Anaerobic *Shewanella* physiology: An unusual respiratory substrate and an unusual respiratory partner

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Abstract

With an elegant and flexible electron transport chain, *Shewanella oneidensis* strain MR-1, is the most versatile respiratory organism known to date. MR-1 is a diverse respiratory heterotroph that lives in complex aquatic communities, often in association with other microorganism or eukaryotes, such as fish and algae. Fish produce TMAO as an osmoprotector, which also serve as a respiratory substrate for *Shewanella* isolates that are able to respire it. Although, TMAO is readily found in the aquatic environments where MR-1 is known to be found, MR-1 metabolism under TMAO respiring conditions is not fully understood. In addition, bacteria are usually studied as monocultures in laboratory conditions, however, microorganisms exist in nature as members of communities that interact with each other. Therefore, the factors that shape microbial behavior and interactions in communities remain largely undefined. The work presented in this thesis aims to further elucidate the metabolic strategy of MR-1 under TMAO respiring conditions, as well as, in a commensal interaction with *Geobacter sulfurreducens*.

Coupled to the reduction of terminal electron acceptors, MR-1 has an aerobic branch, as well as, an anaerobic branch for the oxidation of carbon sources. However, in conditions where TMAO is the sole electron acceptor, the oxidation pathway for carbon sources is not fully understood. Furthermore, at the electron transport chain level, TMAO is reduced differently from other anaerobic compounds. Therefore, we aim to understand electron and carbon flux under growth conditions with this important electron acceptor. We have made gene deletions of key enzymes in both aerobic and anaerobic metabolic pathways in MR-1, and assayed for growth under conditions where TMAO is the sole terminal electron acceptor.

We aim to begin to explore MR-1 metabolism in a more complex system with two organisms instead of one. For this purpose, we have engineered a close physical association

between *S. oneidensis* and *Geobacter sulfurreducens. G. sulfurreducens* is an anaerobic subsurface bacterium and another well-characterized organism capable of metal reduction and extracellular electron transfer. By performing laboratory evolution of this synthetic co-culture we aim to identify genes implicated in community interaction and understand how these genes influence their metabolism.

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Introduction

Introduction

Shewanella oneidensis is a facultative anaerobe, mainly found in marine and fresh water environments (Myers & Nealson 1988; Hau & Gralnick 2007; Fredrickson et al. 2008). Being able to respire a wide array of terminal electron acceptors, *S. oneidensis* is the most versatile respiratory organism known to date (Hau & Gralnick 2007; Fredrickson et al. 2008). Among the respiratory substrates are soluble and insoluble electron acceptors including heavy metals, organic acids, oxyanions, and oxygen. *S. oneidensis* has been primarily studied for its capacity to respire solid substrates that lay on the outside of the cell. Electrons that are generated from oxidative metabolism inside the cell are transferred to the extracellular space in a process called extracellular electron transfer (EET). This EET respiratory mechanism is useful for *S. oneidensis* use in waste water treatment, biofuel generation, and bioremediation of toxic pollutants (Flynn et al. 2010; Bretschger et al. 2007; Carpentier et al. 2003).

Shewanella oneidensis respiratory pathways

S. oneidensis has a variety of respiratory pathways that broadly categorize as aerobic respiration and anaerobic respiration. The aerobic respiratory pathway consists of the traditional complexes I through IV coupled to ubiquinone electron carriers (Figure 1.1). Electrons that are generated via the oxidation of carbon sources are transferred to complex I or complex II (NADH oxidoreductase and succinate dehydrogenase, respectively), and transferred to a pool of redox-active ubiquinone (UQ) electron carriers; subsequently, an alternative complex III called ACIII oxidizes the ubiquinone pool and transfers the electrons to cytochrome-C; ultimately (Refojo et al. 2012), complex IV (cytochrome *c*-oxidase) transfers electrons from cytochrome C to an oxygen molecule (Figure 1.1) (Fredrickson et al. 2008; Hau & Gralnick 2007) . In contrast, anaerobic respiration in MR-1 utilizes a wide variety of pathways dependent on the substrates

available, however, a common theme exists with electrons that originate from organic carbon oxidation being transferred dumped into a pool of redox-active menaquinones (MK) (Fujimoto et al. 2012) and a tetrahaem cytochrome (CymA) that oxidizes the quinone pool and transfers electrons to periplasmic terminal oxidases or to periplasmic electron carriers which transfer electrons to outer membrane terminal oxidases (Myers & Myers 2000; Schuetz et al. 2009) (Figure 1.2).

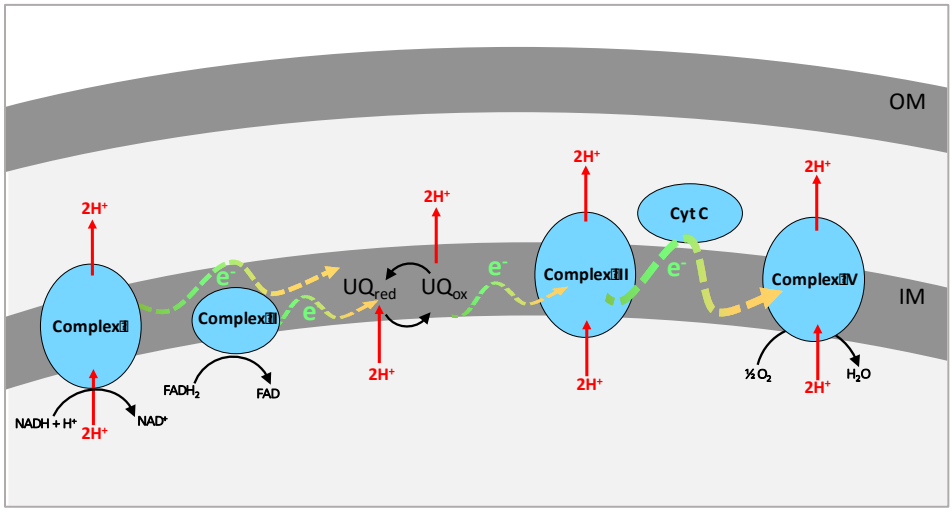

Figure 1.1. Schematic representation of the aerobic electron transport chain in *S. oneidensis.*

Quinones are cyclic ketones that are derived from aromatic compounds, are lipid soluble and shuttle electrons between static membrane complexes in respiratory chains. Lipid-soluble electron carriers, are divided in UQ and MK, with their use being dependent of either aerobic or anaerobic growth. Ubiquinones have a redox potential of +0.1V, higher than their counter part menaquinones, are mostly utilized in aerobic respiratory chains, and are the only redox-active electron carriers utilized by mammalian organisms. On the other hand, menaquinones have a redox potential of -0.1V, which makes them more suitable electron carriers for anaerobic growth where substrates have lower electron potentials than oxygen (Fujimoto et al. 2012).

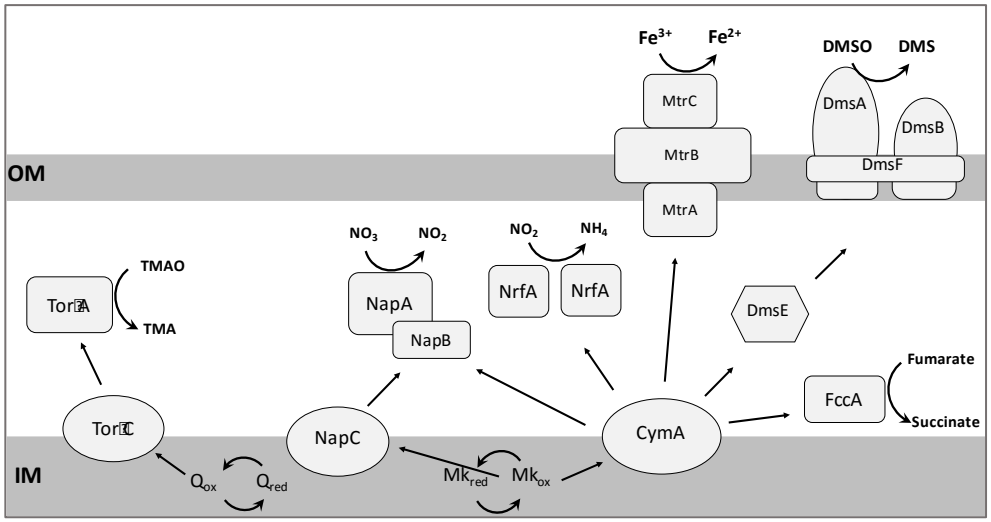

Figure 1.2. Schematic representation of the most studied anaerobic respiratory pathways in *S. oneidensis* (Chen & Wang 2015).

TMAO respiration

Timethylamine–*N* oxide (TMAO) has been found to be associated with marine environments and organisms in significant concentrations, moreover, TMAO has been intensely studied for its role as an osmolyte in marine organisms (Ma et al. 2014; Yancey et al. 1982; Yancey et al. 2002; Dos Santos et al. 1998). Aquatic bacterial species such as *S. oneidensis* have been found to be able to respire TMAO and the byproduct of TMAO respiration, trimethylamine (TMA), is a volatile compound that causes a deteriorating flavor and odor in rotten fish, and is considered to be responsible for fish spoilage and the fishy odor that dead fish exude (Dos Santos et al. 1998).

S. oneidensis TMAO respiratory operon *torECAD* is organized as follows (Figure 1.2). *torE* encodes for a short protein of unknown function that is similar to proteins in the NapE superfamily which consists of several bacterial periplasmic nitrate reductases (Gon et al. 2002). *torC* encodes a pentaheme *c*-type cytochrome that functions as quinone-oxidoreductase. *torA* encodes for a periplasmic terminal reductase of the molybdenum containing dimethyl sulfoxide

(DMSO) family of proteins, and *torD* encodes for a TorA-specific chaperon.

Electrons that are generated from the oxidation of organic carbon sources are transferred to lipid-soluble electron carriers, ubiquinone. The quinone pool is further oxidized by a periplasmic pentaheme *c*-type cytochrome, TorC. TorC transfers electrons to the terminal reductase TorA, which converts TMAO to TMA.

Expression of the TMAO respiratory genes is controlled through a two-component system. Upon binding TorT, a TMAO binding protein, undergoes a conformational change, that activates TorS, a histidine kinase, which phosphorylates the response regulator TorR (Moore and Hendrickson 2005). TorR then binds to the promoter region of the *torECAD* operon, which turns on expression (Bordi et al. 2004; Simon et al. 1995).

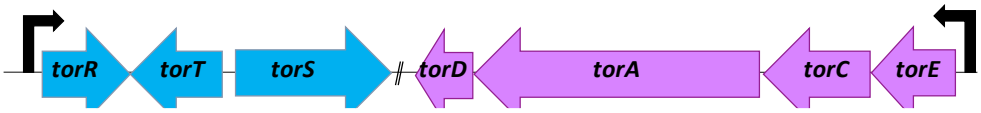

Figure 1.3. Genomic organization of genes directly involved in TMAO respiration in MR-1. Genes encoding the three component system that senses and responds to TMAO presence are colored blue. Genes of the *torECAD* operon which encodes for proteins responsible for TMAO respiration are colored purple.

TMAO terminal reductases (TorA)

Reactions catalyzed by DMSO and TMAO reductases follow the oxo-transferase mechanism, in which the oxo-group on the oxidized ion loses a hydroxyl group upon reduction (McCrindle et al. 2005; Morozkina & Zvyagilskaya 2007). The *Escherichia coli* TorA has a stricter substrate specificity than the DMS terminal reductase DmsA being able to only reduce the *N*-oxide form of substrates, in contrast DmsA reductases can reduce the *N*-oxide and the *S*-oxide form (McCrindle et al. 2005; Morozkina & Zvyagilskaya 2007; Iobbi-Nivol et al. 1996). In *E. coli*, TMAO has been shown to be reduced both by TMAO and DMSO pathways, with TorA

shown to be responsible for 90% of its reduction (Iobbi-Nivol et al. 1996). For *S. oneidensis* and *E. coli* TorA has a 52% amino acid identity and DmsA has a 56% amino acid identity, therefore it is hypothesized that both terminal reductases in *S. oneidensis* follow the same activity as *E. coli* terminal reductases.

