Extracellular Electron Transport in *Shewanella*

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Preface

I originally began on this report with a broad focus on methods of uranium reduction. While uranium is toxic on account of its metallic properties, its radioactivity in both the common ^{285}U and highly active ^{235}U forms pose longer lived, potentially ecologically catastrophic risks if the metal is allowed to become present in high concentrations (Wall and Krumholz (2006)). While the metallic properties can be countered through chemistry, radioactivity can not. In sites with high concentrations of anthropogenically generated uranium waste, the aim has thus become fixation of the contamination by creation of an insoluble form of the metal pending physical removal to reduce the likelihood of contamination of water sources. Reading reviews on the topic (You et al. (2021)), I quickly came to realize that the focus within this domain is not on the biochemistry and genetics of this process, rather on the suitability of specific taxa capable of heavy metal reduction to the task. As I began to read about these taxa, one stood out. It is ubiquitous in aquatic environments, resistant to toxins and radiation, and intriguingly seems to engage in every time of electron transfer discussed, from reduction on the cytosolic membrane to through nanowire structures. Now, I am enamored with *Shewanella* and their uniquely robust extracellular electron transport system. This report is a tribute to the beauty of the taxa's utilization of diverse terminal electron acceptors and the dedication of scientists, in particular Charles and Judith Myers, at the Medical College of Wisconsin, Daad Saffarini, at the University of Wisconsin Milwaukee, Marcus Edwards at University of East Anglia, and Alex Belieav at the Pacific Northwest National Laboratory (among the others cited multiple times in the **Works Cited)** in their quest to determine the elements of this pathway and their interactions. Research for this report has given me a deep appreciation for their dedication and inventiveness to unraveling the black box of metabolism.

Shewanella

This report will focus broadly on *Shewanella*, a genus of gram negative, aerobic, γ-proteobacteria comprising approximately 70 species (Lemaire et al. (2020)). *Shewanella* taxa are spread over a large portion of the surface of the Earth and occupy a wide range of ecosystems, though a few characteristics are fairly universal. Nearly all *Shewanella* are aquatic, though their environments may take on many extremes, from hypersaline to fresh, from freezing to tropical, from the surface to its depths. Members of the taxa are known for their resistance to and propensity to degrade toxins, a reflection of their broader metabolic diversity. Analysis of the evolutionary history of the taxa in Zhong et al (2018). has identified *S. loihica* PV-4, a resident of microbial mats associated with hydrothermal vents (Gao et al. 2006), as the closest relative to their common ancestor, revealing their robustness as a product of their ecology. Another common attribute of *Shewanella* is their engagement in symbiotic interactions, almost always leveraging their adaptations to unfavorable conditions to protect a host in exchange for diffused nutrients and organic matter as well as a favorable niche.

For the purposes of this report, most discussion will be of *Shewanella* overall because of how widespread the discussed systems are. However, several strains will be referenced specifically. Among them, *S. oneidensis* MR-1, is notable, not for any physiological characteristic, instead its use as a model organism for the study of metal reduction and *Shewanella*, though members of the genus are generally genetically tractable. It is a freshwater strain, originally isolated from the Oneida freshwater lake in New York, separating it from other *Shewanella*, though this characteristic is infrequently relevant.

A defining element of their physiology, enabling many of the aforementioned metabolic and ecological phenomena, is their ability to engage in extracellular electron transfer in

oxygen-limited environments. *S. oneidensis* MR-1 has been shown to lack expression of the type A oxidase despite its presence in the genome (Le Laz et al. 2016). In spite of this, few *Shewanella* engage in fermentation. This has resulted in *Shewanella* generally residing in redox-stratified waters, or generating low-oxygen microenvironments through aggregation. To support their metabolism in lieu of use of oxygen, *Shewanella* utilize a wide range of terminal electron acceptors, including Fe(III), Mn(IV), many heavy metals, TMAO, DMSO, and nitrogen and sulfur cycle products. Beyond having a diversity of possible electron acceptors, the genus is capable of reduction beyond the surface of the cell.

Motivation

These properties have made *Shewanella* a taxa of interest for applications in bioelectrical production and bioremediation of metals. Application of the genus to these tasks requires, beyond an environmental engineering framework for their utilization, thorough understanding of their extracellular electron transfer system. Without a deep knowledge of the components of this system, their actions and interactions, and their response to regulation by *Shewanella*, successful application will be hindered. With this motivation, throughout the remainder of this report, we will seek to answer questions on this process, namely;

1. By what mechanism is *Shewanella* capable of extracellular electron transfer?
2. How were the elements of this mechanism characterized?
3. What accounts for the robustness of *Shewanella* reduction?
4. How does *Shewanella* genomic structure support electron transfer?

To complete this review, I will extensively reference the primary literature that originally characterized the function of the pathway and the genes and gene products associated with its activity. Through this, disagreements on findings will be addressed where applicable and

development of results demonstrated. That being said, the progression of this review will be linear through the system as opposed to chronological for biological clarity. The review will move from a discussion of metabolism to a description of the mechanism for storage of electrons in and transport through the cytoplasmic membrane to methods of transport through the cytoplasm to the Mtr complex and extracellular electron shuttles. When otherwise not cited, facts come from a comprehensive review from Beblawy et al. (2018) focused on the pathway by which members of the genus reduce solid electron acceptors.

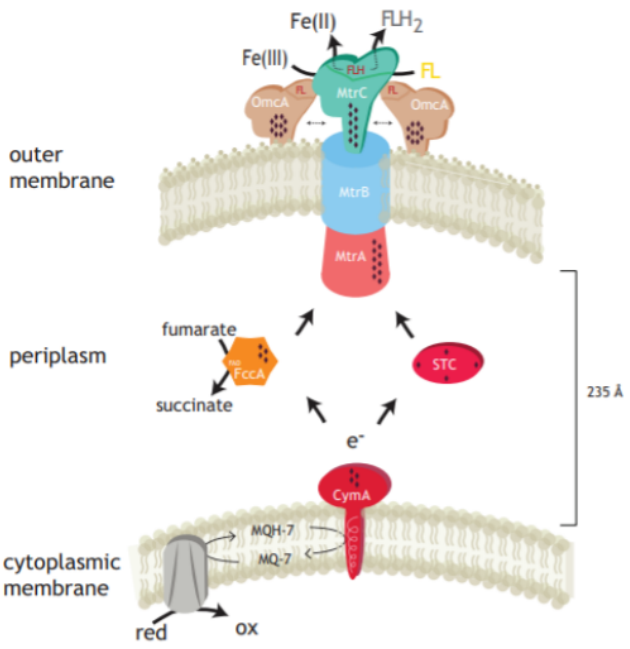

Figure 1: Overview of *Shewanella* extracellular electron transfer system, encompassing the portion of the mechanism from the cytoplasmic membrane onward, from Beblawy et al. (2018)

