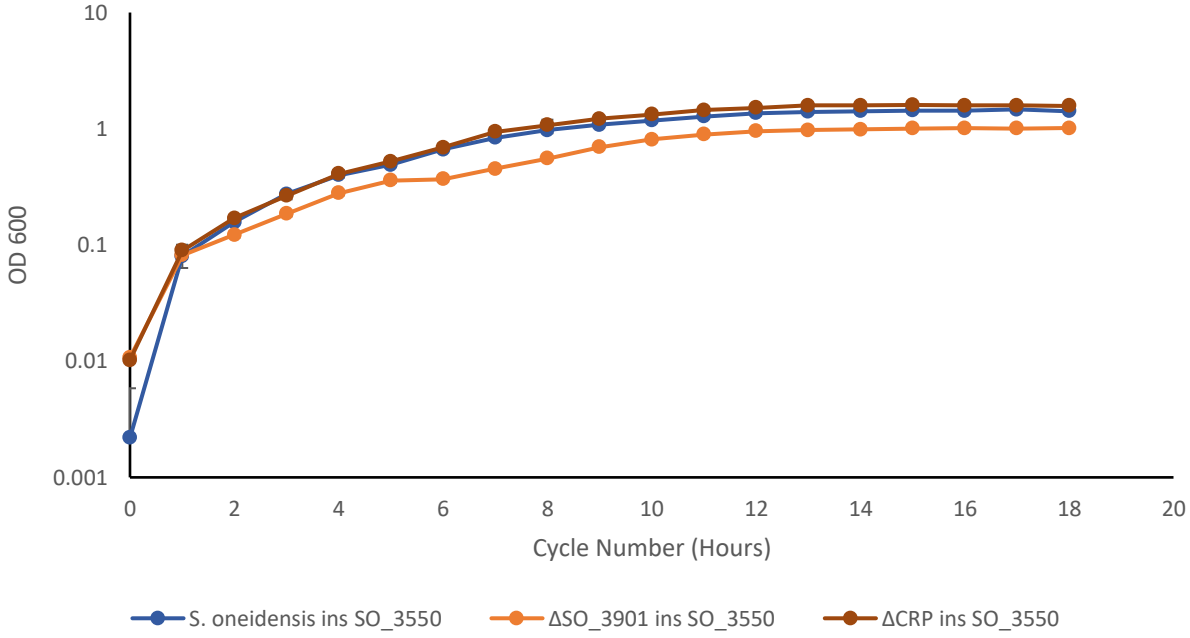


Figure 5.5 Aerobic growth of wild-type *S. oneidensis* and deletion mutants with an insertional mutant in SO_3550 in basal medium supplemented with 30mM lactate and 0.01% casamino acids.

Discussion

As discussed in Chapter 4, a deletion mutant in the cAMP phosphodiesterase (SO_3901) exhibits an aerobic growth deficiency in minimal media but not in rich media. Expression of the terminal oxidase complexes does not appear to be the reason for the growth deficiency in the ΔSO_3901 mutant in basal media; therefore, it must be another metabolic process that is affected. LB medium is comprised of tryptone, yeast extract, and NaCl (42). Growth curves were conducted in basal medium with tryptone and basal medium with yeast extract (data not shown). The growth deficiency was overcome using either component; however, both are still complex and make it difficult to discern the specific reason for growth defects. To assess if addition of excess amino acids could remedy the deficiency, growth was measured with increasing concentrations of casamino acids. Increases amounted to 5x, 10x, 20x, or 50x of the customary concentration used in this lab. The media containing 0.5% casamino acids (50x) nearly remedied the growth deficiency in the ΔSO_3901 mutant, with the culture reaching the same density as wild-type *S. oneidensis* at a slightly slower rate.

Casamino acids are a digest of casein (one of the main proteins in milk). Tryptone and peptone (sometimes used interchangeably) are common casein/protein digests used in microbiological media; however, these are nonspecific and partial digests of proteins, leading to a more complex and undefined medium. Casamino acids are a more complete digest, resulting in mostly single amino acids with the exception of tryptophan, which gets destroyed during processing (102, 103).

The results in Figure 5.1 suggest that amino acid metabolism is impacted in the ΔSO_3901 mutant. This is supported by a recent study by Kasai, et al., in which microarray data indicated decreased expression of genes related to amino acid metabolism, in particular

methionine, s-adenosylmethionine (a methionine derivative that is a methyl donor for other metabolic processes), and histidine. Addition of these amino acids partially restored the growth of the mutant in this study (101, 104). Deletion of adenylate cyclases did not affect the phenotype of this mutant; therefore, the effects of this deletion are independent of cAMP concentrations (101).

