RECOMBINEERING FOR PRECISE GENOME EDITING

IN *SHEWANELLA ONEIDENSIS*

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Engineering

by

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ABSTRACT

With the rapid growth in renewable energy systems to fulfill the increasing global electricity demand, we must establish methods for low-cost and large-scale electrical energy storage. The key to solving our energy storage problems may lie in biological systems, and we could use these systems to our advantage. One such system of interest is the extracellular electron transfer process in *Shewanella oneidensis,* which could potentially be used for electrosynthesis and allow for electrical energy to be converted and stored as microbial fuel cells. Genetically engineering microbes such as *S. oneidensis* may help address the lack of energy storage solutions, and we can study and engineer these systems using molecular and synthetic biology tools. This paper explores a method that allows us to knock out genes quickly and precisely using recombination-mediated genetic engineering, or recombineering, as well as discusses possible areas for future research in *S. oneidensis*.

ACKNOWLEDGEMENTS

First and foremost, I am grateful to Professor Buz Barstow for his support through the course of my MEng project. I was first introduced to synthetic biology during the 2nd year of my undergraduate studies while taking his course in molecular and cellular bioengineering (BEE 3600), and with his encouragement, I was able to pursue my interests in biological engineering as a member of his lab group. I would also like to thank Farshid Salimijazi, who provided guidance in the creation of this MEng project and gave me valuable advice to help me build a strong foundation for a future career in science. Brooke Pian taught me most of the skills I needed in order to succeed in the lab, and I am immensely grateful for her positive energy and infinite patience while answering the deluge of questions I always had. Dr. Alexa Schmitz and Sean Medin provided me with encouragement and advice throughout my studies, and they always let me bounce ideas off of them. I would like to thank current lab members Sabrina Marecos, David Specht, Katie Randolph, and Finn Kennedy for always saying hello and brightening my day while working in the lab. Dr. Eric Richards, my mentor at the Boyce Thompson Institute, taught me the fundamentals of biology research and helped me grow into the scientist that I am today. Dr. Jean Hunter, Dr. Sunghwan Jung, Dr. Shogo Hamada, and all of my previous professors in the Department of Biological and Environmental Engineering taught me the fundamentals of biological engineering and pushed me to explore and to never stop learning. Finally, I am grateful for all the friends I found during my four years at Cornell, and I can’t wait to see what the next step in life brings for us all.

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INTRODUCTION

As the global demand for energy increases, we must find novel ways to produce and store energy. In 2021, the global electricity demand grew by 6%, corresponding to the largest ever increase in absolute terms of over 1,500 TWh (Fig. 1). This rapid increase in demand has pushed power prices to a record high, and CO_2 emissions from electricity rose by 7%. During 2022–2024, the International Energy Agency expects a rapid growth in renewable energy by about 8% per year in order to match demand growth, and by 2024, renewables could provide more than 32% of the world's electricity supply [IEA2021a].