Electron Transport into the Cytoplasmic Membrane

Metabolic Capacity

Shewanella primarily absorb lactate, pyruvate, and N-acetyl-glucosamine under anaerobic conditions as well as a wider set of organic compounds, including amino acids, in oxic conditions. Under neither condition, however, does *S. oneidensis* MR-1 utilize glucose. However, a selection study completed by Howard et al. (2012) demonstrated that the strain could quickly become capable of some glucose catabolism by removal of regulation of the N-acetyl-glucosamine kinase and permease, both with some activity toward glucose. Under anaerobic conditions, *Shewanella* does not utilize glycolysis, instead the EntnerDoudoroff pathway for N-acetyl-glucosamine metabolism. This pathway does not result in oxidative

phosphorylation, resulting in a lower energetic yield, though the pathway itself is thermodynamically more favorable.

Additionally, while the TCA cycle is downregulated in *Shewanella* under anaerobic conditions, Tang et al. (2007) were able to identify activity across all branches of the path under anaerobic conditions, an uncommon characteristic. They also found notable catabolism of provided serine and glycine, demonstrating the broad range of organic compounds that could be used by the genus under varying redox conditions to support growth. Catabolism of carbon-based compounds required oxidation to generate energy, reducing NADH and NADPH in the process. The build-up of reduced dinucleotides is unsustainable, as cycling is required to ensure the presence of electron acceptors for continued function. Thus, even in the absence of oxidative phosphorylation under anaerobic conditions, excess electrons must be transferred out of the cytoplasm.

Menaquinone-7

Quinones are a class of cyclic organic compounds with two carbonyl groups and a lipophilic tail, used for sequestration of the compound within membranes (McMillan et al 2012). Reduction of quinones converts the carbonyl groups to carboxyls, meaning that a quinone can hold a maximum of two electrons. Quinone oxidoreductases, specifically Nrq1, catalyzes the oxidation of NADH and NADPH generated during carbon cycling, converting quinones to hydroquinones. Menaquinone-7 (MQ-7) is hypothesized to be the most important compound of this class in *Shewanella*. In Saffaraini et al (2002), *Shewanella putrefaciens* mutants with Tn-5 insertions in the *menB* and *menD* genes were shown to have significantly decreased capacity to reduce Fe(III), shown to have high homology to genes found in E. coli associated with MQ-7 biosynthesis. After the addition of MQ-7 function was restored. A study of the activity of

alternative quinones present in *Shewanella oneidensis* (McMillian et al. 2012) demonstrated that, only in the presence of MQ-7, other electron donors could be utilized to supply electrons for periplasmic and extracellular reduction. Structural analysis of the CymA protein discovered the presence of two sites, a tight-binding site occupied by MQ-7, and a low-affinity site where other quinones, generally those expressed under aerobic conditions, could bind, providing evidence for the function of MQ-7 as a cofactor for CymA.

CymA

CymA is a c-type tetraheme cytochrome bound to the outside of the cytoplasmic membrane. In Myers and Myers (1997), a TnphoA insertion resulting in a *Shewanella putrefaciens* MR-1 deficient in metal, nitrate, and fumarate reduction determined the location of a gene coding for a protein with partial amino acid homology to NapC, CymA. During separation of cellular components, this protein was found in both the periplasm and cytoplasmic membrane, though subcellular fractionation demonstrated a higher likelihood of membrane binding as the usual state albeit with low affinity, similar to other similarly located proteins. The separated component also showed high NADH oxidation capability, consistent with c-cytochrome activity, further corroborated by heme staining paired with SDS-PAGE. This study concluded that expression of CymA occurred under the same conditions as MQ-7 as compared to other quinones, supporting their association.

In Schwalb, Chapman, and Reid (2003), a soluble mutant of CymA missing the N-terminal membrane anchor was shown to bind directly to the fumarate reductase, a soluble enzyme in *Shewanella* as opposed to membrane-bound as in many other bacteria, and to be required for reduction of nitrite and DMSO. Knowing that these compounds have well-characterized terminal reductases, namely Nir and the DMS complex, the importance of

CymA across many electron transfer pathways and thus its associated promiscuity were demonstrated.

Periplasmic Transport

Many of the papers referenced in this report focused on CymA hypothesize on direct electron transfer between CymA and MtrA. However, in Dohnalkova et al. (2011), direct imaging of *S. oneidensis* MR-1 reported a periplasmic thickness of 235 Å, 25% thicker than originally believed. This greater distance demonstrated the low likelihood of direct interaction between proteins localized to the inner and outer membranes. Six putative soluble c-cytochromes were identified through proteomic analysis in Meyer et al. (2004), out of the 42 total identified cytochromes in *S. oneidensis* MR-1. They include the ScyA Monoheme c5, FccA, BCCP, Small Tetraheme C, Monoheme C, and Diheme c4, though ScyA, FccA, and STC were found to be the most abundant in the periplasm. ScyA is known to transfer electrons to the cytochrome peroxidase CcpA, FccA has dual functions in fumarate reduction and in electron storage for reduction, and STC is a relatively nonspecific electron carrier between CymA and a wide set of terminal oxidoreductases. However, none of these proteins alone are necessary for iron reduction per the results of knock-outs conducted in Schuetz et al. (2009). This supports the hypothesis that periplasmic transport between CymA and the Mtr complex is highly robust. Sturm et al. (2015) found that *stc* and *fccA* single knockouts had