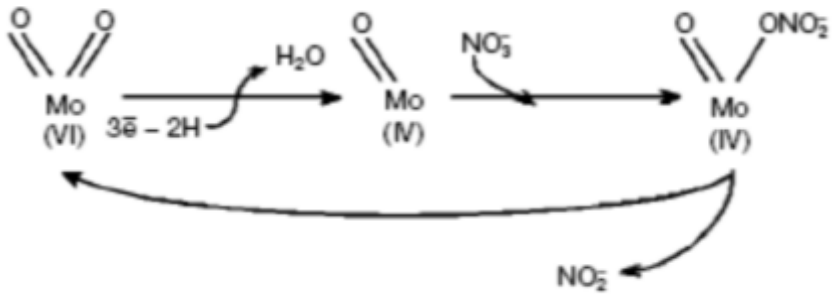

Figure 1.4. Scheme of the oxo-transfer mechanism catalyzed by DMSO and TMAO reductases (Morozkina & Zvyagilskaya 2007).

Carbon metabolism pathways

Lactate and pyruvate are the preferred carbon and energy sources for *S. oneidensis* studies under laboratory conditions in minimal medium. A constraint based metabolic model of *S. oneidensis* has been constructed using the annotated genome in combination with physiological studies and metabolic flux analysis (Flynn et al. 2012a; Tang et al. 2007; Feng et al. 2012; Brutinel & Gralnick 2012a). In addition, a high-throughput sequencing (Tn-seq) study helped to further delineate the *S. oneidensis* metabolic network (Brutinel & Gralnick 2012a). The proposed metabolic pathways are divided in aerobic and anaerobic growth. Lactate is oxidized to pyruvate and the fate of pyruvate depends on whether oxygen is present ((Flynn et al. 2012a). Under aerobic conditions pyruvate is converted to acetyl coenzyme A (acetyl-coA) by the pyruvate dehydrogenase complex (PDH), while 2 electron equivalents are generated. Acetyl-CoA is further incorporated into the tricarboxylic acid cycle and oxidized to CO_2 (Tang et al. 2007; Feng et al.

2012; Ringo Stenberg, E., Strom, A.R. 1984). On the other hand, when oxygen is not present pyruvate-formate lyase (PFL) converts pyruvate to acetyl-CoA and formate. Formate is transported to the periplasmic space and fully oxidized to CO_2 and H^+ by the action of formate dehydrogenase complex (FDH). Acetyl-CoA is directed towards acetate production, which is the primary source of ATP generation under anaerobic conditions in *S. oneidensis*, and the TCA cycle runs in a broken manner as a reductive branch and an oxidative branch ((Hunt et al. 2010; Brutinel & Gralnick 2012b). An incomplete TCA cycle has been reported for many fermentative organisms, where the primary source of ATP generation is substrate level phosphorylation and not oxidative phosphorylation(Hunt et al. 2010; Brutinel & Gralnick 2012a), as a means to balance redox reactions with the oxidative branch and avoiding unnecessary generation of NAD(P)H with the reductive branch. However, growth conditions with TMAO as terminal electron acceptor are an exception where carbon flux is directed towards acetate production, but runs a full TCA cycle (Figure 1.3) (Tang et al. 2007; Feng et al. 2012; Ringo Stenberg, E., Strom, A.R. 1984).

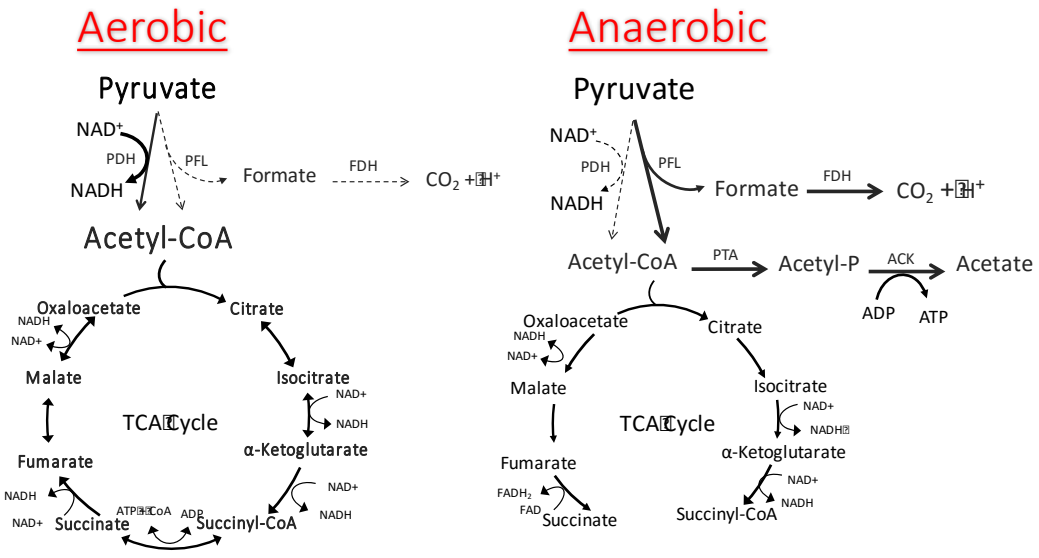

Fig. 1.5: Metabolic map describing *S. oneidensis* central metabolism under aerobic and anaerobic conditions.

Microbes in Communities

Microorganisms are mostly studied as monocultures of single species under laboratory conditions, however, in nature, they act as interacting mixed microbial communities that have a significant role in the geochemical fate and cycling of important elements (De Roy et al. 2014; Wade 2002). To put it differently, less than 2% of microorganisms are readily culturalble by using the conventional culturing methodologies in laboratory (Wade 2002). Moreover, that 2% culturable microorganisms exist as interacting communities in their natural niche, where their behavior differs from that of pure culture (De Roy et al. 2014). Genome sequencing of widely characterized and extensively studied bacterium have identified the presence of a vast number of genes with unknown biological functions, which have been suggested to be essential for life in conditions that have not been tested to date (Serres et al. 2001). The study of microbial communities in laboratory conditions can help meet the disengage that exists between the study of microorganisms in their natural environment and the pure culture studies in the laboratory conditions. Furthermore, these studies may potentially elucidate the function of unknown genes that might be involved in community life styles, and allow the study of currently unculturable microorganisms.

Studies of microbial communities have been performed *in situ* (Tringe & Rubin 2005). Diversity can be identified through metagenomics (Raes, Harrington, et al. 2007; Harrington et al. 2007; Tringe & Rubin 2005), but cultivation of individual members is challenging; nor is it possible to identify the individual role of each species and the interactions in the communities (Raes & Bork 2008; Raes, Foerstner, et al. 2007). One particular disadvantage for the study of communities *in situ* is the extensive number of unknown variables, which make it difficult to develop testable hypothesis. These unknown variables include fluctuating factors and unquantifiable or untestable factors. Fluctuating factors range from: temperature, pH, spatial

organization, humidity, nutrient availability over time, species abundance, among others (Goers et al. 2014). Unquantifiable factors include initial point of contact between partners of a community, evolutionary trajectory of a community, and individual interactions between species (Marx 2009; Hillesland & Stahl 2010; Rozen et al. 2005). Same disadvantage applies for a different approach that studies microbial communities through cultivation of microbial enrichments from environmental samples (Escalante et al. 2015). Enrichment efforts combined with metagenomics, transcriptomics, and proteomics have provided insight into the diversity, functionality, and resilience within microbial communities (VerBerkmoes et al. 2009; Temperton & Giovannoni 2012; Raes & Bork 2008; Tringe & Rubin 2005; Harrington et al. 2007; Raes, Harrington, et al. 2007). However, these studies have often been unsuccessful in efforts to isolate all individual members of the microbial community and thus to understand the system using the classical pure culture methodologies. Though, much can be learned from enriched microbial communities using "omics", these techniques provide limited understanding of contributions and roles of each member of the community, or of the specific interactions that allow those communities to persist (Raes & Bork 2008; De Roy et al. 2014).

A different strategy to the study of microbial communities called synthetic co-culture consists of building communities with two or more species in a controlled environment (Goers et al. 2014; De Roy et al. 2014). This new approach provides the study of communities from a bottom-up approach in a simplified representation of natural ecosystems. In addition, co-cultures are initiated with pure cultures of species that are well-characterized, genetically tractable, and whose sequenced genomes are available. Therefore, synthetic co-cultures provide an opportunity to study: interactions from the initial point of contact, specific interactions between partners, and the evolutionary processes and factors that affect and allow a community origin and maintenance. A common design for synthetic co-cultures is to link the metabolism of two or more organisms by a process called cross-feeding (Estrela et al. 2012; Escalante et al. 2015). Metabolic cross-feeding

is an interaction commonly identified within naturally occurring microbial communities (Estrela et al. 2012). Through engineered cross-feeding several interactions can be established: cooperation, commensalism, or parasitism. In a cooperative cross-feeding co-culture, both partners beneficiate from the presence of each other. Both partners produce a product or service required for the growth or survival of the other partner in the co-culture. Commensalism, on the other hand, also called a one-way cross-feeding, consists of one producer and one cross-feeder, where the producer provides a metabolic by-product that is utilized by its partner, but the producer is neither benefitted nor affected by the presence of the partner. In this case, the producer is unaffected, whereas the consumer is dependent and relying on the producer for growth. A parasitic cross-feeding is often built on the same principles of a one-way cross-feeding interaction, but the producer is negatively affected by the presence of a consumer partner.

From a biotechnology perspective, synthetic communities that are linked by metabolic cross-feeding are advantageous for efforts that include: industrial fermentation, biodegradation and generation of biofuels (Bader et al. 2010; Goers et al. 2014; De Roy et al. 2014). In industrial fermentation, the production of bulk chemicals that are not produced by the metabolism of a single species can be achieved through cross-feeding (Bader et al. 2010). A similar theme exists with bioremediation efforts, where linking the metabolism of more than one microorganism allows for the successful degradation of compounds that are not degraded by a single species(Bader et al. 2010). In addition, cross-feeding allows for bacterial electricity production to be performed with a broader array of organic compounds (Bader et al. 2010; Strycharz-Glaven et al. 2013) .

Geobacter sulfurreducens* and *S. oneidensis

To better understand microbial interactions, the Gralnick laboratory has engineered a synthetic co-culture between two previously non- interacting bacteria: *S. oneidensis* and *Geobacter sulfurreducens.* Both bacterial species have become model organisms to study

extracellular electron transfer (Richter et al. 2012). *Shewanella* and *Geobacter* are found in different environments that are stratified either permanently or seasonally. *Shewanella* are found in aquatic systems, whereas *Geobacter* are found in diverse soils and sediments(Hau & Gralnick 2007; Nealson & Scott 2006). *Shewanella* is a facultative anaerobe that can respires over 20 substrates, whereas *Geobacter* is a strict anaerobe able to reduce a smaller number of substrates. Even though these two bacterial species are not typically found together in nature, their individual environments have a common theme of being oxic/anoxic stratified environments where both organisms have to adapt and respond to the available respiratory substrate (Mahadevan et al. 2011). In such oxic/anoxic environments, both organisms play an important role in the biogeochemical cycling of important metals like, iron, manganese, uranium, and chromium (Richter et al. 2012). In order to respire these insoluble electron acceptors, electrons that are generated from the oxidation of carbon sources have to be transported to the outside of the cell surface (Richter et al. 2012; Von Canstein et al. 2008; Hau & Gralnick 2007; Hansen et al. 2007; Weber et al. 2006). Both *Shewanella* and *Geobacter* have unique biochemical pathways to transfer their electrons in a process called extracellular electron transfer. Both organisms utilize quinone electron carriers and *c*-type cytochromes in their inner membrane that then transfer electrons to multi-haeme proteins that are anchored in the outer membrane of the cell. However, the final step of metal reduction differs. *G. sulfurreducens* requires direct contact between the multi-haeme outer membrane proteins and the substrate (Nevin and Lovley 2000, Nevin and Lovely 2002), whereas *Shewanella* utilizes flavin shuttles that transfer the electrons to the substrate (Von Canstein et al. 2008; Brutinel & Gralnick 2012b). These two strains are the most studied organisms on extracellular electron transfer that utilize different biochemical pathways for EET, and are found in two different environments. A synthetic co-culture consisting of *S. oneidensis G. sulfurreducens* will therefore provide an opportunity to understand the evolutionary principles and molecular basis of interaction. In addition, their unique respiratory capacity

coupled to their well-characterized nature of their extracellular electron transport pathways makes these two species excellent candidates for initial investigations into bioremediation of toxic chemicals and energy production.