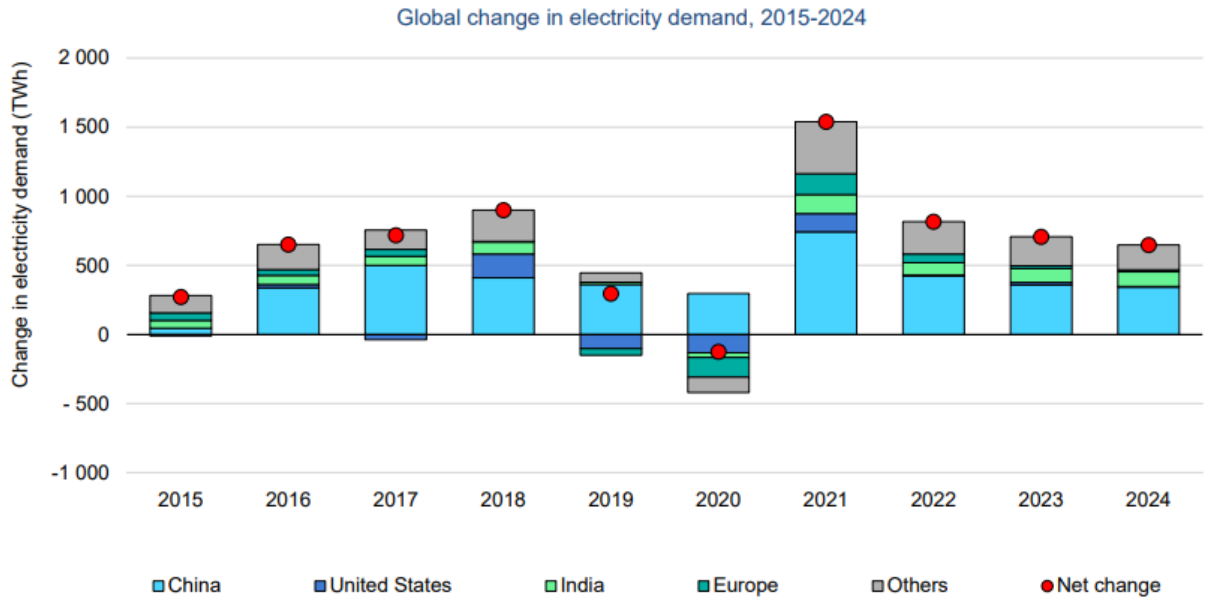


Figure 1: Global electricity demand increased by 6% in 2021 and is expected to continue rising in the upcoming years [IEA2021a].

While the growth of renewables will help address the problem with rising emissions, the power output of most renewables is intermittent. Before renewables can take over a large fraction of the current electricity supply, methods for low-cost and large-scale electrical energy storage must

first be established [Salimijazi2019a]. Energy storage technologies provide support for renewable energy and help balance out power production and energy demand. Some examples of current energy storage technologies include mechanical (pumped energy storage, compressed air), electrochemical (batteries, capacitors), thermal (sensible and latent heat storage), and chemical (hydrogen, biofuels). As of 2018, about 96% (169,557 GW) of the installed energy storage capacity worldwide came from pumped hydro storage (PHS) systems, which store energy in the form of gravitational potential energy of water [Olabi2020a]. PHS systems can last about 70–100 years, and the efficiency can very between 70–80% [Tan2021a].

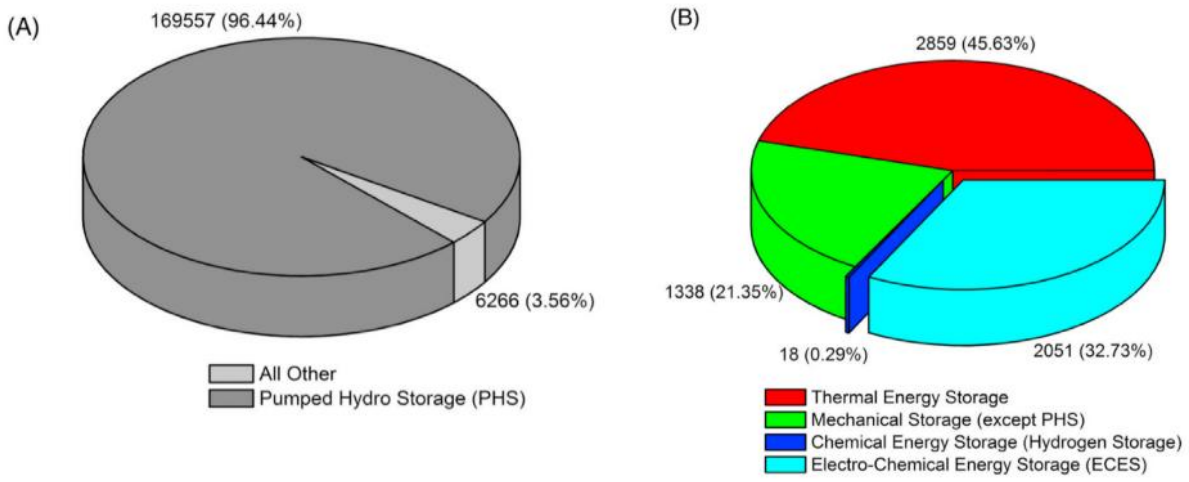


Figure 2: A) PHS systems make up the vast majority of the installed energy storage capacity worldwide. B) Breakdown of all other major types of energy storage systems [Olabi2020a].

However, constructing these systems can be quite expensive and damaging to the environment. Batteries are another popular and portable method for energy storage, though more research needs to be done in order to improve cell capacity, energy density, and lifespan [Tan2021a]. Lithium-ion and lithium-polymer batteries, which are dominant in consumer electronic products, may contribute substantially to environmental pollution due to the presence of toxic materials

[Kang2013a]. The solution to our energy storage problems may lie in biological systems–after all, nature is the best engineer there is. Over the course of billions of years, nature has come up with biological systems that can harvest and store energy from the environment, and we can study these systems using molecular and synthetic biology tools in order to use them to our advantage.