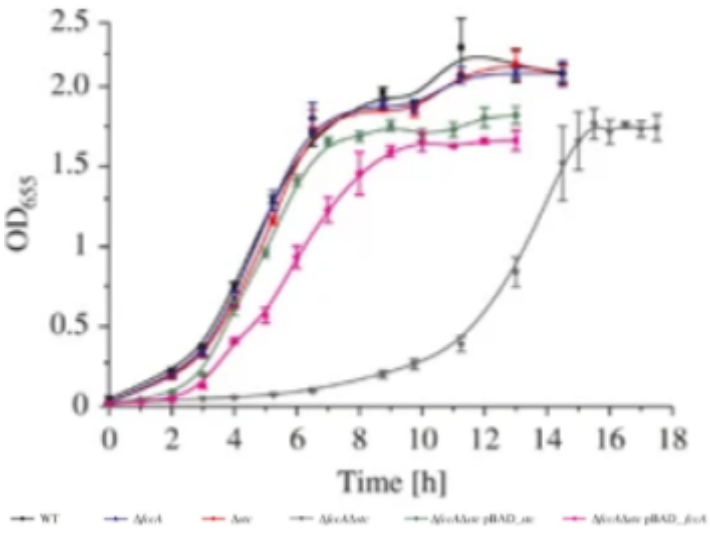

Figure 2: Growth, measured by optical density, for the wild type, stc, fccA, and double knockout mutants as well as back-complemented strains, demonstrating high robustness to single knockouts and decreased growth in the double knockout, from Sturm et al. (2015)

slightly lower growth than wildtype, and a double knockout for those genes had significantly lower growth initially, though was able to grow to the same density as the single knockouts after a longer period of time, with a similar maximum rate of growth. This supports the hypothesis that STC and FccA act in parallel to shuttle electrons across the periplasm and in combination are the primary proteins involved in this process, but that in their absence promiscuous behavior of CymA and MtrA allows for other redox-active compounds, including other c-cytochromes found in the periplasm, to continue the chain.

The Mtr System

The evolutionary origins of the Mtr system are questionable. While it has been studied particularly in *Shewanella* since the discovery of reduction of and respiration enabled solely by manganese for *S. oneidensis* MR-1 in Nealson and Myers (1988), a recent study of comparative microbial genetics found the Mtr system in phylogenetically distant taxa, though highly varied pathways for transfer of electrons into the periplasm, as well as cases in which the orientation of the complex on the membrane is flipped to instead allow for extracellular oxidation. The case for horizontal transfer is supported by the existence of conserved passenger genes, unassociated with the cluster but found close on the chromosome. Most taxa with the complex also include an additional copy of the *mtrC* gene, *mtrF*.

Discovery of the relative structure of the Mtr complex was first achieved through a protein cross-linking study conducted by Ross et al. (2007). Here, MtrC/A/B and OmcA were characterized individually, followed by development of polyclonal antibodies for accurate tagging via exposure to rabbit immune cells. *S. oneidensis* MR-1 in culture were then treated with 1% formaldehyde for between 0 and 120 minutes. Addition of formaldehyde results in the conversion of amine groups to Schiff bases, capable of spontaneous bonding, though this

bonding is reversible when heat is applied. By targeting cross-linked complexes containing the associated proteins utilizing the generated antibodies followed by boiling, the authors were able to accurately characterize direct binding between proteins. Using SDS-PAGE and heme staining on the protein product, they definitely proved that MtrC/A/B were closely associated, forming a complex in a 1:1:1 stoichiometric ratio. This finding was particularly important, as other papers had theorized that MtrA was loosely associated with the inner face of the outer membrane, and may have been capable of direct association with CymA during dissociation. Purification followed by reduction of the MtrC/A/B complex demonstrated high ability to oxidize ferric citrate in solution, confirming independent terminal reductase capabilities of the complex. MtrC and OmcA were also shown to associate, albeit much less strongly than the MtrCAB complex.

X-ray crystallography of the MtrCAB complex, as expressed in *Shewanella baltica* by Edwards et al. (2020) has provided deeper insight into the function of the complex and confirmation of the relationship between its components (to be described individually in the coming sections). They showed the presence of a decaheme cytochrome with linearly connected heme centers, MtrA, oriented from the interior of a transmembrane porin, MtrB, out toward the periplasm on one end and the exterior of the cell, where it was found to be bound to a decaheme cytochrome with centers arranged over a wider surface area, MtrC (already characterized). Based on the highly connected electrical structure of the complex, the authors theorized that the complex was capable of electron transfer 10 to 100 times quicker than had been measured

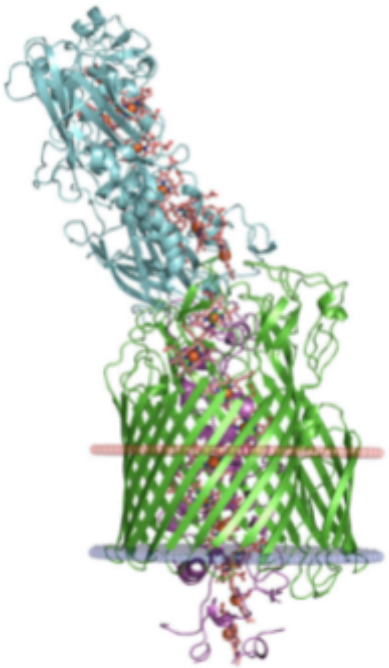

Figure 3: Crystal structure of MtrCAB complex (from Edwards et al. (2020))

experimentally.

MtrA

MtrA is a decaheme c-cytochrome, fixed to the inner portion of the outer membrane through its association with MtrB. Beliaev et al. (2001) utilized knockouts in *S. putrefaciens* to determine that reduction of Fe(III) and Mn(IV) was significantly decreased in its absence. Fractionalization showed localization of the protein to the periplasm. They theorized that it was engaged in transport from the periplasm to the complex embedded in the outer membrane. Surrogate genetics in e. coli completed in Pitts et al. (2003) demonstrated independent c cytochrome activity of the protein when coexpressed with native cytochrome c maturation genes, resulting in transfer of electrons from the electron transport chain to host oxidoreductases and iron.

Interestingly, many papers other from the early 2000's suggested that MtrA was localized to the periplasm. Analysis of its location through fractionalization generally yielded bands in both the periplasm and outer membrane, with the assumption being that transient interaction between the protein and complexes embedded in the membrane resulted in this discrepancy. It was not until Ross et al. (2007) that its association with MtrB and C was definitely demonstrated. Since then, experiments on its relationship with MtrB (see the next section) and X-ray crystallography including the Edwards et al. (2020) study have confirmed the importance of MtrA's association with the rest of the complex.