As adjustments to media components remedy the growth deficiency of Δ*cpd*A mutants in minimal media, we attempted to assess genetic components that may suppress the growth deficiency. Transposon mutagenesis using the mini*Himar* RB1 was carried out on the Δ*cpd*A mutant. As this transposon inserts into the genome at random and only once, we are able to determine single gene mutation candidates that compensate for the Δ*cpd*A phenotype (46). The anti-sigma factor encoded by SO_3550 was determined to be a viable candidate. Figure 5.4 indicates that the transposon insertion rescued the deletion mutant from its deficiency and that complementing the transposon mutant with *cpd*A did not negatively affect the growth phenotype, suggesting that the transposon mutant did not negatively impact growth. To further test this anti-sigma factor, insertional mutants were generated as described above. There does not appear to be a CRP binding site upstream of this operon according to the predicted CRP binding motif, and ECF sigma factors such as RpoE (SO_1342) are active under anaerobic conditions; therefore, exploring the possibility that this yet unclassified sigma factor SO_3551 could rescue the phenotype of the Δ*crp* mutant was in question (99, 100). The insertion in SO_3550 does not affect the Δ*crp* mutant in aerobic or anaerobic conditions, suggesting that the genes transcribed by this sigma factor are not negatively impacted by a *crp* deletion, are not activated under anaerobic conditions, or the cause for the Δ*crp* phenotype is unrelated to the action of this sigma factor. This targeted insertion in SO_3550 does have a compensatory effect on the phenotype of

the ΔSO_3901 mutant, reinforcing the validity of the observed phenotype for the transposon mutant.

The supplemental microarray data by Kasai, et al. do not indicate up or down regulation of any of the five ECF sigma factors or their cognate anti-sigma factors in the Δ*cpd*A mutant (101). Therefore, the action of the sigma factor SO_3551, while mitigating under these conditions, is not directly affected by the deletion of *cpd*A and has a yet undetermined role in growth conditions for wild-type *S. oneidensis*. As mentioned above, ECF sigma factors are typically employed in stress responses (including stress responses to nutrient concentrations in minimal media), and the likely role of this sigma factor is to ameliorate deficiencies in amino acid metabolism under stress conditions (41). However, further testing will be needed.

CHAPTER 6

CONCLUDING REMARKS

Shewanella oneidensis is an organism that demonstrates a remarkable degree of respiratory versatility. Its ability to respire using toxic substrates and produce electricity from extracellular electron transfer has generated much research interest. Little work has been done on how this organism respires aerobically and accordingly is the topic of this work.

The beginning of this study focused on the terminal aerobic oxidase complexes and how they are employed in aerobic growth. The *S. oneidensis* genome encodes three terminal oxidase complexes, aa_3- and cbb_3-type cytochrome *c* oxidases, and a *bd*-type quinol oxidase. The low-affinity aa_3-type cytochrome *c* oxidase is often used by other organisms as the primary oxidase complex in oxygen-replete environments. In *S. oneidensis*, this protein does not appear to have a role in aerobic respiration. The cbb_3-type cytochrome *c* oxidase and the *bd*-type quinol oxidase both have a high affinity for oxygen and are usually employed under low oxygen tensions. The work presented here confirms that the cbb_3-type cytochrome *c* oxidase is the primary oxidase used in this organism. Surprisingly, a deletion of the intermediate ubiquinol:cytochrome *c* oxidoreductase and the *bd*-type quinol oxidase was viable, but grew poorly under all conditions tested and likely survived by fermentation of amino acids. Carbon source has little effect on the growth phenotype of any oxidase deletion mutant. Based on the ecological niche occupied by *S. oneidensis*, these results reinforce the model of respiratory flexibility – this organism survives in environments with a highly variable supply of electron acceptors.

Respiration in *S. oneidensis* is regulated by three well-studied systems, EtrA, ArcAB, and CRP. EtrA is an FNR homologue and functions in regulation of anaerobic respiration. The

ArcAB two-component system functions anaerobically to regulate the reduction of DMSO. Deletions of the response regulator ArcA have a negative effect on aerobic growth in *S. oneidensis*, likely due to stationary phase defects. The cAMP-binding protein CRP has an uncommon role in *S. oneidensis* in its regulation of respiration. Mutants of this gene are deficient in using a variety of electron acceptors, including fumarate and metal oxides. Aerobically, *crp* mutants have the most pronounced growth defect when lactate is the sole carbon source. Therefore, it appears that CRP plays a role in regulation of respiration and, to a lesser extent, carbon source usage in *S. oneidensis*. Two CRP homologues were assessed in this study: SO_2550 and SO_2551. They both contain cyclic nucleotide binding domains and the helix-turn-helix motif associated with CRP family proteins. They do not share much sequence similarity with the *S. oneidensis* or *E. coli* CRP and they are unable to complement an *E. coli* CRP mutant. However, they do share similarity with a CRP family protein of *S. putrefaciens* and a deletion of both genes negatively affects aerobic growth, suggesting they work in concert as an additional regulatory system in *S. oneidensis*.

To assess if growth deficiencies of regulatory proteins were due to lack of production of terminal aerobic oxidase complexes, the plasmid pKMB1 was generated for this study. It uses a variant mCherry that fluoresces blue and then red based on time of translation. This allowed the study of promoter expression with growth over time. These assays supported conclusions drawn using a β-galactosidase assay – promoter expression of the *cco* and *cyd* genes was increased in the deletion mutants of regulatory proteins. The only decreased expression was the *cyd* promoter of the Δ*crp* mutant. The results obtained using this plasmid indicate that the expression of terminal aerobic oxidases remains consistent throughout the growth cycle of *S. oneidensis*.