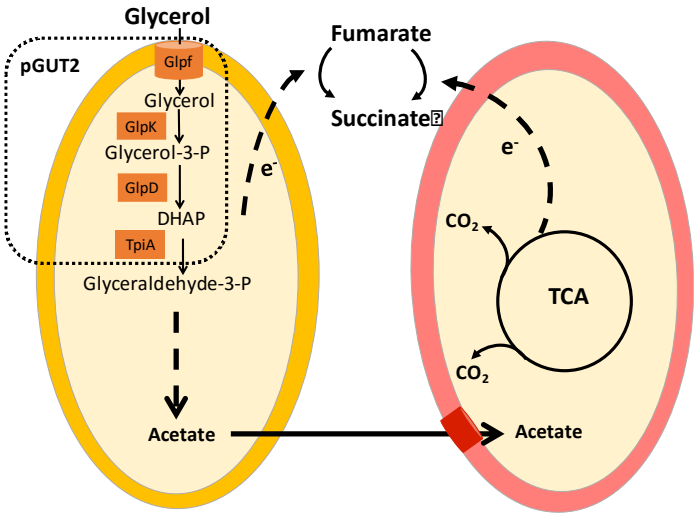

Figure 1.6. Schematic of *G. sulfurreducens* (red) and *S. oneidensis* pGUT2 (orange) in co-culture. Glycerol utilization pathway encoded by pGUT2 is highlighted by the dotted rectangle.

G. sulfurreducens and *S. oneidensis* co-culture

In efforts to better understand microbial interactions, we have developed s synthetic co-culture between *G. sulfurreducens* and *S. oneidensis*, by linking their metabolisms to allow the complete oxidation of glycerol to CO_2 (figure 1.4). *S. oneidensis* metabolizes glycerol and secretes acetate as part of its anaerobic metabolism, whereas *G. sulfurreducens* is able to utilize the byproduct acetate as sole carbon and energy source (Kane and Gralnick, unpublished data). This is a one way cross-feeding interaction where *S. oneidensis* does not depend on its partner for growth, but *G. sulfurreducens* depends on its partner's ability to produce acetate. This system serves as a suitable platform where we can study the basis of this one-way cross-feeding interaction and use it as a model for other cross-feeding communities.

Chapter 2

S. oneidensis Central Metabolism under TMAO Respiring Conditions

Introduction

Trimethyl amine *N*-oxide is a compound readily available in marine and fresh water systems (Dos Santos et al. 1998). It has been extensively studied for its role as a nitrogen source in marine heterotrophic bacteria like *Ruegeria pomeroyi* (Lidbury et al. 2014), however it is also utilized as terminal electron acceptor in bacteria like *E. coli*, *Shewanella*, *Rhodobacter* and *Vibrio* (Iobbi-Nivol et al. 1996; Gon et al. 2002; Bordi et al. 2004; Bordi et al. 2003; Baraquet et al. 2006; Ansaldi et al. 2007; Moore & Hendrickson 2009). *E. coli*, *S. oneidensis* and *Vibrio* have separate respiratory chains for the reduction of DMSO and TMAO, on the other hand *Rhodobacter* have only one pathway for the respiration of both substrates (McCrindle et al. 2005; Morozkina & Zvyagilskaya 2007). TMAO and DMSO reductases are molybdoenzymes, members of the DMSO superfamily and follow the oxo-transferase reaction mechanism (McCrindle et al. 2005; Morozkina & Zvyagilskaya 2007). TMAO reductase has a stricter substrate specificity and can only reduce TMAO, whereas the DMSO reductase can reduce both substrates (Iobbi-Nivol et al. 1996; McCrindle et al. 2005). Expression of the TMAO reduction operon *torECAD* is positively regulated by TMAO via a two-component system, where TMAO induces a cascade of conformational changes from a periplasmic receptor, to a histidine kinase and ultimately to a response regulator that activates expression of *torECAD* (Bordi et al. 2004; Bordi et al. 2003; Baraquet et al. 2006; Ansaldi et al. 2007; Moore & Hendrickson 2009; Simon et al. 1995).

For *S. oneidensis* TMAO is an unusual respiratory substrate due to the fact that it is respired in a unique anaerobic respiratory chain due to the use of a specific *c*-type cytochrome and UQs instead of MKs as lipid-soluble electron carriers. In addition, growth under conditions where TMAO is the respiratory substrate follows an uncommon central carbon flux

(Ringo Stenberg, E., Strom, A.R. 1984; Tang et al. 2007). From the respiratory chain perspective, TMAO respiration utilizes its own quinone-oxidase protein, called TorC and ubiquinone electron carriers, instead of menaquinones. The *S. oneidensis* genome encodes for TorC and CymA, two homologous tetra-heme *c*-type cytochromes that perform similar quinone oxidizing reactions, however, TorC is directly linked to TMAO respiration by being expressed and regulated in the same operon as TorA, whereas CymA is encoded in its own operon, thus regulated separately from other electron acceptors (Breuer et al. 2015). In addition, CymA has been shown to be involved in the reduction of a vast majority of the anaerobic terminal electron acceptors used by *S. oneidensis* and to be linked to the menaquinones form of electron carriers and not ubiquinones. TorC, on the other hand, utilizes ubiquinone as lipid soluble electron carrier, which has a higher redox potential than menaquinone, thus are more efficient when oxygen is the terminal electron acceptor due to it being the highest electronegative substrate known. In addition, the reduced form of menaquinones is highly reactive with molecular oxygen and is subjected to non-catalytic oxidation, which makes it an inefficient cofactor in oxygen-containing environments (Schoepp-Cothenet et al., 2009, Fujimoto et al. 2012). Utilization of ubiquinones during TMAO respiration may confer a competitive advantage that allows *S. oneidensis* to utilize TMAO as a respiratory substrate in microaerophilic conditions. TMAO respiration by *S. oneidensis* has been detected in conditions where oxygen concentrations can become limited or depleted. Due to the fact that the *torECAD* expression is activated by TMAO presence and not by anaerobic conditions in *S. oneidensis* (Bordi et al. 2004; McCrindle et al. 2005), it is possible that TMAO can be respired simultaneously with oxygen and follow an aerobic carbon metabolism with higher biomass yield and a broader spectrum of consumable carbon sources. From the carbon source perspective, it has been established that during *S. oneidensis* anaerobic growth the major source of ATP production is substrate-level phosphorylation through acetate production (Hunt et al. 2010) and that carbon sources are

metabolized through a broken TCA cycle as a means to balance redox equations while keeping the levels of electron carrying coenzymes (NADH) inside the cell low (Flynn et al. 2012a; Brutinel & Gralnick 2012a). However, central carbon metabolism in anaerobic conditions with TMAO as terminal electron acceptor in *S. oneidensis* is unconventional as it runs a full TCA cycle but carbon flux has been shown to be directed towards acetate production, thus generating ATP via substrate level phosphorylation (Tang et al. 2007; Ringo Stenberg, E., Strom, A.R. 1984). In addition, pyruvate is known to be converted to acetyl-CoA by PFL under anaerobic conditions, reaction which generates formate or by PDH under aerobic conditions (Flynn et al. 2012b; Kane et al. 2016). Currently, it is not known whether pyruvate is converted to acetyl-CoA by PDH or PFL during TMAO respiring conditions, and thus the effect of electrons in the form of NADH or in the form of formate is not fully understood thus the full metabolic network of *S. oneidensis* under TMAO respiring conditions remains poorly understood. The work presented in this chapter seeks to fill in the gaps of *S. oneidensis* metabolism during TMAO respiration under strict anaerobic conditions and simultaneously with oxygen.

Importance

Even though respiration of TMAO is important for *Shewanella* and the physiology of other marine bacteria, there is little evidence that help elucidate substrate preferences, catabolic processes, and energy conservation during anaerobic or microaerophilic TMAO respiring conditions. Our limited knowledge is due to the fact that more interest has been given to the immediate applications of extracellular terminal electron acceptors, their pathways, and related aspects. However, in order to tap into the full potential of *S. oneidensis* physiology under environmentally relevant conditions, like fish gut, and for biotechnological and bioremediation applications it is important to understand the metabolic strategies under TMAO respiring conditions.

Materials and Methods

Reagents

Primers were ordered from Integrated DNA Technologies via the University of Minnesota Genomics Center (UMGC). Phusion High- Fidelity DNA polymerase enzymes were purchased from New England Biolabs (Ipswich, MA). Reactions were set up according to the manufacturer's protocol. Chemicals were obtained from Sigma-Aldrich (St. Louis, MO) with the exception of glycerol, which was purchased from Fisher Chemical (Pittsburgh, PA). Medium components were purchased from Becton, and Dickinson and Company (Sparks, MD).

Culturing conditions

Strains used in this study are listed in table 2.1. *E. coli* strains were cultured aerobically at 37°C on either lysogeny broth (LB) agar plates or LB liquid cultures shaking at 250rpm and supplemented with either 50 µg/mL kanamycin (Km) or diaminopimelic acid (DAP). *S. oneidensis* strains were cultured aerobically at 30°C on either LB agar plates, or shaking liquid cultures of LB and/or *Shewanella* Basal Medium (SBM) at 250rpm.

Growth assays

Growth assays were performed in SBM. Strains stored in 15% glycerol at -80°C were freshly streaked at 37°C for *E. coli* and 30°C FOR *Shewanella* into LB agar plates to obtain isolated colonies under aerobic conditions. LB liquid medium was then inoculated with single colonies for aerobic overnight propagation, shaking at 250 rpm and a temperature of 30°C. Afterwards, overnight LB cultures were sub-cultured into aerobic SBM shaken at 250 rpm for ~16 hours at 30°C. Cells were then washed and added to a final optical density of ~0.02 (OD600) into aerobic or anaerobic SBM, as necessary. SMB was composed of 5 mL/L vitamin mix, 5 mL/L mineral mix (Hau & Gralnick, 2007), 0.05% casamino acids, and supplemented with pyruvate as sole carbon source. For anaerobic growth assays, SBM was supplemented with

fumarate, or trimethylamine *N*-oxide as terminal electron acceptor, while Balch tubes were degassed with argon headspace and stoppered with butyl rubber (Balch & Wolfe, 1976). Growth rate and biomass yield were monitored by measuring optical density at 600 nm (OD_{600}).

Table 2.1. Strains and plasmids used in this chapter.