One such organism of interest is *Shewanella oneidensis*. *S. oneidensis* was first described in 1988 as a bacteria that could grow with manganese as the sole electron acceptor using manganese reduction. The *Shewanella* genus is known for having high respiratory versatility, and *S. oneidensis*, a gram-negative bacteria first isolated from Lake Oneida in Upstate New York, is one of the best understood models for studying the extracellular electron transfer (EET) process [Beblawy2018a]. *S. oneidensis* is able to anaerobically respire metals via the EET pathway. The metal respiratory (Mtr) pathway consists of multiple multiheme *c*-type cytochromes, which serve as electron transfer proteins, and flavins, which facilitate the electron transfer process (Fig. 3). Specifically, cytoplasmic membrane protein CymA is regarded as the electron hub, and it oxidizes quinol in the cytoplasmic membrane and transfers electrons to periplasmic cytochromes FccA and STC. In the periplasm, electrons are transferred using the monoheme cytochrome ScyA, the fumarate reductase FccA, and the small tetraheme cytochrome STC. FccA and STC then transfer electrons to metal reducing protein MtrA, and a trans-outer membrane protein complex consisting of MtrA, MtrB, and MtrC transfer the electrons to the extracellular space, where MtrC serves as the terminal reductase that interacts with metal substrates. With its unique metal-reducing capabilities, *S. oneidensis* can potentially be applied for bioremediation of metal-

polluted environmental sites and also as microbial fuel cells for use in space and on Earth [Corts2019b, NASA2018a].

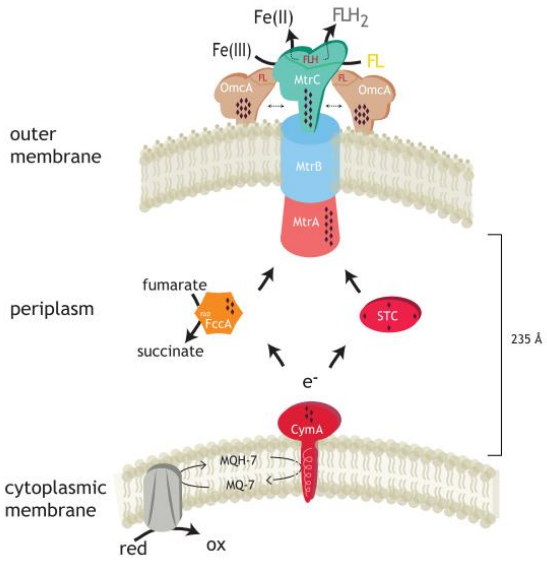


Figure 3: Diagram of the metal respiratory pathway in *S. oneidensis* MR-1 [Beblawy2018a].

Compared to other pathways, the Mtr pathway in *S. oneidensis* is very promiscuous and can reduce soluble substrates that form solids upon reduction, including vanadium (V), uranium (VI) and technetium-(VII), as well as 9,10-anthraquinone-2,6-disulfonic acid (AQDS) and carbon electrodes [Corts2019b]. While much is still to be learned about the molecular mechanisms involved in the Mtr pathway and its promiscuous nature, *in vivo* studies of *S. oneidensis* MR-1 have shown that the bacteria can use CymA to facilitate electron flow from a cathode into its quinone pool, using oxygen as the terminal electron acceptor [Beblawy2018a]. Based on electron transport chain inhibitor studies and the higher cellular ATP levels measured in cathode-respiring cells, there is evidence that the Mtr pathway generates a proton motive force, which is then used for cellular processes under aerobic and cathodic conditions. This process has significant

environmental and technological implications, especially in electromicrobiology applications such as waste treatment, bioremediation, CO_2 mineralization, and especially electrosynthesis [Rowe2018a, Marecos2022a]. Electromicrobiology and electrosynthesis involves EET between microbes and solid-phase electron-active redox compounds, and this allows for electrical energy to be converted to microbially synthesized products or stored as microbial fuel cells or biofuels [Rowe2018a].