Mutants of the cAMP phosphodiesterase CpdA, encoded by SO_3901, had a severe growth deficiency in basal media, regardless of carbon source tested. The expression of terminal oxidase complexes is not decreased in this mutant. Recovery of the growth phenotype was assessed through nutrient supplementation and transposon mutagenesis. Growth is recovered when increased amounts of casamino acids are added to the culture medium, suggesting a downregulation of amino acid metabolic pathways in this mutant. Transposon mutagenesis resulted in disruption of an anti-sigma factor (SO_3550) that ameliorated the growth deficiency. Targeted disruption of SO_3550 did not negatively affect growth of wild-type *S. oneidensis* or the Δ*crp* mutant. This anti-sigma factor is in an operon with an ECF-like sigma factor. ECF sigma factors are typically employed during stress response. Disruption of this anti-sigma factor allowed the function of the sigma factor SO_3551 to activate genes that relieve basal medium growth deficiencies.

This work elaborates on the roles of the terminal oxidase complexes in aerobic respiration in *S. oneidensis*. Lack of strict regulation is not uncommon in this organism due to its inhabitation of redox-stratified environments. The ability of *S. oneidensis* to rapidly shift its electron acceptor usage drives its success in this niche. This work also describes new regulatory proteins, SO_2550 and SO_2551. Further work will be needed to fully elucidate their role in regulation of respiration. In addition, the anti-sigma factor SO_3550 allows growth of the CpdA mutant in basal media. Future work will include further study of this anti-sigma factor as well as the role of its cognate sigma factor in stress response.

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Education

- University of Wisconsin – Madison
  - B.S. in Genetics and Bacteriology
  - Fall 2001 – Spring 2006
- Mayo Clinic Graduate School – Rochester, MN
  - 16 graduate credits, including courses in Chemical Principles of Biological Systems, Molecular Cell Biology, Molecular Genetics, and Scientific Writing
  - Fall 2009 – Spring 2011
- University of Wisconsin – Milwaukee
  - Ph.D. in Microbiology, Secondary concentration in Molecular Biology
  - Fall 2012 – Spring 2020
  - Dissertation Title: Mechanisms and Regulation of Aerobic Respiration in *Shewanella oneidensis* MR-1

Awards

- Undergraduate Research Scholar 2003-2004
- Wisconsin Idea Fellowship Recipient 2004-2005
- Chancellors Award 2012-2016 (Awarded yearly)

Teaching experience

- Course Assistant: Sociology/Women's Studies 200
  - Discussion
  - Fall 2003 and Fall 2004
- Teaching Assistant: Biological Sciences 383: Introduction to Microbiology
  - Laboratory
  - Fall 2012 – Spring 2020 (Fall and Spring Semesters)
- Guest Lecturer: Biological Sciences 383: Introduction to Microbiology
  - Lecture
  - Fall 2015 (Four 75-minute lectures)

Presentations

- University of Wisconsin – Madison: Undergraduate Research Symposium –
  - Poster Presentation
  - 2004 and 2005
- University of Wisconsin – Milwaukee: Department of Biological Sciences Symposium
  - Poster Presentation
    - 2014
  - Seminar Presentation
    - 2016 and 2017

- University of Wisconsin – Milwaukee: Department of Biological Sciences Colloquium
    - Seminar Presentation
        - 2019

Professional Memberships

- American Society for Microbiology

Work History

- Scarab Genomics – Madison, WI
    - Researcher: May 2006-September 2006
        - Troubleshooting and execution of DNA extraction, plasmid preparation, PCR
        - Educating lab assistants in culture technique and reagent preparation
- Mayo Clinic Cytogenetics Laboratory– Rochester, MN
    - Technologist: September 2006-February 2009
        - Preparation of samples for Fluorescence in Situ Hybridization (FISH)
        - Using fluorescence microscopy to analyze hematological, oncological, and congenital patient samples on multiple tissue types
        - Karyotyping and chromosome identification
    - Technical Specialist I – FISH: February 2009-August 2012
        - Developing training programs and materials for testing
        - Presenting educational lectures for certification program
        - Organizing multiple case review presentations at lab-wide education meetings – topics include chronic lymphocytic leukemia and multiple myeloma
        - Reviewing and editing of Standard Operating Procedures
        - Validating specific reagent concentrations within our protocols
        - Troubleshooting FISH setup processes, analysis, and reporting of clinical casework

Publications

- Richard W. Wang, Renee M. Olson, Jack L. Spurbeck, Kristen M. Bertling, Elyse B. Mitchell, Daniel L. Van Dyke. "ISCN (2009) – A reference guide." The Journal of the Association of Genetic Technologists Volume 37, Number 2 (2011): pp 85-94.

- Banerjee, A and Bertling, K "Analysis of the cAMP phosphodiesterase CpdA, and its role in aerobic growth of *Shewanella oneidensis.*" (In preparation)