***E. coli* Strains**	**Genotype/Characteristic**	**Reference**
UQ950	DH5α λ(*pir*) cloning host; F^-Δ(*argF-lac*)169 Φ80d*lacZ58*ΔM15 *glnV*44(AS) *rfbD1 gyrA96*(Nal^R) *recA1 endA1 spoT1 thi-1 hsdR17 deoR λpir*$^+$	(Saltikov & Newman 2003)
WM3064	Donor strain for conjugation; *thrB*1004 *pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD)567 ΔdapA*1341::[*erm pir*(wt)]	(Saltikov & Newman 2003)
***S. oneidensis* Strains**	**Genotype/Characteristic**	**Reference**
JG274	WT MR-1	(Myers & Nealson 1988)
JG619	Δ*pfl*	(Flynn et al. 2012b)
JG422	Δ*aceE*	(Flynn et al. 2012a)
JG2957	Δ*fdh*	(Kane et al. 2016)
JG2655	Δ*dms*	(West, 2016)
JG3266	Δ*torECAD*	(West, 2016)
JG3516	Δ*dms* Δ*torECAD*	(West, 2016)
JG3881	Δ*dms* Δ*pfl*	This Study
JG3880	Δ*dms* Δ*pdh*	This Study

Generation of deletion mutants

PCR primers were developed by targeting deletion target flanking regions, which were then amplified and cloned into the pSMV3 suicide vector using PCR. Deletion constructs were then moved into *S. oneidensis* by conjugal transfer from *E. coli* donor strain WM3064. Homologous recombination was then used to generate scar less, in-frame gene deletions as previously described (Coursolle et al., 2010). All plasmid constructs and gene deletions were verified by

sequencing (University of Minnesota Genomics Center).

Results and Discussion

S. oneidensis has a different metabolic strategy for aerobic vs anaerobic growth conditions

Growth rate and yield for wild type MR-1 and three deletion strains were examined with oxygen, fumarate, or TMAO as sole electron acceptors respectively. The three strains had a deletion on genes encoding for key metabolic enzymes: pyruvate dehydrogenase (*pdh),* pyruvate formate lyase (*Δpfl),* and formate dehydrogenase *(Δfdh)*. Under conditions with oxygen as electron acceptor and pyruvate as carbon source deletion strains *Δfdh* and *Δpfl* had same growth phenotypes as wild type MR-1, *Δpdh* strain resulted in decreased growth rate and yield (Figure 2.1A). In MR-1 as well as in *E. coli*, conversion of pyruvate to acetyl-CoA can be catalyzed by either PDH or PFL (Flynn et al. 2012a). In *E. coli*, PFL has been shown to be inactivated in the presence of oxygen (Knappe & Sawers 1990). *E. coli* PFL and MR-1 PFL have 81% protein identity, therefore it is hypothesized that MR-1 PFL is also inactive during aerobic conditions. Here, the results showed that *S. oneidensis Δpdh* strain, which contains only PFL, was able to generate biomass under aerobic conditions, but was not able to rescue MR-1 phenotype since it showed lower yield and longer stationary phase than MR-1. These results, support previous studies on the role of PDH during aerobic growth in *S. oneidensis* (Flynn et al. 2012a).

Under anaerobic conditions with fumarate as sole electron acceptor, deletion of *pfl* or *fdh* conferred a growth defect, where *Δpfl* strain showed absence of growth and *Δfdh* strain showed a decrease in growth rate and yield (Figure 2.1B). Additionally, *Δpdh* strain did not result in a growth defect, confirming that this enzyme was not necessary for growth under the anaerobic conditions tested. Although previous studies have shown that PDH is expressed from early log phase to stationary phase with pyruvate as sole carbon sources and fumarate as terminal electron acceptor (Meshulam-simon et al. 2007), *Δpfl* did not grow under these conditions (Flynn et al.

2012a). One possible explanation for absence of growth for *Δpfl* strain, under anaerobic conditions, could be the generation of $NADH+H^+$. Coenzyme NADH is known to be re-generated by the NADH-ubiquinone oxidoreductase complex, which is a reaction coupled to the reduction of UQ electron carriers (Spero et al. 2016). One possible hypothesis that explains the absence of growth for *Δpfl* strain under anaerobic conditions with fumarate as TEA is the reduction of UQ pools by PDH activity which is not able to transfer electrons to fumarate, due to the fact that the fumarate respiratory chain is not capable of UQ oxidation (Koland et al. 1984).

TMAO is respired simultaneously with oxygen

Natural settings where TMAO is present as a respiratory substrate for *S. oneidensis*, like fish scale, are not strict anaerobic environments. Therefore, to determine the physiological significance of TMAO respiration along with oxygen respiration, growth of MR-1 and the three deletion strains were examined with both oxygen and TMAO present together as respiratory substrates. Under this condition, *Δpfl* and *Δfdh* strains, which have gene deletions in key enzymes for anaerobic growth, showed a biphasic growth phenotype that was also seen in MR-1 (Figure 2.1D). During the first phase all the strains showed faster doubling times than treatments with either TMAO or oxygen alone (Figure 2.1A and D). The second phase was absent for *Δpdh* which remained in stationary phase, whereas MR-1, *Δpfh*, and *Δfdh* showed that growth rate was lower than the first phase, and was similar to aerobic doubling time. The results represent a simultaneous use of TMAO and oxygen, whereas the second phase shows a different growth strategy that could be hypothesized to be driven by TMAO depletion, however further HPLC analysis is needed to confirm this hypothesis.

Role of PDH and PFL under TMAO respiring conditions

Interestingly, deletion of *pfl*, *pdh* or *fdh* did not confer a growth defect as compared to MR-1 under conditions where TMAO was the sole electron acceptor (Figure 2.1 C). A possible

hypothesis is that TMAO being an unusual respiratory substrate that utilizes UQ instead of MK as part of the respiratory chain, allow for growth of *Δpfl* strain under this anaerobic condition, whereas the absence of oxygen allows for *Δpdh* a strain using PFL to grow under this conditions.

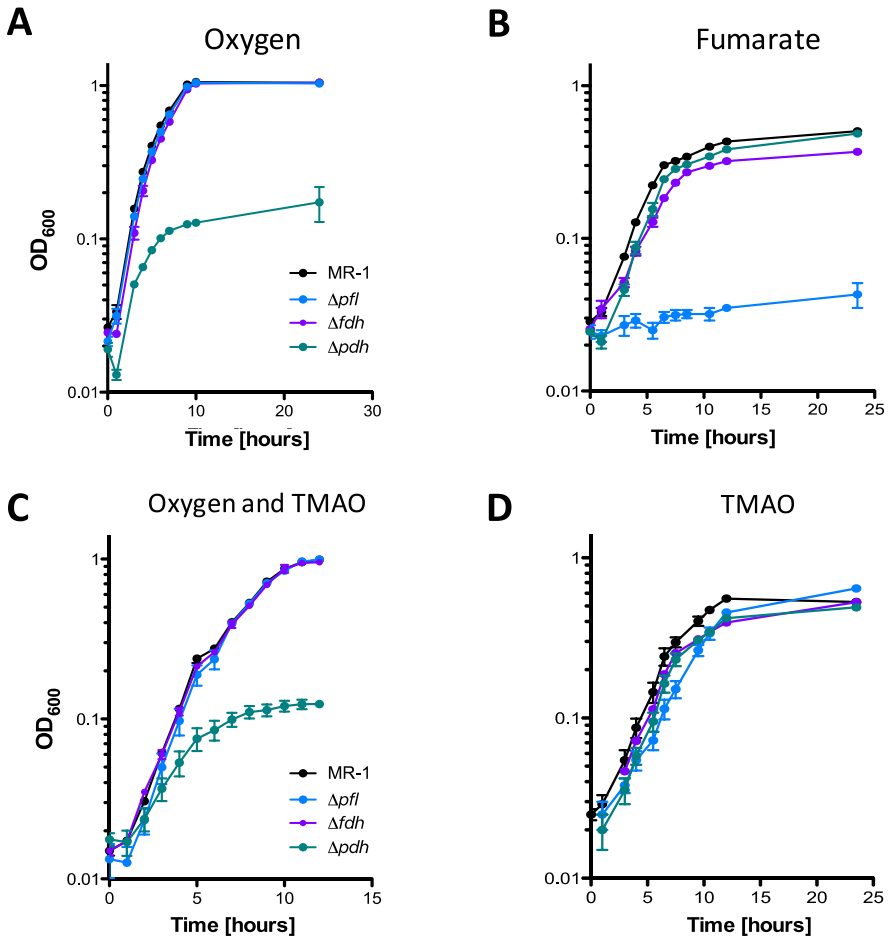

Figure 2.1. Growth curves of *S. oneidensis* MR-1 (WT), *Δpfl*, *Δpdh*, and *Δ*f*dh* were performed in minimal medium (SBM) with 20 mM pyruvate under aerobic conditions (A and C) and anaerobic condition with 40 mM fumarate (B) or 40 mM TMAO (D) as terminal electron acceptor (right). Growth curve C was performed under aerobic conditions and amended with 40 mM TMAO. Reported values are the average of three independent experiments and error bars represent standard error of the mean (SEM).

MR-1 can respire TMAO with the DMSO respiratory machinery

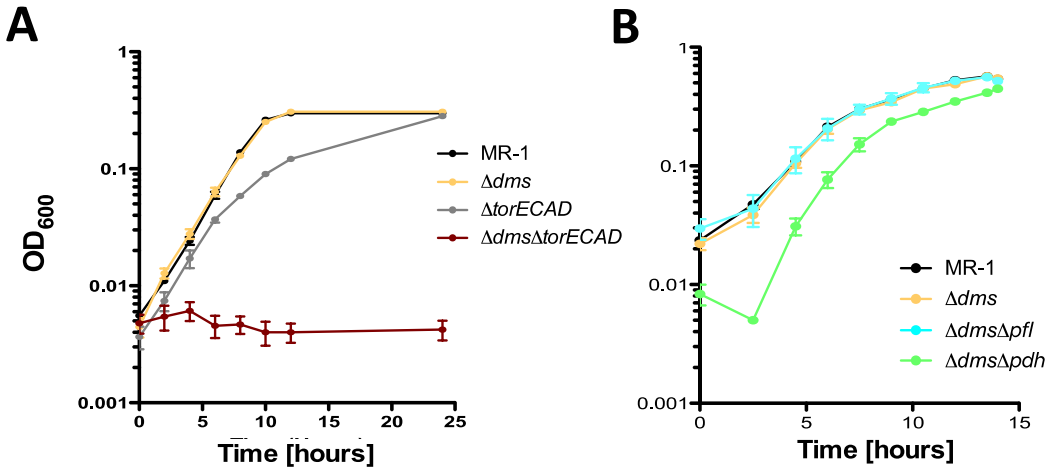

Figure 2.2. Growth curves of *S. oneidensis* MR-1 (WT*), Δdms*, *ΔtorECAD*, and *ΔtorECADΔdms* were performed under anaerobic condition with40 mM TMAO as terminal electron acceptor and 20 mM pyruvate. Reported values are the average of three independent experiments and error bars represent standard error of the mean (SEM) (West et al, manuscript in preparation).

Shewanella and *E. coli* are both gamma-protebacteria and thus are phylogenetically close. *E. coli* and *Shewanella*, are able to couple energy yielding reactions to TMAO reduction (Iobbi-Nivol et al. 1996). TMAO respiration in *E. coli* has been shown to be catalyzed by the TMAO reductase TorA and by the DMSO reductase DmsA (Iobbi-Nivol et al. 1996). In addition, TorA from *E. coli* and *S. oneidensis* have a 52% amino acid identity and DmsE from *E. coli* and *S. oneidensis* have a 56% amino acid identity. Deletion of *S. oneidensis torECAD* operon was not sufficient for preventing growth under conditions with TMAO as sole electron acceptor. Therefore, deletion of *torECAD* along with deletion of *dmsEFAB* was needed to inhibit growth with TMAO (Figure 2.2A).