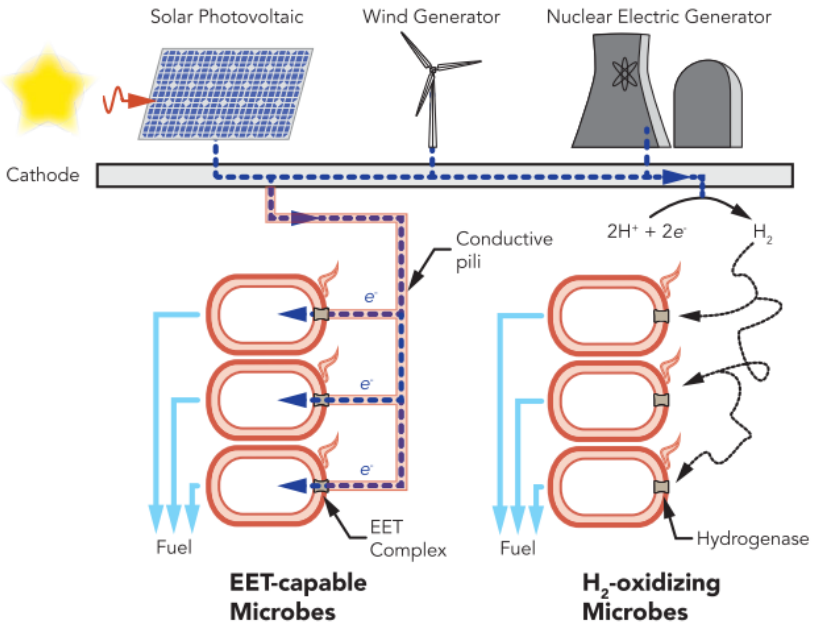


Figure 4: Electroactive microbes like *S. oneidensis* can accept electricity from many energy sources. Solid-matrix EET-capable microbes H_2-oxidizing microbes are able to absorb electricity into its carbon-fixing metabolism and can be used for fuel synthesis [Adesina2017a].

Genetically engineering microbes such as *S. oneidensis* may be a viable option to address the lack of energy storage solutions. Using molecular and synthetic biology tools, we can learn more about how the extracellular electron uptake process works in *S. oneidensis*, allowing us to engineer microbially-catalyzed processes on electrodes to produce microbial fuel cells. Currently, much of our genetic engineering toolkit is geared towards *Escherichia coli*, and there

is a lack of methodologies for large-scale genome engineering in *S. oneidensis.* The most reliable method in *S. oneidensis* has been bacterial conjugation, which is quite tedious and time-consuming compared to electroporation-based methods. Transposon mutagenesis and targeted knockouts by suicide vectors are also used, however these methods are better for gene disruption applications rather than metabolic engineering purposes [Corts2019a].

Recently, *in vivo* homologous recombination-mediated genetic engineering, or recombineering, using single-stranded DNA (ssDNA) oligonucleotides (oligos) has emerged as a powerful tool for precise genome editing. First developed in *E. coli*, recombineering enables efficient and rapid construction of mutant genes by taking advantage of bacteriophage proteins that allow for homologous recombination, such as the λ Red system from phage λ and the RecET system from the Rac prophage. Linear DNA fragments (dsDNA or ssDNA) can be designed with homology sequences to allow for accurate insertion, deletion, or modification of a genomic target without needing convenient placement of restriction sites. The λ Red system consists of three proteins, including Exo, Beta, and Gam. Exo, a dsDNA exonuclease, creates 3' ssDNA overhangs and is needed for dsDNA recombineering. Recombinase Beta, a single strand annealing protein, binds to ssDNA overhangs and pairs them with complementary ssDNA targets. Finally, Gam protects the exogenous DNA from being degraded by inhibiting nuclease activities in the host. Compared to other established methods for genome editing in *S. oneidensis*, ssDNA recombineering allows for precise genome editing in only a few days, and the use of ssDNA does not leave any extraneous genetic scars and only requires the expression of the recombinase Beta [Corts2019a].

Recombineering can be applied in a diverse range of bacterial species. Since the recombineering process was first applied in *E. coli*, a few other phage homologous recombination systems were found in *Pseudomonas, Vibrio, Lactobacillus, Mycobacteria, Photorhabdus, Staphylococcus*, and even *Shewanella*. In a study by Corts *et al*., the authors identified λ Red recombinase homologs in *Shewanella oneidensis* and demonstrated the use of *Shewanella* sp. W3-18-1 recombinase (W3 Beta), which shares 55% identity to the λ Red Beta recombinase. They developed a stepwise approach to ssDNA recombineering in *S. oneidensis* as seen below (Fig. 5).