MtrB

MtrB is a transmembrane porin, characterized by a prominent beta barrel motif. In a transposon mutagenesis study conducted by Beliaev and Saffarini. (1998) in *S. putrefaciens*, one knockout performed results in loss of Fe(II) and Mn(IV) reduction, without effect on other

compounds known to be reduced in the periplasm. Analysis of the insertion revealed its presence within an open reading frame coding for a protein with a sequence typical of secreted proteins, and analysis of isolated cell fractions demonstrated presence primarily in the outer membrane. However, they did not predict its specific function, though they identified *mtrA* as a gene coding for a putative decaheme cytochrome in close proximity.

Myers and Myers (2002) studied the localization of OmcA and MtrC (here called OmcB), in a directed *mtrB* knockout strain of *S. putrefaciens*. They found significantly decreased concentrations of cytochromes generally in the outer membrane fraction, and mislocalization of OmcA and MtrC to the cytoplasmic membrane and within the soluble fraction. The authors remained unsure the exact role of the protein, believing it might have been a chaperone, but were clear that it was essential for proper cytochrome localization.

Spectral characterization of MtrB conducted in Hartshone et al. (2009) demonstrated with high specificity that the protein had a beta-barrel structure. Further, with a set of knockout strains for components of the complex, they demonstrated that a MtrAB complex could stabally form, whereas MtrCB was unstable. Further, they used phylogeny to demonstrate higher levels of conservation for *mtrA* and *mtrB* than for other genes associated with the complex. This resulted in the hypothesis that MtrA associates strongly with MtrB and passes electrons to MtrC on the exterior of the membrane through it.

In Schicklberger et al. (2011), surrogate genetics of the system in E. coli was used to investigate the relationship between MtrA and B. They found little expression of the MtrB in the absence of MtrA under normal conditions, though they noticed significant levels of transcription. This phenomena could be countered by knockout of *degP*, coding for a protease, with the associated *degP/mtrA* double knock-out properly localizing MtrB to the outer membrane. From

these results, the authors theorized that MtrA acts as a scaffold for MtrB folding when in the periplasm, followed by insertion of the complex into the outer membrane.

MtrC

MtrC is a decaheme c-cytochrome fixed to the outer membrane through its association with MtrB. A transposon mutagenesis study conducted by Belieav et al. (2001) generated a library of mutants of *S. putrefaciens* MR-1 varying in their capability to express MtrC/A/B. They found that the purified total membrane fractions of strains with a *mtrC* knockout had approximately ⅓ the rate of Fe(III) reduction as compared to mutants lacking some combination of MtrA and B alone. Furthermore, they localized MtrC to the outer membrane. With this information and putative sequence analysis suggesting that the *mtrC* gene encodes a decaheme c cytochrome, the authors postulated that the MtrC protein was likely a component of a terminal reductase.

Characterization of MtrC completed in Hartshorne et al. (2007) with EPR spectroscopy and voltammetry demonstrate redox capability over a large range of voltages, from 100 to -500 mV. They found that this range was minimally impacted by engagement with a solid mineral surface, as would be expected in reduction of insoluble salts. Furthermore, they found the range for purified MtrC very closely matched that for whole cells, meaning that MtrC is likely the primary terminal reductase engaging with solid substrates.

In Edwards et al. (2015), X-ray crystallography was utilized to determine the physical structure of MtrC. Their analysis returned a nonlinear path between heme centers, increasing the surface area over which compounds could be reduced, indicative of a broad-range terminal reductase. The authors also characterized binding between flavins and the complex associated with reduction, to be discussed later in the **Flavins** section of this report.

OmcA

OmcA is a decaheme c cytochrome. After identification of the protein in 1997, Myers and Myers (2001) utilized a knock-out mutant of *Shewanella putrefaciens* MR-1 to determine that, in its absence, reduction of Mn(IV) was decreased by 45%, significant but lower than the 75% decrease associated with the knockout of MtrC (again called OmcB in this relatively early paper). They also found that, in the absence of MtrC, OmcA localized to the cytoplasmic membranes and soluble fractions as opposed to the outer membrane, where it had been characterized to be found in the wild type. This suggested that both cytochromes directly engaged in terminal reduction of Mn(IV), though MtrC likely did so with a higher affinity, and that the two discussed proteins were associated on the outer membrane, with MtrC likely binding before OmcA. In a similar study conducted by Belchik et al. (2011), a related phenomena was found with respect to Cr(VI), where a mutant lacking *mtrC* had a 44% decrease in reduction while a *omcA*-deficient mutant had a 53% decrease, suggesting that OmcA was the dominant terminal reductase. In fact, since the Myers and Myers (2001) study, the terminal reductase associated with reduction of many compounds and metals have been elucidated (see Table 1), with OmcA engaging in similar levels to MtrC in many cases. These results are not surprising – OmcA shares a very high structural similarity to MtrC (Edwards et al. (2011)).

Table 1: The terminal reductases associated with many *Shewanella* extracellular electron acceptors and the original study in which this relationship was characterized. Adapted from Beblawy et al. (2018)

Extracellular electron acceptor	Main terminal reductase	Study
DMSO	DmsA/B	Gralnick et al. (2006)
Fe(III) minerals	MtrC	Coursolle and Gralnick (2010)
Soluble Fe(III) complexes	MtrC	Coursolle and Gralnick (2010)
Mn(IV) minerals	MtrC, OmcA, MtrF	Bretschger et al. (2007); Gao, Barua et al. (2010)
Soluble Co(III) complexes (in presence of $MgSO_4$)	MtrC, OmcA	Hau et al. (2008)
U(VI)	MtrC, OmcA	Marshall et al. (2006)

Cr(VI)	OmcA, MtrC	Gao, Barua et al. (2010); Belchik et al. (2011)
V(V)	MtrC, OmcA	Myers et al. (2004)
Tc(VII)	MtrC, OmcA, HyaB	Marshall et al. (2007)
Tellurite	MtrC, OmcA	Kim et al. (2012)
AQDS	MtrC	Lies et al. (2005)
Flavins	MtrC	Marsili et al. (2008); Coursolle et al. (2010)
Electrodes Graphite, glassy carbon, graphene oxide	MtrC, OmcA	Bretschger et al. (2007); Coursolle et al. (2010); Jiao et al. (2011)