In order to examine the physiological significance of PDH and PFL with the TMAO respiratory pathway, deletion of each enzyme, *Δpdh* and *Δpfl*, was needed to be constructed on a *Δdms* deletion background. All strains have the same growth rate under conditions with TMAO

as terminal electron acceptor, however strain *ΔdmsΔpfl* showed an initial decrease in OD and a longer stationary phase. The conversion of pyruvate to acetyl-CoA generates two electron equivalents in the form of formate when the reaction is catalyzed by PFL or in the form of NADH when the reaction is catalyzed by PDH. Based on the results presented here it can be speculated that acetyl-CoA can be generated by either PDH or PFL under TMAO respiring conditions. In addition, it can be inferred that the electrons generated by PDH in the form of NADH+ H+ are equivalent to the electrons generated by PFL in the form of formate, which are ultimately oxidized by FDH. This speculation is reinforced by the absence of a cumulative effect on growth of MR-1 from having both enzymes expressed under this conditions.

Conclusion

The work presented in this chapter helps elucidate *S. oneidensis* metabolic strategy for growth conditions with Oxygen, fumarate, and TMAO as TEA. Under aerobic conditions *Δpdh* strain shows a growth defect with oxygen as TEA as compared to MR-1. Under anaerobic conditions with fumarate as TEA *Δpfl* strain showed absence of growth. Results here show the first evidence of PDH activity in *S. oneidensis* under strict anaerobic conditions with TMAO as TEA. In addition, growth with TMAO as sole TEA is the only condition in which conversion of pyruvate to acetyl-CoA is converted by *Δpdh* and *Δpfl* in similar growth phenotype as MR-1.

Chapter 3

Geobacter sulfurreducens as a Respiratory Partner

Introduction

In nature microorganisms are found as interacting communities, however, in laboratory they are mostly studied as isolated cultures. During the last decade, in order to model microbial interactions, academic research has seen an increase in the utilization of synthetic communities (Dunham 2007; Goers et al. 2014; De Roy et al. 2014), where two or more organisms are grown in co-culture. Advantageous applications of synthetic communities range from bioremediation, production of bulk chemicals, enzyme food additives, antimicrobial substances and biofuels (Brenner et al. 2008; Bader et al. 2010). One of the most common designs is a cross-feeding interaction- where a metabolic byproduct of one partner serves as substrate for the metabolism of the other. However, previous studies have demonstrated that when two organisms are put together to interact for the first time, partners might not be optimized for the interaction (Summers et al. 2010; Hillesland & Stahl 2010; Hillesland et al. 2014; Zhang & Reed 2014; Hansen et al. 2007; Seth & Taga 2014; Mee et al. 2014). In nature, metabolic cross-feeding can broadly shape microbial communities (Seth & Taga 2014; Doebeli 2002), which contribute significantly to the global cycling of nutrients (9,10). Before the previously mentioned advantageous applications can be utilized fully, there is a compelling need to understand the specific principles that drive the origin and maintenance of individual partners in the community. This gap in our knowledge limits our understanding over how microorganisms in a community have adapted for many generations and how we can harness the full potential that communities have on industrial applications.

Evidence of improved interactions by laboratory evolution

Recent studies have evidenced that although synthetic communities are efficient for rational engineering, partners in the community can optimize for the interaction, and produce

stronger interactions (Summers et al. 2010; Hillesland & Stahl 2010; Hillesland et al. 2014; Zhang & Reed 2014; Hansen et al. 2007; Seth & Taga 2014; Mee et al. 2014). One such study performed laboratory evolution of a cross-feeding interaction between *G. sulfurreducens* and *G. metallireducens* (Summers et al. 2010). Laboratory evolution selected for a mutation in *G. metallireducens* that enhanced extracellular electron transfer, increased physical association, and accelerated ethanol metabolism by at least 70%. Another study of laboratory evolution with a cooperative synthetic community between *Desulfovibrio vulgaris* and *Methanococcus maripaludis,* selected, surprisingly, for a *D. vulgaris* mutant that lost the primary physiological attribute of its genus: sulfate reduction (Hillesland et al. 2014). Evolution improved this interaction by selecting for higher growth rate and higher biomass yield (Hillesland & Stahl 2010). In a different study using two *Escherichia coli* auxotroph strains (leucine or lysine), adaptively evolution resulted in improved growth performance by increase in growth rate by three fold and optical densities (Zhang & Reed 2014).

***S. oneidensis* and *G. sulfurreducens* engineered commensalism**

In efforts to better understand microbial interactions, we have developed a co-culture system between two previously non-interacting gram-negative bacteria, *Shewanella oneidensis* strain MR-1 and *Geobacter sulfurreducens*, strain PCA (Kane and Gralnick, manuscript in preparation). In this synthetic association, *G. sulfurreducens* growth is enabled by the secretion of acetate as a byproduct of *S. oneidensis* metabolism, while both respire fumarate as sole electron acceptor. Utilization of glycerol as sole carbon and energy source has been engineered by the incorporation of a glycerol utilization plasmid (pGUT2) in *S. oneidensis* by that contains the first three enzymes in the pathway for glycerol utilization in *E. coli* (Flynn et al. 2010). These enzymes are *glpF*, *glpK*, and *glpD*, which encode a glycerol facilitator, a glycerol kinase, and a membrane-bound quinone-linked glycerol-3-phosphate dehydrogenase, respectively. Co-culture media does not contain a source of carbon for *G. sulfurreducens*, therefore *G. sulfurreducens* is

able to grow only when co-cultured with *S. oneidensis* pGUT2 (Figure 3.1A). *S. oneidensis* is able to grow as a monoculture in co-culture media, however, higher growth yield and growth rate is obtained when both organisms are present. In addition, we are able to assay for individual growth of the members of the community by plating in selective media and measuring colony forming units (CFUs) per milliliter (Figure 3.1B).

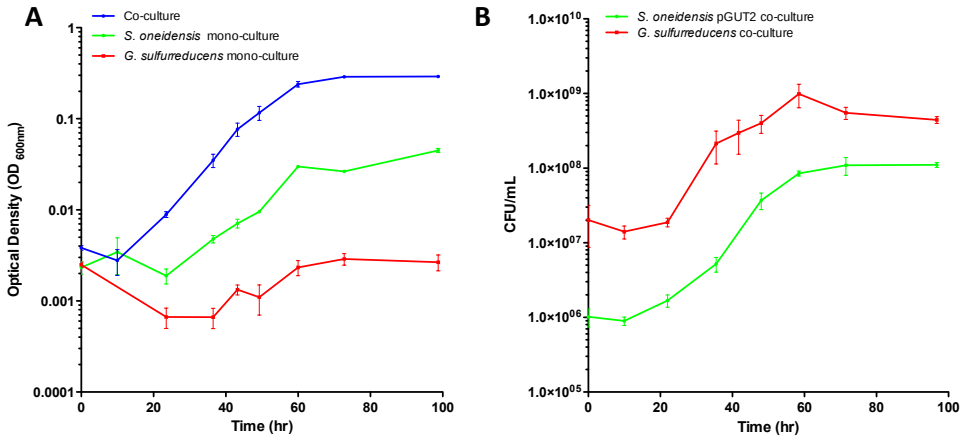

Figure 3.1. A) Shows growth, as measured by optical density, for *S. oneidensis* pGUT2 in monoculture, *G. sulfurreducens* in monoculture, and commensal co-culture of *S. oneidensis* pGUT2 and *G. sulfurreducens* under conditions with glycerol as electron acceptor and fumarate as carbon source (NBFA). **B)** Shows growth of the individual members of the community measured by colony forming units (CFU) per milliliter in selective media (Kane and Gralnick, unpublished work).

The purpose of the work presented in this chapter is to expose *S. oneidensis* pGUT2 and *G. sulfurreducens* to a new growth condition, a co-culture, to which both organisms must adapt via an evolutionary process. Followed by evolution, physiological examination of evolved strains will allow us to understand the adjustments that both partners make to each other's presence.

Materials and Methods

Reagents

Medium components were purchased from Becton, and Dickinson and Company (Sparks,

MD). Glycerol was purchased from Fisher Chemical (Pittsburgh, PA).

Culturing conditions

S. oneidensis pGUT2 strains were cultured aerobically at 30°C on either lysogeny broth (LB) and/or Shewanella Basal Medium (SBM) shaking at 250 rpm supplemented with 50 µg/mL kanamycin (Km). SBM is composed of 5 mL/L vitamin mix, 5 mL/L mineral mix (Hau & Gralnick, 2007), 0.05% casamino acids, and supplemented with glycerol as sole carbon source. For anaerobic growth assays, SBM was supplemented with fumarate as terminal electron acceptor, while Balch tubes were degassed with argon headspace and stoppered with butyl rubber (Balch & Wolfe, 1976). *G. sulfurreducens* strains were cultured at 30°C anaerobically using an anaerobic chamber with a 5% H_2/75%N_2/20%CO_2 atmosphere (Coy Lab Products; Grass Lake, MI) on nutrient broth (NB) plates or liquid. NB is composed of the following per liter: 0.38g KCl, 0.2g NH_4Cl, 0.069g $NaH_2PO_4 \cdot H_2O$, 0.0 g $CaCl_2 \cdot 2H_2O$, 0.2g $MgSO_4 \cdot 7H_2O$, 2.0g $NaHCO_3$ (pH 6.8), 10mL/L of a mineral mix (Hau et al., 2008) and supplemented with 0.1% trypticase (wt/vol), 1 mM cysteine, 20 mM acetate, 40 mM fumarate (NBFA+TC) and solidified with 1.5% agar.

Co-culture growth assays

Growth assays were performed in NB medium as follows. *S. oneidensis* strains stored in 15% glycerol at -80°C were freshly streaked on LB agar plates and incubated at 30°C to obtain isolated colonies under aerobic conditions. LB liquid medium was then inoculated with single colonies for aerobic overnight propagation, shaking at 250 rpm and a temperature of 30°C. Overnight LB cultures were sub-cultured into aerobic SBM shaken at 250 rpm for ~16 hours at 30°C, prior to co-culture experiment. *G. sulfurreducens* strains stored in 10% dimethyl sulfoxide (DMSO) stocks at -80°C were freshly streaked at 30°C on NB agar plates in an anaerobic chamber (5%:75%:20% H_2:N_2:CO_2) to obtain isolated colonies. One mL of NB supplemented with fumarate and acetate (NBFA) liquid medium was then inoculated with single colonies for

propagation during 48 hours at a temperature of 30°C. This culture was sub-cultured by transferring into ten milliliters anaerobic NBFA non-shaking for 24 hours at 30°C, prior to co-culture experiment. For co-culture experiment *S. oneidensis* and *G. sulfurreducens* cells were washed in SBM aerobically and added to a final optical density of 1.0 (OD600) into anaerobic NB supplemented with casamino acids, glycerol as carbon source, and fumarate as terminal electron acceptor, hereafter referred to as NBFG. Growth rate and biomass yield of the co-culture was monitored by optical absorbance at OD_{600}. Growth rate and biomass yield of the individual members of the community were monitored by colony-forming units (CFU) in selective media as follows. De-gassed needles were used to extract 100μL of co-culture media from Balch and used for serial dilutions and plated on aerobic LB or NBFA for *S. oneidensis* or *G. sulfurreducens*, respectively.

Laboratory evolution

Co-cultures of *S. oneidensis* and *G. sulfurreducens* were evolved in Batch tubes through serial transfers to fresh medium every time stationary phase is reached. Evolution was stopped when increase in growth rate and biomass yield plateaued over time. Growth rate and biomass yield were monitored by optical density. Freezer stocks were saved throughout several transfers and for the final evolved strains. Freezer stocks were made by saving co-cultures in 10% DMSO under strict anaerobic conditions in an anaerobic chamber (5%:75%:20% H_2:N_2:CO_2). *S. oneidensis* and *G. sulfurreducens* were isolated by plating on selective medium (aerobic LB and anaerobic NBFA) followed by picking up single colonies to grow in liquid cultures and then stored in 10% DMSO for *G. sulfurreducens* and 15% glycerol for *S. oneidensis*. Further co-culture growth experiments for analysis of evolved strains were started with their respective isolated freezer stocks and following the previously described propagation and growth assay methods.