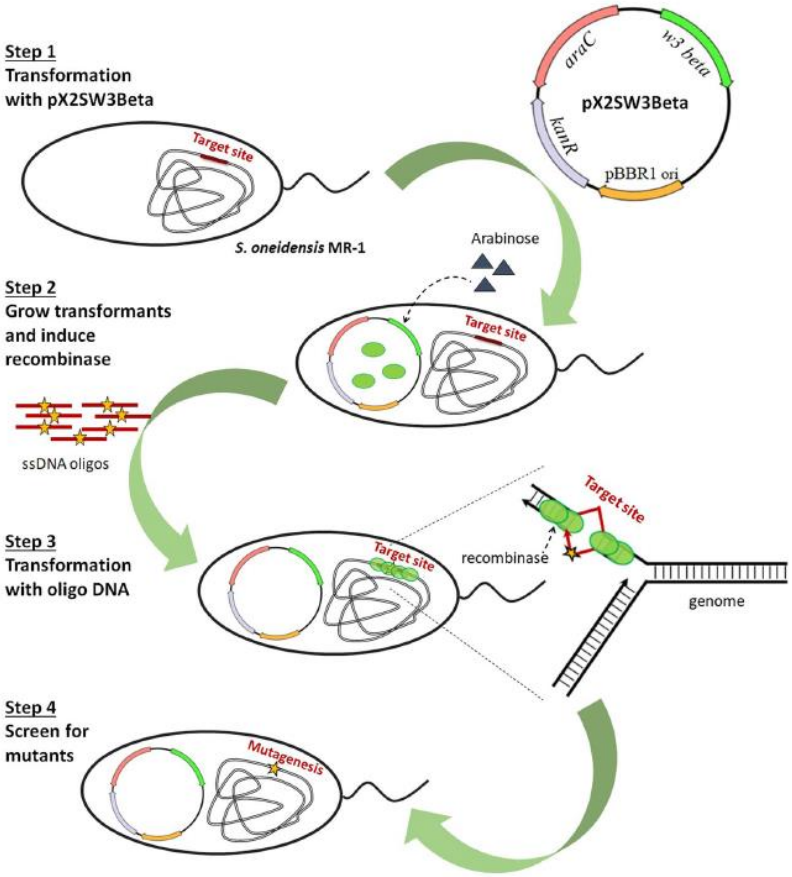


Figure 5: Diagram of the stepwise approach used for recombineering in *S. oneidensis* as developed by Corts *et al*. In this project, the plasmid pX2SW3Beta was replaced by pX2C9pLacW3Beta. The plasmid is introduced into *S. oneidensis* using electroporation, then grown on LB+Km50 plates at 30 °C. Next, ssDNA is introduced using the same electroporation protocol, then grown on LB+Km50 plates at 30 °C after a period of recovery. Transformants are screened using PCR and gel electrophoresis [Corts2019a].

Using ssDNA recombineering methods, Corts *et al.* found a recombination efficiency of about 5% recombinants among total cells, while Ronda *et al.* found a recombination efficiency of about 0.68% to 5.4% recombinants among total cells [Corts2019a, Ronda2015a]. While the overall recombination rate of the system is quite low, recombineering can be rapidly implemented at a large scale, and future research in this area will advance high-throughput methods for investigating and genetically engineering *S. oneidensis* and other environmental bacteria.

The overall goal of this project is to optimize a recombineering protocol in order to knock out genes in *S. oneidensis*. The first semester of work on this project was spent learning wet lab techniques, conducting literature research on current scientific progress in recombineering, and doing preliminary recombineering experiments to knock out *SO_0181*, a gene associated with EET. During the second semester of this project, recombineering experiments were done using the *S. oneidensis* Δfdh strain acquired from the Gralnick Lab. This strain contains a clean deletion of the three formate dehydrogenase (FDH) operons (*SO_0101, SO_0102, SO_0103*), preventing the oxidation of formate to carbon dioxide and therefore eliminating the use of formate as an energy source in *Shewanella* [Kane2016a, Serre2006a]. Using the *S. oneidensis* Δfdh strain, the goal was to further knock out two genes related to acetate and formate metabolism: *SO_AceA* and *SO_pflB. SO_AceA*, which encodes for isocitrate lyase, is a part of the glyoxylate cycle and is used for glyoxylate and dicarboxylate metabolism. Knocking out *SO_AceA* prevents the glyoxylate shunt, which is a variant of the tricarboxylic acid cycle (TCA cycle) and is essential for utilizing acetate and fatty acids [Ahn2016a]. *SO_pflB* encodes for pyruvate formate-lyase PflB, which is used in the pyruvate metabolism pathway to convert acetyl-CoA and formate to pyruvate. If carried out successfully, the new mutant strain should no

longer be able to grow well on either formate or acetate. Though the goal to knock out *SO_AceA* and *SO_pflB* does not directly contribute to research on EET or the Mtr pathway, the work done in this project explores the efficacy of the general recombineering process and will hopefully serve as an inspiration for future genetic engineering experiments relating to recombineering in *S. oneidensis.*

MATERIALS AND METHODS

Strains and Growth Conditions

Wild type *S. oneidensis* MR-1 was recovered from the Barstow Lab glycerol stock (Box 1, #11), struck out on plain LB agar plates, then grown overnight in an incubator at 30 °C. *S. oneidensis* Δfdh (Box 1, #82) was struck out on plain LB agar plates and grown at 30 °C. The *S. oneidensis* Δfdh was previously acquired from the Gralnick Lab, and this strain contains knockouts for all three formate dehydrogenase gene clusters present in *S. oneidensis* MR-1. *S. oneidensis* +PX2 (Box 1, #44), was struck out on LB+Km50 µM plates at grown at 30 °C. *S. oneidensis* +PX2 contains the pX2C9pLacW3Beta editing plasmid with kanamycin (Km) resistance (Fig. 6), acquired from the Gralnick Lab as well. Single colonies for the wild type and Δfdh strains were picked into 3 mL plain LB liquid media and grown in 20 mL culture tubes at 30 °C with 200 rpm shaking. Single colonies for the +PX2 strain were picked into 3 mL LB liquid media supplemented with 3 µL Km50 µM and grown in culture tubes at 30 °C with 200 rpm shaking in order to confirm the presence of the plasmid. All strains that were used and/or created during this project are listed in *Table 3* in the Appendix.