Extracellular Reduction

Flavins

Marsili et al. (2008) sought out to characterize the previously known mechanism by which *Shewanella* are capable of reduction to metals 50 μm from their cell surface. First, they cultured *S. oneidensis* MR-1 on an electrode until a stable current was found, followed by replacement of media. Immediately after, reduction dropped by 73%, a much larger effect than generally found in other taxa of metal reducers, though over 72 hours the current was restored. Secondary mass spectroscopy analysis found that, between new and old media, there was a significant increase in concentration of riboflavin, though at early time points FMN accounted for approximately half of present flavins. Addition of riboflavin to sterile electrodes and fresh growth medium, similar electrical properties were observed to the cultured case. Furthermore, they characterized riboflavin adsorption to electrode surfaces as well as to substrates containing Fe(III) and Mn(IV). This study provided strong evidence that flavins are essential to extracellular reduction, with riboflavin putatively being the dominant molecule in this class.

von Canstein et al. (2008) conducted a similar study in parallel, using reduction of azo dye applied to the fractionalized product of cultured *S. oneidensis* MR-1. They identified flavins as the dominant reducing agents in the redox active fraction, and FMN and riboflavin in particular using mass spectroscopy. They then characterized this across 11 *Shewanella* strains,

and compared to *Pseudomonas* and *Escherichia*, incapable of reduction of the dye. To demonstrate that these flavins were specifically responsible for Fe(III) reduction at a distance from the cell surface, they suspended cultured cells in a bicarbonate buffer with lactate as an electron donor and crystalline Fe(III) oxide and citrate as an acceptor, with and without added flavins, and found 18 times the rate of reduction in the presence of flavins.

Coursolle et al. (2010) characterized the impact of knockouts of Mtr complex proteins on flavin reduction. They found a limited decrease in reduction rates for riboflavin and FMN in the absence of OmcA, but a 50% drop in the absence of MtrC and a 75% drop in the absence of both terminal reductases. Furthermore, they characterized highly reduced biofilm production capacity for mutant strains with these knockouts. Together, these results suggested that reduction by the Mtr system accounts for approximately 90% of flavin reduction and elements of the complex are likely involved in adhesion.

Edwards et al. (2015) utilized X-ray crystallography of the MtrC protein to identify a CX_8C disulfide group required for growth under aerobic conditions. Further, they found that in the presence of flavins, reductive cleavage of the disulfide bond could form a flavocytochrome, allowing for increased reduction of bound metals. However, in the presence of oxygen, the bond is lost, freeing the flavins to chelate metals in the environment and allowing for and preventing the formation of reactive oxygen species.

Genetics

Structure

Belieav and Saffarini et al. (2001), in the aforementioned study that originally characterized the effects of MtrA and C through the use of transposon mutagenesis, utilized sequence analysis of the disrupted region to first characterize the *mtr* operon in *S. putrefaciens* MR-1. This analysis demonstrated that *mtrC* was transcribed first, followed by *mtrA* and then *mtrB*. This was supported by directed knockout and back-complementation, demonstrating disruption within *mtrA* generally also disrupted *mtrB* expression, a polar effect. They found only one promoter, upstream of *mtrC*, making unlikely the alternative hypothesis that *mtrC* and *mtrAB* were individually regulated. This promoter was found 119 bases upstream of the start sequence for *mtrC* and had relatively low sequence similarity, matching 2 of the 6 positions for the consensus sequence for the strain at the -35 position and 4 out of 6 at the -10 position.

They also found two potential hairpin-loop structures, between *mtrC* and *mtrA* and after *mtrB*. To characterize the impact of the first on transcription, they generated mutant strains carrying *mtrC-lacZ* and *mtrB-lacZ* mutants and measured their β-galactosidase activity. They found that, consistent across cultures with nitrate, fumarate, and Fe(III) as terminal electron acceptors, that the *mtrC-lacZ* fusion-carrying strain had approximately 12 times the activity during mid-log phase, likely on account of destabilization of the RNA polymerase by formation of the hairpin-loop structure.

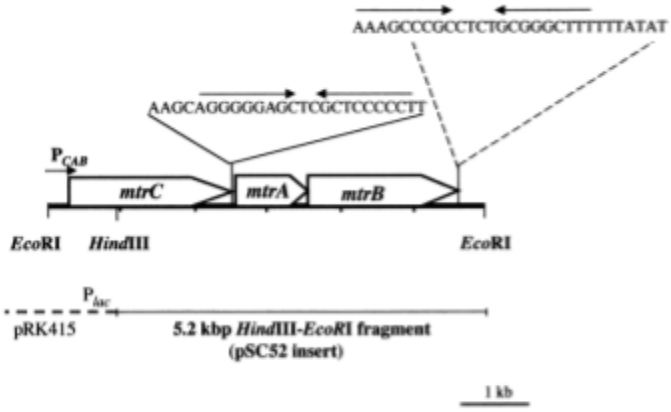

Figure 4: structure of the *mtr* operon from Belieav and Saffarini et al. (2001)

The *omcA* gene is found directly upstream of the *mtr* operon, having its own promoter and being followed by a putative hairpin loop structure (per Kasai et al. (2015)). Together with the *mtr* operon, they form the *mtr* cluster.

Expression

Saffarini and Schultz et al. (2003) first characterized the impact of the *crp* gene, encoding a cyclic AMP receptor protein, through generation of mutant strains of *S. oneidensis* through transposon mutagenesis. The CRP protein is found across many bacteria and is involved in regulation of carbon cycling and stress response, as depletion of ATP and ADP reserves in the presence of adenylyl cyclase results in production of cAMP, required for its binding to DNA. The two strains they generated were deficient in Fe(III) and Mn(IV) reduction, and could not grow under anaerobic conditions on most terminal electron acceptors, with the exception of TMAO. Analysis of heme c content by a pyridine hemochrome assay demonstrated a significant reduction in cytochrome expression. To demonstrate dependence on cAMP as an activator of anaerobic response, wild-type cells were incubated with high concentrations of cAMP, resulting in an increase in Fe(III) and fumarate reduction, though no change in DMSO or nitrate reduction. However, CRP consensus sequences were found upstream of the genes coding for the later reductases.