Results and Discussion

Laboratory evolution of commensal co-culture

Commensal co-cultures of *S. oneidensis* and *G. sulfurreducens* were initiated and laboratory evolution was performed through serial transfers into fresh new medium during stationary phase for a total of 17 transfers (Fig 4.2). After five transfers biomass yield started to increase, and the yield increase plateaued at sixteenth transfer. On average, ancestral co-cultures had a final optical density at OD_{600} of 0.66 and evolved co-cultures had a final optical density of 0.85.

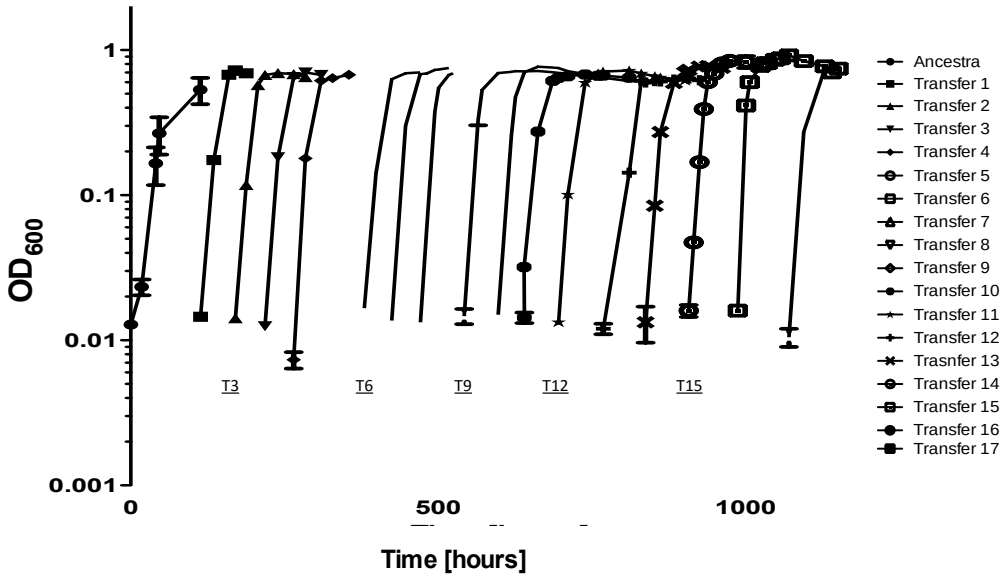

Figure 3.2. Growth rate and final biomass yield of *S. oneidensis*-*G. sulfurreducens* co-culture as measured by optical density (OD_{600}) during laboratory evolution by serial transfers.

Flip in species ratio during evolution

As engineered, co-cultures start in a 1:2 ratios (1 G. *sulfurreducens*: 2 *S. oneidensis*) and this ratio is maintained over the entirety of the growth experiment (Figure 3.1A and 3.3A). In order to monitor individual growth of *S. oneidensis* pGUT2 and *G. sulfurreducens* in the midst of

evolution, colony-forming-units in selective medium were determined during growth of twelfth transfer (Fig 3.2B). The results showed an increase in growth rate for both evolved partners compared to partners in ancestral co-cultures (Fig. 3.2). In addition, the results showed that laboratory evolution lead to a flip in species ratio, where *S. oneidensis* pGUT2 had higher growth rate and higher yield than during ancestral co-cultures (Figure 3.3A and B).

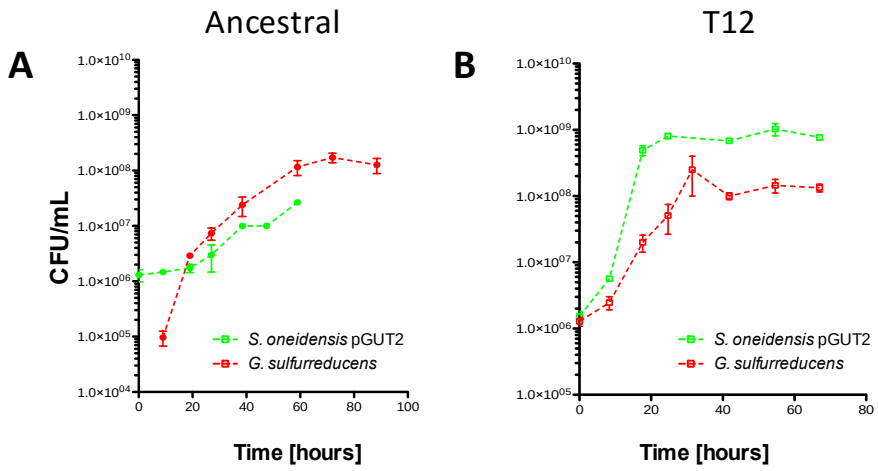

Figure 3.3. Growth rate and final biomass yield as measured by colony-forming-units of individual members of the *S. oneidensis* pGUT2-*G. sulfurreducens* commensal co-culture. **A)** shows growth of ancestral co-cultures, and **B)** shows growth assayed during transfer #12 of the evolution experiment evolved co-cultures.

Growth in monoculture of evolved strains compared to ancestral strains

Mono-cultures of ancestral and evolved *S. oneidensis* pGUT2 strains were assayed for growth under co-culture medium containing glycerol as carbon source and fumarate as terminal electron acceptor (NBFG) (Figure 3.4A). Growth of *G. sulfurreducens* ancestral and evolved strains were compared in minimal medium with acetate as carbon source and fumarate as terminal electron acceptor (NBFA) (Figure 3.4B). *S. oneidensis* pGUT2 evolved strains showed an improved phenotype of higher final biomass yield, whereas *G. sulfurreducens* evolved strain showed no difference in growth rate or biomass yield as compared to WT.

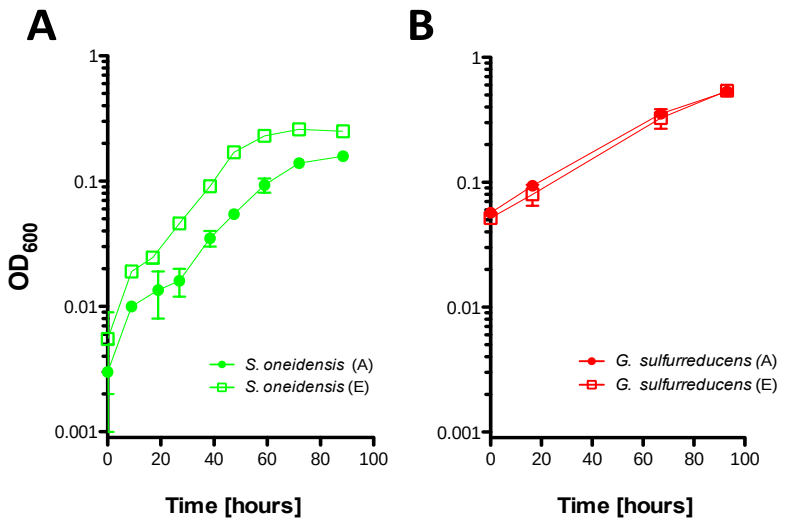

Figure 3.4. Growth in mono-culture measured by optical absorbance for **A)** *S. oneidensis* pGUT2 ancestral and evolved strains (10mM glycerol and 60mM Fumarate) and for **B)** *G. sulfurreducens* ancestral and evolved strains (20mM acetate and 40mM fumarate).

Evolved *S. oneidensis* pGUT2 responsible for increased growth rate and yield of evolved co-culture

In order to analyze the contribution of each evolved partner, partnerships were constructed between ancestral *S. oneidensis* pGUT2-*G. sulfurreducens* (A:A) and between isolated *S. oneidensis*-*G. sulfurreducens* evolved strains (E:E) in the following combinations: S_E:G_E, and S_A:G_A. Co-cultures constructed with both evolved strains (S_E:G_E) showed higher growth rates and higher yields than co-cultures constructed with ancestral strains (S_A:G_A). The individual growth of all members of the community (S_E, G_E, S_A, G_A) were assayed as CFU in selective medium (Figure 3.3B and C). Growth phenotype of evolved *G. sulfurreducens* grown in co-culture showed similar to growth of ancestral *G. sulfurreducens*. For *S. oneidensis* growth of evolved strain S_E showed higher yield than its ancestral counterpart S_A.

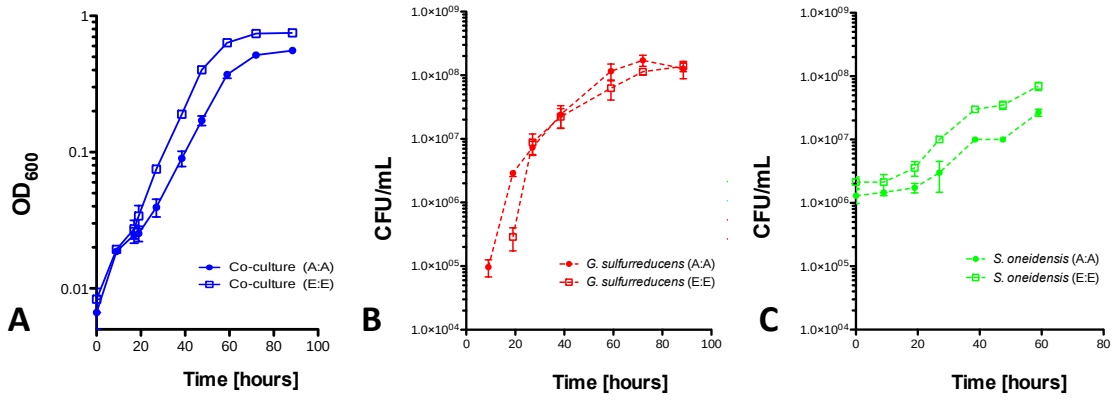

Figure 3.5. A) Growth rate and final biomass yield of co-cultures built with ancestral *S. oneidensis* and ancestral *G. sulfurreducens* (A:A) or co-culture built with evolved *S. oneidensis* and evolved *G. sulfurreducens* (E:E). Growth measured by colony-forming-units of **B)** ancestral and evolved *G. sulfurreducens* and **C)** ancestral and evolved *S. oneidensis* pGUT2.

Conclusion

In conclusion, using experimental evolution of a model microbial commensalism, we were able to demonstrate an evolutionary response where the co-dependent partner, *G. sulfurreducens* did not showed a phenotype of adaptations to the co-culture conditions, whereas the independent partner, *S. oneidensis* pGUT2 showed adaptations to the culturing medium, but not to the presence of its respiratory partner. Examination of the evolutionary process of an obligate interaction, such as cooperation or competition, between these two organisms will help to broaden our knowledge on behavior and interactions of *S. oneidensis* and *G. sulfurreducens* in community.

References

Ansaldi, M. et al., 2007. Aerobic TMAO respiration in *Escherichia coli*. *Molecular Microbiology*, 66(2), pp.484–494.

Bader, J. et al., 2010. Relevance of microbial coculture fermentations in biotechnology. *Journal of Applied Microbiology*, 109(2), pp.371–387.

Baraquet, C. et al., 2006. TorT, a member of a new periplasmic binding protein family, triggers induction of the Tor respiratory system upon trimethylamine *N*-oxide electron-acceptor binding in *Escherichia coli*. *Journal of Biological Chemistry*, 281(50), pp.38189–38199.

Bordi, C. et al., 2003. Effects of ISSo 2 Insertions in structural and regulatory genes of the trimethylamine oxide reductase of *Shewanella oneidensis*., *Journal of Bacteriology*, 185(6), pp.2042–2045.

Bordi, C. et al., 2004. Genes regulated by TorR , the trimethylamine oxide response regulator of *Shewanella oneidensis* genes regulated by TorR,the trimethylamine oxide response regulator of *Shewanella oneidensis*, *Journal of Bacteriology*, 186(14), pp.4502–4509.

Brenner, K., You, L. & Arnold, F.H., 2008. Engineering microbial consortia: a new frontier in synthetic biology. *Trends in Biotechnology*, 26(9), pp.483–489.