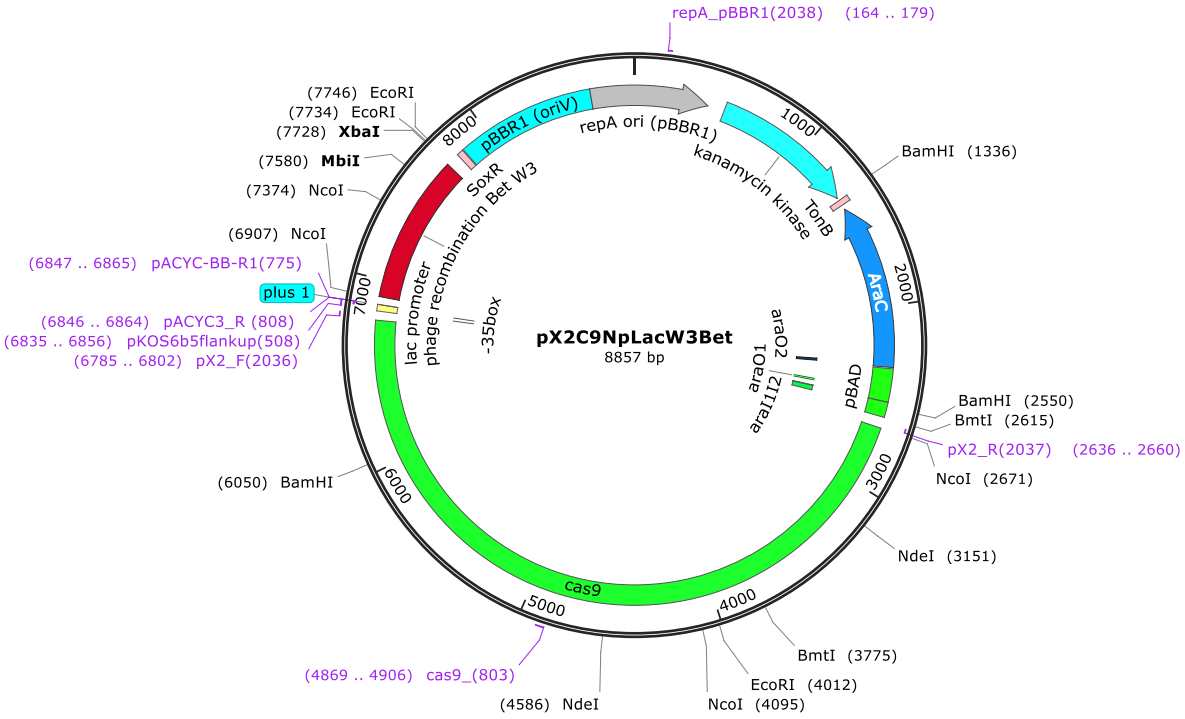


Figure 6: The pX2C9pLacW3Beta editing plasmid contains kanamycin resistance, the *cas9* gene under the arabinose inducible promoter, and the W3 Beta recombinase gene under a constitutive *lac* promoter.

Electroporation Protocol for Plasmid Transformation in S. oneidensis MR-1

For the recombineering experiments using the *SO_0181* gene, *S. oneidensis* +PX2 was grown from glycerol stock using LB+Km50 media in order to verify the presence of the pX2C9NpLacW3Beta editing plasmid. However, the *S. oneidensis* Δfdh strain, which does not contain this plasmid, was used for the recombineering experiments using the *SO_AceA* and *SO_pflB* genes. A modified version of the Gralnick Lab high efficiency electroporation protocol for plasmid transformation in *S. oneidensis* MR-1 was used to introduce this plasmid into the Δfdh strain.