Kasai et al. (2015) studied transcriptional differences between the *mtr* operon and the adjacent *omcA* gene under aerobic and anaerobic conditions utilizing fumarate as a terminal electron acceptor. Using q-RT-PCR, they found 2.3 fold higher *omcA* expression under anaerobic conditions and 2.7 fold higher *mtr* expression under aerobic conditions. In a *crp* knockout strain provided with aerobic conditions, expression of both operons was markedly reduced. Furthermore, the strain could not grow under anaerobic conditions. The authors then generated

plasmids for expression of *lacZ* with the associated promoters. They found increased expression under the *mtr* promoter during early stationary growth than in mid-logarithmic phase under aerobic conditions, increased expression during mid-log with increased fumarate under anaerobic conditions, but the opposite during early stationary phase. The *omcA* promoter engaged in similar phenomena, though expression was lower and less varied under aerobic conditions. Generating mutants with portions of the promoter sequences removed, they found that 203 to 101 bases before *mtr* initiation, a sequence was involved in constitutive repression, and from 100 to 41, a sequence also found upstream of *omcA* was required for activation.

Barchinger et al. (2016) utilized RNA-seq to characterize differential expression during microaerobic culturing of *S. oneidensis* MR-1. From their original data, they characterized a 2-fold increase in levels of the extracytoplasmic function sigma factor RpoE, associated with vesicle formation under stress. In *Shewanella* in particular, RpoE is associated with response to temperature shifts, high salinity, and antibiotic administration. Using similarity to binding sites for the protein from E. coli, a binding site was found upstream of the *mtr* operon.

Duplication

S. oneidensis MR-1 contains several homologs of the genes associated with the core complex, namely *omcA* and *mtrF* for *mtrC*, *mtrD, dmsE,* and *SO4360* for *mtrA* and *mtrE, dmsF,* and *SO4359* for *mtrB*. On account of high sequence similarity to their better known genes, all are hypothesized to be the result of gene duplication events (Coursolle and Gralnick, 2010). Coursolle and Gralnick (2010) sought to determine if the high similarity of these homologs allowed for compatibility in electron transfer using a large set of knockout strains. They found that *mtrF* and *omcA* knockouts generally lacked a distinct phenotype with respect to ferric citrate reduction, but double knockout of *mtrC* and *mtrF* resulted in significantly decreased reduction,

meaning that *mtrF* could provide redundant function in the event of loss of *mtrC.* With respect to *mtrA* homologs, loss of *mtrD* decreased past the lowered level found in the *mtrA* knockout. *dmsE* was also found to account for some reduction in the event of a *mtrA/mtrD* knockout. Using iron oxide as a terminal electron acceptor, the result for *mtrF* was similar to the previous case, though loss of *omcA* accounted for a greater decrease in reduction than in the other case. Loss of *mtrA* homologs in any combination did not change the rate of reduction whereas loss of *mtrA* resulted in a lack of reduction. The authors also demonstrated an increase in paralog expression in knockout strains for central genes.

Current Directions

From the studies cited here, it is clear that the vast majority of the groundwork for our understanding of the *Shewanella* metal reduction occurred over about 10 years, from 1997 to 2007, with Ross et al. (2007) providing convincing evidence of the pathway's structure. Since then, advancements have primarily been made in determination of the physical structure of the proteins, their regulation, and the relationship of the pathway to other forms of terminal reduction used by the genus.

Kasai et al. (2015) noted that, while the relationship between *omcA* and *mtr* expression and CRP was well characterized, the regulation of cAMP levels in *Shewanella* had not yet been characterized. Given the complexity of carbon cycling, especially with its unique variations in the genus, combined with cAMP regulation, it is difficult to fully predict what conditions might promote reduction. This broad theme appeared throughout the discussions of articles included here. While the metal reduction system is well understood and direct imaging and structural analysis has confirmed its mechanism, the regulatory dynamics of the genus are not fully understood. How does the presence of specific terminal electron acceptors impact the expression

of their reductases and other upstream elements of the extracellular electron transport system? Do they have a unique effect, or does the robustness of the system combined with more generalizable mechanisms of sensing allow for efficient reprogramming?

Further research into the exact path of electrons through the periplasm is also required. While understanding of the path has progressed, with researchers originally hypothesizing that MtrA was either periplasmic or interfaced directly across the periplasm with CymA, the full mechanism has not been elucidated. While knockout of *stc* and *fccA* does substantially reduce reduction, it does not entirely stop it, and other electron carriers are produced after some delay allowing for continuation of reduction at near-wild type levels. Answering this question will provide further insight into the versatility of the system.

Shewanella were also first shown to engage in direct extracellular electron transport over distances through the use of "nanowires" in El-Naggar et al. (2010), characterized to be similar to those in *G. sulfurreducens* originally, though additional research has demonstrated that the phenomena utilizes a chain of vesicles linked by cytochromes in the genus, as opposed to being physiologically related to pili (Pirbadian et al. 2014). This discovery complicated some earlier findings, especially with respect to flavins, that assume no physical connection between cells and electron acceptors at a distance. Characterization of this phenomenon remains in its early stages (ergo its exclusion from the body of this report), though since its discovery research on the extracellular electron transport has generally accounted for nanowires and sought to determine their structure and genetic regulation.

Returning to the motivation behind this report, much of the cutting edge work being done with *Shewanella* has been for development of biotechnological applications, particularly in bioremediation and energy generation. *Shewanella* biofilm formation has increased in

importance, as novel applications involve culturing on electrodes or other electron-accepting surfaces on account of the genus's need to develop a microaerobic environment (Mukherjee et al. 2020). Recent work has attempted to characterize utilization of c-di-GMP and quorum sensing in the process of biofilm formation, the impact of knockouts and mutants in biofilm biosynthesis genes, and the role of viral infection in determination of the composition of biofilms. The electrical properties of members of the genus, while discussed in only a limited manner here, are the focus of research meant to develop microbial fuel cells utilizing *Shewanella* as a current source. Such studies (Wang et al. (2021), Cao et al. (2021)) utilize novel growth substrates and cell configurations to yield higher currents and overall electrical yields from cultured.

Conclusion

The study of *Shewanella* extracellular electron transfer has not taken a linear path, though synthesis in that form was attempted in this report. Early researchers relied on the random insertion of transposons within elements of the genome critical to the system to first determine how specific losses of function modulated reduction. Most of the conclusions made then were shown to be incorrect in some form, though their results have been foundational to our current understanding of the process. It is certainly not the fault of those researchers – as this report has demonstrated, while the pathway feeds nearly directly from the cytoplasm to the outer membrane, functional redundancy and complex regulation obfuscate the exact nature of the underlying system.