Bretschger, O. et al., 2007. Current production and metal oxide reduction by *Shewanella oneidensis* MR-1 wild type and mutants. *Applied and Environmental Microbiology*, 73(21), pp.7003–7012.

Breuer, M. et al., 2015. Multi-haem cytochromes in *Shewanella oneidensis* MR-1: structures, functions, and opportunities. *Journal of Royal Society, Interface*, 12: 20141117.

Brutinel, E.D. & Gralnick, J.A., 2012a. Anomalies of the anaerobic tricarboxylic acid cycle in *Shewanella oneidensis* revealed by Tn-seq. *Molecular Microbiology*, 86(2), pp.273–283.

Brutinel, E.D. & Gralnick, J.A., 2012b. Shuttling happens: Soluble flavin mediators of extracellular electron transfer in *Shewanella*. *Applied Microbiology and Biotechnology*, 93(1), pp.41–48.

Von Canstein, H. et al., 2008. Secretion of flavins by *Shewanella* species and their role in extracellular electron transfer. *Applied and Environmental Microbiology*, 74(3), pp.615–623.

Carpentier, W. et al., 2003. Microbial reduction and precipitation of vanadium by *Shewanella oneidensis*. *Applied and Environmental Microbiology*, 69(6), pp.3636–3639.

Chen, Y. & Wang, F., 2015. Insights on nitrate respiration by *Shewanella*. *Frontiers in Marine Science*, 1(80), pp.1–9.

Doebeli, M., 2002. A model for the evolutionary dynamics of cross-feeding polymorphisms. *Population Ecology*, 44, pp.59–70.

Dunham, M.J., 2007. Synthetic ecology: a model system for cooperation. *Proceedings of the National Academy of Sciences of the United States of America*, 104(6), pp.1741–1742.

Escalante, A.E. et al., 2015. Ecological perspectives on synthetic biology: Insights from microbial population biology. *Frontiers in Microbiology*, 6(FEB), pp.1–10.

Estrela, S., Trisos, C.H. & Brown, S.P., 2012. From metabolism to ecology: cross-feeding interactions shape the balance between polymicrobial conflict and mutualism. *The American Naturalist*, 180(5), pp.566–76.

Feng, X. et al., 2012. Integrating flux balance analysis into kinetic models to decipher the dynamic metabolism of *Shewanella oneidensis* MR-1. *PLoS Computational Biology*, 8(2), pp. 1-11.

Flynn, C.M. et al., 2012a. Construction and elementary mode analysis of a metabolic model for *Shewanella oneidensis* MR-1. *BioSystems*, 107(2), pp.120–128.

Flynn, J.M., Ross, D.E. & Hunt, K. a, 2010. Enabling Unbalanced Fermentations by Using Engineered Electrode. *American Society Microbiology, mBio*, 1(5), pp.1–8.

Fredrickson, J.K. et al., 2008. Towards environmental systems biology of *Shewanella*. *Nature Reviews, Microbiology*, 6(8), pp.592–603.

Fujimoto, N., Kosaka, T. & Yam, M., 2012. Menaquinone as well as ubiquinone as a crucial component in the *Escherichia coli* respiratory chain. *Chemical Biology*, 10, pp.187–208.

Goers, L., Freemont, P. & Polizzi, K.M., 2014. Co-culture systems and technologies: taking synthetic biology to the next level. *Journal of the Royal Society, Interface*, 11(96), pp. 1-13.

Gon, S. et al., 2002. Reconstitution of the trimethylamine oxide reductase regulatory elements of *Shewanella oneidensis* in *Escherichia coli*. *Journal of Bacteriology*, 184(5), pp.1262–1269.

Hansen, S.K. et al., 2007. Evolution of species interactions in a biofilm community. *Nature*, 445(7127), pp.533–536.
Harrington, E.D. et al., 2007. Quantitative assessment of protein function prediction from metagenomics shotgun sequences. *Proceedings of the National Academy of Sciences of the United States of America*, 104(35), pp.13913–13918.
Hau, H.H. & Gralnick, J. a, 2007. Ecology and biotechnology of the genus *Shewanella*. *Annual Review of Microbiology*, 61: pp.237–58.
Hendrickson, J.O.M. and W.A., 2005. An asymmetry-to-symmetry witch in signal transmission by the histidine kinase receptor for TMAO. *Biophysical Chemistry*, 257(5), pp.2432–2437.
Hillesland, K.L. et al., 2014. Erosion of functional independence early in the evolution of a microbial mutualism. *Proceedings of the National Academy of Sciences of the United States of America*, 111(41), pp.14822–14827.
Hillesland, K.L. & Stahl, D. a, 2010. Rapid evolution of stability and productivity at the origin of a microbial mutualism. *Proceedings of the National Academy of Sciences of the United States of America*, 107(5), pp.2124–2129.
Hunt, K.A. et al., 2010. Substrate-level phosphorylation is the primary source of energy conservation during anaerobic respiration of *Shewanella oneidensis* strain MR-1. *Journal of Bacteriology*, 192(13), pp.3345–3351.
Iobbi-Nivol, C. et al., 1996. High substrate specificity and induction characteristics of trimethylamine-*N*-oxide reductase of *Escherichia coli*. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 1294(1), pp.77–82.
Kane, A.L. et al., 2016. Formate metabolism in *Shewanella oneidensis* generates proton motive force and prevents growth without an electron acceptor. *Journal of Bacteriology*, 198(8), pp. 1337-1346.
Knappe, J. & Sawers, G., 1990. A radical-chemical route to acetyl-CoA: the anaerobically induced pyruvate formate-lyase system of *Escherichia coli*. *Federation of European Microbiological Societies, Microbiology Reviews*, 75, pp.383–398.
Koland, JG., Miller, MJ., & Gennis, RB., 1984. Reconstitution of the membrane-bound, ubiquinone-dependent pyruvate oxidase respiratory chain of *Escherichia coli* with the cytochrome d terminal oxidase. *American Chemical Society, Biochemistry*,23(3), pp. 44-53.
Lidbury, I., Murrell, J.C. & Chen, Y., 2014. Trimethylamine *N*-oxide metabolism by abundant marine heterotrophic bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 111(7), pp.2710–5.
Ma, J., Pazos, I.M. & Gai, F., 2014. Microscopic insights into the protein-stabilizing effect of trimethylamine *N*-oxide (TMAO). *Proceedings of the National Academy of Sciences of the United States of America*, 111(23), pp.8476–81.
Mahadevan, R., Palsson, B.Ø. & Lovley, D.R., 2011. In *situ* to in *silico* and back: elucidating the physiology and ecology of *Geobacter* spp. using genome-scale modelling. *Nature Reviews, Microbiology*, 9(1), pp.39–50.
Marx, C.J., 2009. Getting in touch with your friends. *Science*, 324(5931), pp.1150–1151.
McCrindle, S.L., Kappler, U. & McEwan, A.G., 2005. Microbial dimethylsulfoxide and trimethylamine-*N*-oxide respiration. *Advances in Microbial Pgysiology*, 50, pp. 147- 198.
Mee, M.T. et al., 2014. Syntrophic exchange in synthetic microbial communities. *Proceedings of the National Academy of Sciences of the United States of America*, 111(20), pp.E2149-56.
Meshulam-simon, G. et al., 2007. Hydrogen metabolism in *Shewanella oneidensis* MR-1. *Applied and Environmental Microbiology*, 73(4), pp.1153–1165.
Moore, J.O. & Hendrickson, W.A., 2009. Structural analysis of sensor domains from the TMAO-responsive histidine kinase receptor torS. *Cell Press, Structure Article*, 17(9), pp.1195–1204.
Morozkina, E. V & Zvyagilskaya, R.A., 2007. Nitrate reductases: structure, functions, and effect of stress factors. *Biochemistry. Biokhimiia*, 72(10), pp.1151–1160.
Myers, C.R. & Nealson, K.H., 1988. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. *Science (New York, N.Y.)*, 240(4857), pp.1319–1321.
Myers, J.M. & Myers, C.R., 2000. Role of the tetraheme cytochrome CymA in anaerobic electron transport in cells of *Shewanella putrefaciens* MR-1 with normal levels of menaquinone. *Journal of*

Bacteriology, 182(1), pp.67–75.
Nealson, K.H. & Scott, J., 2006. Ecophysiology of the genus *Shewanella*. *The Prokaryotes*, pp.1133–1151.
Nevin, K.P. & Lovley, D.R., 2000. Lack of production of electron-shuttling compounds or solubilization of Fe (III) during reduction of insoluble fe (III) oxide by *Geobacter metallireducens* lack of production of electron-shuttling compounds or solubilization of Fe (III) during reduction. *Applied and Environmental Microbiology*, 66(5), pp.2248–2251.
Raes, J., Harrington, E.D., et al., 2007. Protein function space: viewing the limits or limited by our view? *Current Opinion in Structural Biology*, 17(3), pp.362–369.
Raes, J. & Bork, P., 2008. Molecular eco-systems biology: towards an understanding of community function. *Nature Reviews Microbiology*, 6(9), pp.693–699.
Refojo, P. N., Teixeira, M., & Pereira, M. M. (2012). The alternative complex III: Properties and possible mechanisms for electron transfer and energy conservation. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1817*(10), 1852–1859.
Richter, K., Schicklberger, M. & Gescher, J., 2012. Dissimilatory reduction of extracellular electron acceptors in anaerobic respiration. *Applied and Environmental Microbiology*, 78(4), pp.913–921.
Ringo Stenberg, E., Strom, A.R., E., 1984. Amino acid and lactate catabolism in trimethyl oxide respiration of *Alteromonas putrefaciens* NCMB 1735. *Applied and Environmental Microbiology*, 47(5), pp.1084–1089.
De Roy, K. et al., 2014. Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. *Environmental Microbiology*, 16(6), pp.1472–1481.
Rozen, D.E., Schneider, D. & Lenski, R.E., 2005. Long-term experimental evolution in *Escherichia coli*. XIII. Phylogenetic history of a balanced polymorphism. *Journal of Molecular Evolution*, 61(2), pp.171–180.
Saltikov, C.W. & Newman, D.K., 2003. Genetic identification of a respiratory arsenate reductase. *Proceedings of the National Academy of Sciences of the United States of America*, 100(19), pp.10983–8.
Dos Santos, J.-P. et al., 1998. Molecular analysis of the trimethylamine *N*-oxide (TMAO) reductase respiratory system from a *Shewanella* species. *Journal of Molecular Biology*, 284(2), pp.421–433.
Schoepp-Cothenet, B., et al., 2009. Menaquinone as pool quinone in a purple bacterium, *Proceedings of the National Academy of Sciences of the United States of America*, 106(21), pp. 8549-8554
Schuetz, B. et al., 2009. Periplasmic electron transfer via the c -Type cytochromes MtrA and FccA of *Shewanella oneidensis* MR-1. *Applied and Environmental Microbiology*, 75(24), pp.7789–7796.
Serres, M.H. et al., 2001. A functional update of the *Escherichia coli* K-12 genome. *Genome Biology*, 2(9), pp. 1-7.
Seth, E.C. & Taga, M.E., 2014. Nutrient cross-feeding in the microbial world. *Frontiers in Microbiology*, 5, pp.1–6.
Simon, G. et al., 1995. The torR gene of *Escherichia coli* encodes a response regulator protein involved in the expression of the trimethylamine *n*-oxide reductase genes. *Journal of Bacteriology*, 176(18), pp.5601-5606.
Spero, M.A. et al., 2016. Different functions of phylogenetically distinct bacterial complex I isozymes. *Journal of Bacteriology.* 198(8), pp. 1268-1280.
Strycharz-Glaven, S.M. et al., 2013. Electrochemical investigation of a microbial solar cell reveals a nonphotosynthetic biocathode catalyst. *Applied and Environmental Microbiology*, 79(13), pp.3933–3942.
Summers, Z.M. et al., 2010. Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. *Science (New York, N.Y.)*, 330(6009), pp.1413–5.
Tang, Y.J. et al., 2007. Anaerobic central metabolic pathways in *Shewanella oneidensis* MR-1 reinterpreted in the light of isotopic metabolite labeling. *Journal of Bacteriology*, 189(3), pp.894–901.
Temperton, B. & Giovannoni, S.J., 2012. Metagenomics: Microbial diversity through a scratched lens. *Current Opinion in Microbiology*, 15(5), pp.605–612.
Tringe, S.G. & Rubin, E.M., 2005. Metagenomics: DNA sequencing of environmental samples. *Nature reviews, Genetics*, 6(11), pp.805–14.
VerBerkmoes, N.C. et al., 2009. Systems biology: Functional analysis of natural microbial consortia using

community proteomics. *Nature Reviews, Microbiology*, 7(3), pp.196–205.
Wade, W., 2002. Unculturable bacteria—the uncharacterized organisms that cause oral infections. *Journal of the Royal Society of Medicine*, 95, pp.81-83.
Weber, K.A., Achenbach, L.A. & Coates, J.D., 2006. Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. *Nature Reviews*, 4(3), pp.752–764.
Yancey, P.H. et al., 1982. Living with water stress: evolution of osmolyte systems. *Science (New York, N.Y.)*, 217(4566), pp.1214–1222.
Yancey, P.H., Blake, W.R. & Conley, J., 2002. Unusual organic osmolytes in deep-sea animals: Adaptations to hydrostatic pressure and other perturbants. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, 133(3), pp.667–676.
Zhang, X. & Reed, J.L., 2014. Adaptive evolution of synthetic cooperating communities improves growth performance. *PLoS ONE*, 9(10), pp. 1-12.