1. Obtain miniprepped plasmids. Plasmids should be at about 100 ng/µL measured with a Nanodrop.
2. Plate *S. oneidensis* Δfdh on plain LB agar plates, then incubate at 30 °C overnight.
3. Transfer a single colony in a 20 mL culture tube with 3 mL plain LB liquid media. Culture overnight at 30 °C with shaking (~200 rpm). For best results, allow culture to grow for about 18 hours. Allowing the culture to grow for too long may result in poor results, as the culture will be approaching stationary phase.
4. Subculture cells to a starting OD of 0.08 in a new 20 mL culture tube with 3 mL plain LB. Grow for about 1-1.5 hours, or until cells are at OD 0.5-0.6.
5. Once cells are at OD 0.5-0.6, place 1 mL of culture each into three 1.7 mL Eppendorf tubes, then spin at 8000 rcf for 1 minute.
6. Remove the supernatants for all three tubes, then resuspend one tube with 1 mL 1 M sorbitol. Wash by gently pipetting up and down, then transfer the resuspension to the second tube. Repeat the washing procedure for all tubes and combine into one Eppendorf.

7. Spin at 8000 rcf for 1 minute.
8. Wash cells with 1 mL 1 M sorbitol two more times, spinning between washes.
9. Pour off supernatant and add back 60-70 µL sorbitol, then resuspend cells.
10. Add ~100 ng of plasmid DNA, gently pipetting/swirling to mix. Incubate for ~5 minutes at room temperature.
11. Transfer the mixture into a 0.1 cm cuvette without making bubbles.
12. Electroporate at 1.2 kV.
13. Quickly add 1 mL plain LB into the cuvette and mix gently while pipetting up and down.
14. Transfer mixture to new Eppendorf tube, then let cells recover at 30 °C with shaking (~200 rpm).
15. Prepare LB+Km50 µM plates. When ready to plate, take out plates and let them dry at room temperature for ~10 minutes by a flame.
16. Make 10× serial dilutions in LB liquid media, then plate on LB+Km50 plates and grow for 24-36 hours until colonies appear.
17. To make the main transformation tube, transfer remaining mixture to 20 mL culture tube, then add 1 mL LB and 2 µL Km50 for a total volume of 2 mL. Grow overnight at 30 °C with shaking at 200 rpm.

Additionally, a glycerol stock tube can be made by mixing an equal amount of culture and 20% glycerol solution, then frozen at -80 °C. The new *S. oneidensis* Δfdh +PX2 strain can be found in the -80 °C freezer in *S.O.* BOX II, #6.

To verify that a culture has the PX2 editing plasmid, the strains were first struck on LB+Km50 plates. Single colonies were then picked into 3 mL LB liquid media supplemented with 3 µL Km50 and grown in culture tubes at 30 °C with 200 rpm shaking. Furthermore, the plasmid was also verified using culture PCR and gel electrophoresis with primers 803/775 and 2037/2038.

Recombineering Protocol

A similar electroporation protocol was used to knock out genes using the recombineering method.

1. Plate *S. oneidensis* with the pX2C9NpLacW3Beta editing plasmid on LB+Km50 plates, then incubate at 30 °C overnight.
2. Transfer a single colony in a 20 mL culture tube with 3 mL LB and 3 µL Km50. Culture overnight at 30 °C with shaking (~200 rpm). Allow culture to grow for about 18 hours.
3. Subculture cells to a starting OD of 0.08 in a new 20 mL culture tube with 700 µL 100 mM arabinose, 2.3 mL LB, and 3 µL Km50. Grow for about 1-1.5 hours, or until cells are at OD 0.5-0.6.
4. Once cells are at OD 0.5-0.6, place 1 mL of culture each into three 1.7 mL Eppendorf tubes, then spin at 8000 rcf for 1 minute.
5. Remove the supernatants for all three tubes, then resuspend one tube with 1 mL 1 M sorbitol. Wash by gently pipetting up and down, then transfer the resuspension to the second tube. Repeat the washing procedure for all tubes and combine into one Eppendorf.
6. Spin at 8000 rcf for 1 minute.
7. Wash cells with 1 mL 1 M sorbitol two more times, spinning between washes.

8. Pour off supernatant and add back 60-70 µL sorbitol, then resuspend cells.
9. Add 5 µg ssDNA and gently pipette/swirl to mix. Incubate for ~5 minutes at room temperature.
10. Transfer the mixture into a 0.1 cm cuvette without making bubbles.
11. Electroporate at 1.2 kV. Set the electroporator so that the actual voltage falls around 1.2 kV and the time constant is ~4. If the time constant is not ~4, the efficiency may be lower.
12. Quickly add 1 mL plain LB into the cuvette and mix gently while pipetting up and down.
13. Transfer mixture to new Eppendorf tube, then let cells recover at 30 °C with shaking (~200 rpm).
14. Make 10× serial dilutions in LB, then plate on LB+Km50 plates and grow for 24-36 hours until colonies appear.
15. Transfer remaining mixture to 20 mL culture tube, then add 2 mL LB and 3 µL Km50 for a total volume of 3 mL. Grow overnight at 30 °C with shaking at 200 rpm.