At the conclusion of this report, I hope readers have developed a thorough understanding of just how members of the *Shewanella* genus move electrons from their cytoplasm to terminal electron acceptors outside of the cell and an appreciation for the methods by which the elements of this process were discovered. I know that, in the process of writing this, I surely have. For

those who enjoyed this review and find the original motivation of uranium bioremediation fascinating, I recommend You et al. (2021)'s review on the topic, introducing the metal reduction systems of a few other well-studied taxa of bacteria and the engineering systems being developed to best utilize their gifts in this domain.

Works Cited

Barchinger, S. E., Pirbadian, S., Sambles, C., Baker, C. S., Leung, K. M., Burroughs, N. J., ... & Golbeck, J. H. (2016). Regulation of gene expression in Shewanella oneidensis MR-1 during electron acceptor limitation and bacterial nanowire formation. Applied and environmental microbiology, 82(17), 5428-5443.

Beblawy, S., Bursac, T., Paquete, C., Louro, R., Clarke, T. A., & Gescher, J. (2018). Extracellular reduction of solid electron acceptors by Shewanella oneidensis. Molecular microbiology, 109(5), 571-583.

Belchik, S. M., Kennedy, D. W., Dohnalkova, A. C., Wang, Y., Sevinc, P. C., Wu, H., ... & Shi, L. (2011). Extracellular reduction of hexavalent chromium by cytochromes MtrC and OmcA of Shewanella oneidensis MR-1. Applied and Environmental Microbiology, 77(12), 4035-4041.

Beliaev, A. S., & Saffarini, D. A. (1998). Shewanella putrefaciens mtrB encodes an outer membrane protein required for Fe (III) and Mn (IV) reduction. Journal of bacteriology, 180(23), 6292-6297.

Beliaev, A. S., Saffarini, D. A., McLaughlin, J. L., & Hunnicutt, D. (2001). MtrC, an outer membrane decahaem c cytochrome required for metal reduction in Shewanella putrefaciens MR‐1. Molecular microbiology, 39(3), 722-730.

Cao, B., Zhao, Z., Peng, L., Shiu, H. Y., Ding, M., Song, F., ... & Huang, Y. (2021). Silver nanoparticles boost charge-extraction efficiency in Shewanella microbial fuel cells. *Science*, *373*(6561), 1336-1340.

Clarke, T. A., Edwards, M. J., Gates, A. J., Hall, A., White, G. F., Bradley, J., ... & Richardson, D. J. (2011). Structure of a bacterial cell surface decaheme electron conduit. Proceedings of the National Academy of Sciences, 108(23), 9384-9389.2

Coursolle, D., & Gralnick, J. A. (2012). Reconstruction of extracellular respiratory pathways for iron (III) reduction in Shewanella oneidensis strain MR-1. Frontiers in microbiology, 3, 56.

Coursolle, D., Baron, D. B., Bond, D. R., & Gralnick, J. A. (2010). The Mtr respiratory pathway is essential for reducing flavins and electrodes in Shewanella oneidensis. Journal of bacteriology, 192(2), 467-474.

Dohnalkova, A. C., Marshall, M. J., Arey, B. W., Williams, K. H., Buck, E. C., & Fredrickson, J. K. (2011). Imaging hydrated microbial extracellular polymers: comparative analysis by electron microscopy. Applied and environmental microbiology, 77(4), 1254–1262. https://doi.org/10.1128/AEM.02001-10

Edwards, M. J., Baiden, N. A., Johs, A., Tomanicek, S. J., Liang, L., Shi, L., ... & Clarke, T. A. (2014). The X-ray crystal structure of Shewanella oneidensis OmcA reveals new insight

at the microbe–mineral interface. FEBS letters, 588(10), 1886-1890.

Edwards, M. J., White, G. F., Butt, J. N., Richardson, D. J., & Clarke, T. A. (2020). The Crystal Structure of a Biological Insulated Transmembrane Molecular Wire. Cell, 181(3), 665–673.e10. https://doi.org/10.1016/j.cell.2020.03.032

Edwards, M. J., White, G. F., Norman, M., Tome-Fernandez, A., Ainsworth, E., Shi, L., ... & Clarke, T. A. (2015). Redox linked flavin sites in extracellular decaheme proteins involved in microbe-mineral electron transfer. Scientific reports, 5(1), 1-11.

El-Naggar, M. Y., Wanger, G., Leung, K. M., Yuzvinsky, T. D., Southam, G., Yang, J., ... & Gorby, Y. A. (2010). Electrical transport along bacterial nanowires from Shewanella oneidensis MR-1. *Proceedings of the National Academy of Sciences*, *107*(42), 18127-18131.

Gao, H., Obraztova, A., Stewart, N., Popa, R., Fredrickson, J. K., Tiedje, J. M., ... & Zhou, J. (2006). Shewanella loihica sp. nov., isolated from iron-rich microbial mats in the Pacific Ocean. International Journal of Systematic and Evolutionary Microbiology, 56(8), 1911-1916.

Hartshorne, R. S., Jepson, B. N., Clarke, T. A., Field, S. J., Fredrickson, J., Zachara, J., ... & Richardson, D. J. (2007). Characterization of Shewanella oneidensis MtrC: a cell-surface decaheme cytochrome involved in respiratory electron transport to extracellular electron acceptors. JBIC Journal of Biological Inorganic Chemistry, 12(7), 1083-1094.

Hartshorne, R. S., Reardon, C. L., Ross, D., Nuester, J., Clarke, T. A., Gates, A. J., ... & Richardson, D. J. (2009). Characterization of an electron conduit between bacteria and the extracellular environment. Proceedings of the National Academy of Sciences, 106(52), 22169-22174.

Howard, E. C., Hamdan, L. J., Lizewski, S. E., & Ringeisen, B. R. (2012). High frequency of glucose-utilizing mutants in Shewanella oneidensis MR-1. FEMS microbiology letters, 327(1), 9-14.

Kasai, T., Kouzuma, A., Nojiri, H., & Watanabe, K. (2015). Transcriptional mechanisms for differential expression of outer membrane cytochrome genes omcA and mtrC in Shewanella oneidensis MR-1. BMC microbiology, 15(1), 1-12.

Le Laz, S., Bauzan, M., Lignon, S., Rousset, M., & Brugna, M. (2016). Expression of terminal oxidases under nutrient-starved conditions in Shewanella oneidensis: detection of the A-type cytochrome c oxidase. Scientific reports, 6(1), 1-11.

Lemaire, O. N., Méjean, V., & Iobbi-Nivol, C. (2020). The Shewanella genus: ubiquitous organisms sustaining and preserving aquatic ecosystems. FEMS Microbiology Reviews, 44(2), 155-170.