Appendix
Growth of *Shewanella oneidensis* in Defined Minimal Media

Introduction

The first described recipe for growth of *S. oneidensis* in minimal media contains 3μg per ml^{-1} of three essential amino acids: L- arginine, L-glutamate and DL-serine (Myers & Nealson 1988). In order to provide for the amino acid requirement to grow MR-1 in laboratory conditions, our current minimal media recipe contains 0.5% of casamino acids. The work presented in this appendix aims to explore the physiology of MR-1 grown as mono-culture and co-culture under anaerobic conditions in minimal media without casamino acids or amended with trace amounts of L- arginine, L-glutamate and DL-serine.

Materials and Methods

For medium recipe and *S. oneidensis* growth condition, refer to chapter 2 of this manuscript. Growth conditions were set up following the Materials and Methods section from chapter 3 of this manuscript, with the except of casamino acid supplementation. When necessary casamino acids where not added, supplemented in 0.5% or only three amino acids L- arginine, L-glutamate and DL-serine where added in 0.01mM. Strains and plasmids used in this study are listed in table 1.

For *G. sulfurreducens* growth conditions, refer to chapter 3 of this manuscript.

Table I. Strains and plasmids used in this study.

Plasmids	Genotype/Characteristic	Reference
pGUT2	pBBR1 MCS-2 with glycerol utilization genes from *E. coli* k-12 *glpD, glpF, glpK, tpiA*	(Flynn et al. 2010)
pGUT2_F	pBBR1 MCS-2 with glycerol utilization genes from *E. coli* k-12 *glpF, glpK, tpiA* and *glpD* from *Glk. subterraneous*	(Joshi et al. 2015)

Strains	Genotype/Characteristic	Reference
JG2067	MR-1, *gfp*::pGUT2	(Flynn et al. 2010)
JG3312	MR-1::pGUT2_F	(Komal et al. 2010)
JG 3440	WT *G. sulfurreducens*, strain PCA	Obtained from Bond Lab

Results and Discussion

Growth of MR-1 in minimal media with or without amino acid supplementation

Growth of MR-1 was examined in minimal media that was amended with 0.5% casamino acids, with L- arginine, L-glutamate and DL-serine, or without amino acids. Growth was compared between *S. oneidensis* strains that contain either pGUT2 or pGUT2_F and in medium supplemented with glycerol as carbon source (10mM or 50mM) and fumarate as terminal electron acceptor. Results show that conditions where medium is amended with 0.5% casamino acids have faster growth rates and higher biomass yields (Figure 1). Results also show that *S. oneidneisis*_pGUT2 and *S. oneidneisis*_pGUT2_F are able to grow in minimal medium without the need of amino acids (Figure 2.B). Amendment with only L- arginine, L-glutamate, and DL-serine show a lower final yield than all the conditions tested and a clear biphasic growth; indicative of a requirement to adapt differently to the combination of the amino acids present (Figure 1.C). In addition, results show a distinctive phenotype for *S. oneidneisis*_pGUT2_F which shows faster growth rate than *S. oneidneisis*_pGUT2 in conditions where 0.5% case amino acids have been removed from the medium (Figure 1).

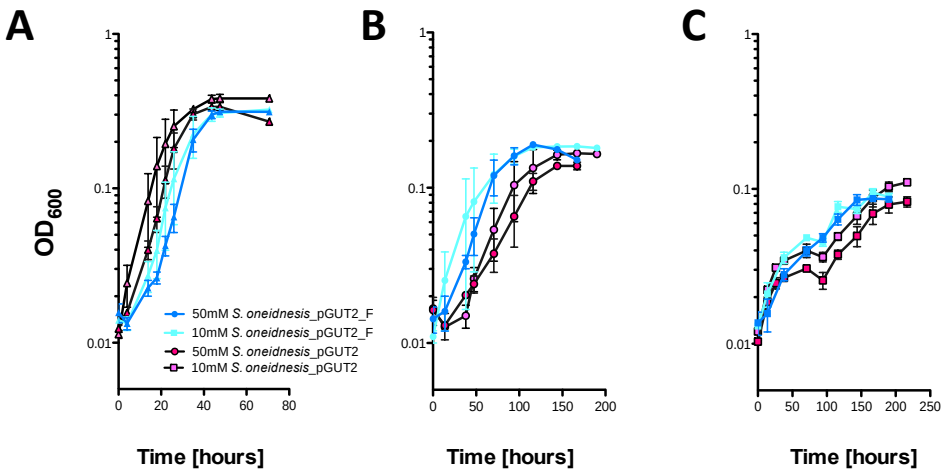

Figure I. Growth curves of *S. oneidneisis*_pGUT2 (pink) and *S. oneidneisis*_pGUT2_F (blue) in minimal medium supplemented with Glycerol as carbon source (10mM or 50mM) and Fumarate (60mM) as terminal electron acceptor. Medium was amended with o.5% casamino acids (A), no amino acids (B), or L- arginine, L-glutamate and DL-serine (C). Reported values are the average of three independent experiments and error bars represent standard error of the mean (SEM).

Serial transfer of *S. oneidensis* in defined minimal medium without amino acids

Adaptation to growth in minimal medium without amino acids was examined by performing serial transfers of *S. oneidneisis*_pGUT2 and *S. oneidneisis*_pGUT2_F. An increase in growth rate was observed during the second transfer and a plateau of optimal levels was reached during the third transfer for *S. oneidneisis*_pGUT2_F, but not for *S. oneidneisis*_pGUT2 (Figure2).

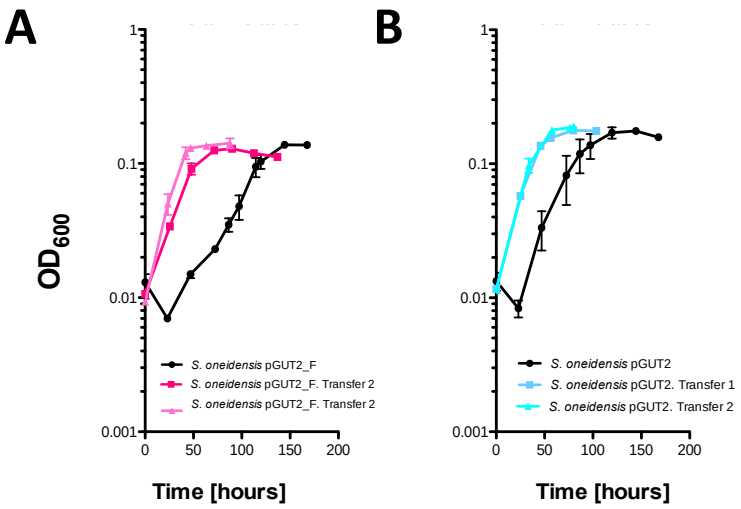

Figure II. Serial transfers of *S. oneidneisis*_pGUT2 (A) and *S. oneidneisis*_pGUT2_F (B) in minimal medium without amino acids and with 50mM Glycerol as carbon source and 60mM fumarate as terminal electron acceptor. Reported values are the average of three independent experiments and error bars represent standard error of the mean (SEM).

*S. oneidneisis*_pGUT2 growth as mono-culture and co-culture in defined minimal medium

Growth of *S. oneidneisis*_pGUT2 was examined in mono-culture and in *S. oneidneisis*_pGUT2 co-culture with *G. sulfurreducens* under anaerobic conditions with 10mM glycerol as carbon and 60mM fumarate as terminal electron acceptor and amended with 0.5% cas-amino acids, no amino acids, or L-glutamate (0.2mM or 0.4mM). Results show that 0.4mM has a benefit for co-culture growth form all the conditions tested, but not during mono-culture.

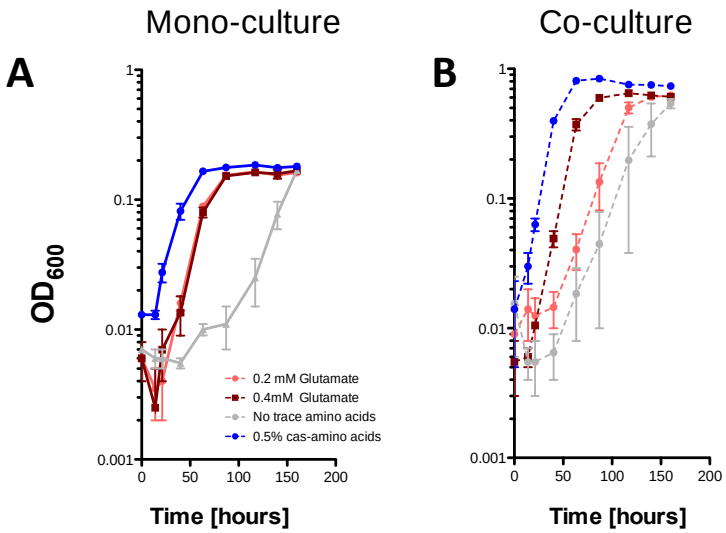

Figure III. *S. oneidneisis*_pGUT2 mono-culture A) and *S. oneidneisis*_pGUT2 co-culture with *G. sulfurreducens* B) in minimal medium with 10mM glycerol as carbon and 60mM fumarate as terminal electron acceptor. Medium was amended with 0.5% casamino acids, no amino acids, or L-glutamate (0.2mM or 0.4mM).

Conclusion

Growth in conditions were minimal medium is amended with 0.5% casamino acids show higher growth rate for *S. oneidneisis*_pGUT2 and *S. oneidneisis*_pGUT2_F. Incorporation of only L-arginine, L-glutamate and DL-serine to provide for the amino acid requirement described in Myers & Nealson 1988 induces a biphasic growth which decreases overall growth rate and final biomass yield for *S. oneidneisis*_pGUT2 and *S. oneidneisis*_pGUT2_F. Moreover, *S. oneidneisis*_pGUT2 and *S. oneidneisis*_pGUT2 are able to grow as mono-culture and *S. oneidneisis*_pGUT2 as co-culture with *G. sulfurreducens* in minimal medium without the need to amend with amino acids. Furthermore, Different L-glutamate concentrations have a phenotypic effect for growth in mono-culture but not for growth in co-culture, indicative of *G. sulfurreducens* utilization of L-glutamate present in the medium.