Marsili, E., Baron, D. B., Shikhare, I. D., Coursolle, D., Gralnick, J. A., & Bond, D. R. (2008).

Shewanella secretes flavins that mediate extracellular electron transfer. Proceedings of the National Academy of Sciences, 105(10), 3968-3973.

McMillan, D. G., Marritt, S. J., Butt, J. N., & Jeuken, L. J. (2012). Menaquinone-7 is specific cofactor in tetraheme quinol dehydrogenase CymA. Journal of Biological Chemistry, 287(17), 14215-14225.

Meyer, T. E., Tsapin, A. I., Vandenberghe, I., De Smet, L., Frishman, D., Nealson, K. H., ... & Van Beeumen, J. J. (2004). Identification of 42 possible cytochrome c genes in the Shewanella oneidensis genome and characterization of six soluble cytochromes. Omics: a journal of integrative biology, 8(1), 57-77.

Mukherjee, M., Zaiden, N., Teng, A., Hu, Y., & Cao, B. (2020). Shewanella biofilm development and engineering for environmental and bioenergy applications. *Current Opinion in Chemical Biology*, *59*, 84-92.

Myers, C. R., & Myers, J. M. (1997). Cloning and sequence of cymA, a gene encoding a tetraheme cytochrome c required for reduction of iron (III), fumarate, and nitrate by Shewanella putrefaciens MR-1. Journal of Bacteriology, 179(4), 1143-1152.

Myers, C. R., & Myers, J. M. (2002). MtrB is required for proper incorporation of the cytochromes OmcA and OmcB into the outer membrane of Shewanella putrefaciens MR-1. Applied and Environmental Microbiology, 68(11), 5585-5594.

Myers, C. R., & Nealson, K. H. (1988). Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. Science, 240(4857), 1319-1321.

Myers, J. M., & Myers, C. R. (2001). Role for outer membrane cytochromes OmcA and OmcB of Shewanella putrefaciens MR-1 in reduction of manganese dioxide. Applied and environmental microbiology, 67(1), 260-269.

Pirbadian, S., Barchinger, S. E., Leung, K. M., Byun, H. S., Jangir, Y., Bouhenni, R. A., ... & El-Naggar, M. Y. (2014). Shewanella oneidensis MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components. *Proceedings of the National Academy of Sciences*, *111*(35), 12883-12888.

Pitts, K. E., Dobbin, P. S., Reyes-Ramirez, F., Thomson, A. J., Richardson, D. J., & Seward, H. E. (2003). Characterization of the Shewanella oneidensis MR-1 decaheme cytochrome MtrA: expression in Escherichia coli confers the ability to reduce soluble Fe (III) chelates. Journal of Biological Chemistry, 278(30), 27758-27765.

Ross, D. E., Ruebush, S. S., Brantley, S. L., Hartshorne, R. S., Clarke, T. A., Richardson, D. J., & Tien, M. (2007). Characterization of protein-protein interactions involved in iron reduction by Shewanella oneidensis MR-1. Applied and Environmental Microbiology, 73(18), 5797-5808.

Saffarini, D. A., Blumerman, S. L., & Mansoorabadi, K. J. (2002). Role of menaquinones in Fe

(III) reduction by membrane fractions of Shewanella putrefaciens. Journal of bacteriology, 184(3), 846-848.

Saffarini, Daad A., Ryan Schultz, and Alex Beliaev. "Involvement of cyclic AMP (cAMP) and cAMP receptor protein in anaerobic respiration of Shewanella oneidensis." Journal of bacteriology 185.12 (2003): 3668-3671.

Schicklberger, M., Bücking, C., Schuetz, B., Heide, H., & Gescher, J. (2011). Involvement of the Shewanella oneidensis decaheme cytochrome MtrA in the periplasmic stability of the beta-barrel protein MtrB. Applied and environmental microbiology, 77(4), 1520–1523. https://doi.org/10.1128/AEM.01201-10

Schuetz, B., Schicklberger, M., Kuermann, J., Spormann, A. M., & Gescher, J. (2009). Periplasmic electron transfer via the c-type cytochromes MtrA and FccA of Shewanella oneidensis MR-1. Applied and environmental microbiology, 75(24), 7789–7796. https://doi.org/10.1128/AEM.01834-09

Schwalb, C., Chapman, S. K., & Reid, G. A. (2003). The tetraheme cytochrome CymA is required for anaerobic respiration with dimethyl sulfoxide and nitrite in Shewanella oneidensis. Biochemistry, 42(31), 9491–9497. https://doi.org/10.1021/bi034456f

Sturm, G., Richter, K., Doetsch, A., Heide, H., Louro, R. O., & Gescher, J. (2015). A dynamic periplasmic electron transfer network enables respiratory flexibility beyond a thermodynamic regulatory regime. The ISME journal, 9(8), 1802-1811.

Tang, Y. J., Meadows, A. L., Kirby, J., & Keasling, J. D. (2007). Anaerobic central metabolic pathways in Shewanella oneidensis MR-1 reinterpreted in the light of isotopic metabolite labeling. Journal of Bacteriology, 189(3), 894-901.

Von Canstein, H., Ogawa, J., Shimizu, S., & Lloyd, J. R. (2008). Secretion of flavins by Shewanella species and their role in extracellular electron transfer. Applied and environmental microbiology, 74(3), 615-623.

Wall, J. D., & Krumholz, L. R. (2006). Uranium reduction. Annu. Rev. Microbiol., 60, 149-166.

Wang, D., Pan, J., Xu, M., Liu, B., Hu, J., Hu, S., ... & Liang, S. (2021). Surface modification of Shewanella oneidensis MR-1 with polypyrrole-dopamine coating for improvement of power generation in microbial fuel cells. *Journal of Power Sources*, *483*, 229220.

You, W., Peng, W., Tian, Z., & Zheng, M. (2021). Uranium bioremediation with U(VI)-reducing bacteria. The Science of the total environment, 798, 149107. https://doi.org/10.1016/j.scitotenv.2021.149107

Zhong, C., Han, M., Yu, S., Yang, P., Li, H., & Ning, K. (2018). Pan-genome analyses of 24 Shewanella strains re-emphasize the diversification of their functions yet evolutionary dynamics of metal-reducing pathway. Biotechnology for biofuels, 11(1), 1-13.