Due to the low efficiency of recovering mutants, some minor tweaks were made to the above protocol in an attempt to boost the success rate. For example, instead of transferring to an Eppendorf tube in Step 13, an alternative method was to transfer the solution to a culture tube to allow for more oxygen in the tube, though it is unclear if any of these modifications contributed to a higher success rate. In addition, adding arabinose in Step 3 is theoretically not needed in the above protocol, since the W3 Beta recombinase is under the constitutive *lac* promoter and *cas9* is under the arabinose inducible promoter. However, during the first couple months of this research project, it was found that not including arabinose or even including old arabinose solution did not

result in successful recombination. After making and including fresh 100 mM arabinose solution in Step 3, all subsequent recombineering attempts resulted with mixtures containing both wild type and recombinant cells.

ssDNA Oligonucleotides and Primers

All of the ssDNA oligos and primers used in this project were designed using SnapGene and ordered from IDT. Information regarding sequence and purpose can be found in *Table 2* in the Appendix, as well as the Barstow Lab Primers Google Sheet. The primers used for verification of pX2C9pLacW3Beta were designed and ordered by Sonia Jaidka and Farshid Salimijazi. The ssDNA oligos and primers used for the *SO_0181* knockout experiment were designed by Farshid Salimijazi, and the ssDNA oligos and primers used for the *SO_AceA* knockout experiment were designed by Linda Li.

For the recombineering process, ssDNA oligos were designed with well-defined modifications used to inactivate protein-coding sequences by introducing nonsense mutations in the open reading frame. To do this, the sequence of the gene of interest was first imported into SnapGene. Excess base pairs at the 3' and 5' ends were also included to aid in primer design. Approximately 100 bp after the beginning of the open reading frame, a sequence of about 5-9 bp in length was overwritten in order to introduce nonsense mutations. Homologous arms of about 50 bp in length were added to the 3' and 5' ends of the overwrite, creating an ssDNA sequence of about 105-109 bp in length. In the *SO_AceA* knockout experiment, 6 base pairs were overwritten, leading to an ssDNA sequence of 106 bp (Fig. 7*).*

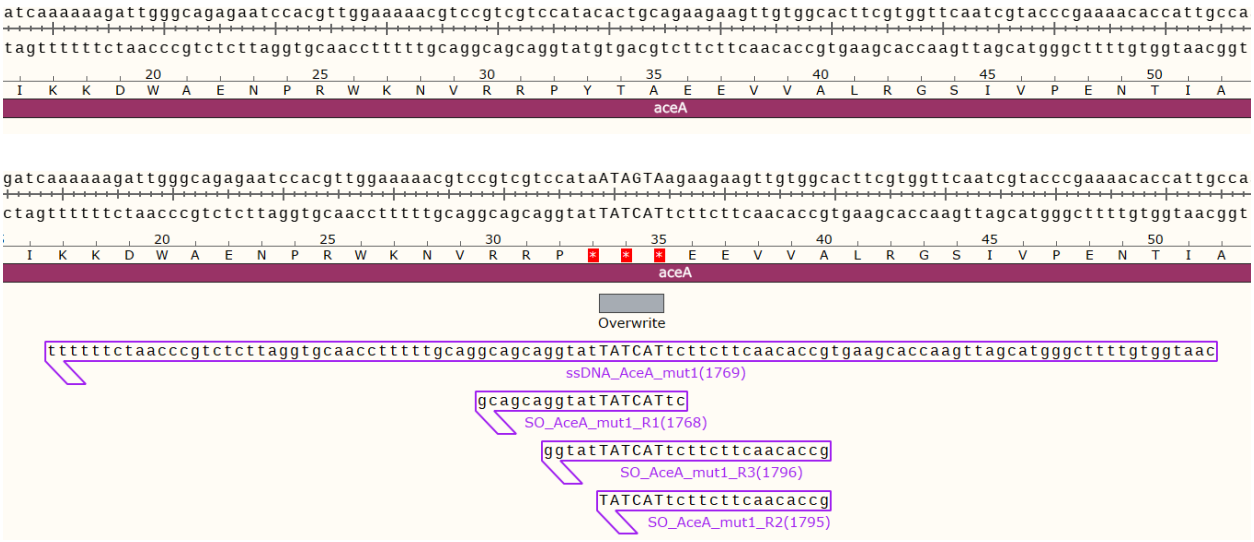


Figure 7: Using the original sequence (above), an ssDNA oligo consisting of a 6-bp overwrite flanked by 50-bp homologous arms was created (below). Reverse primers were designed to overlap with the overwrite.

Primers used to verify knockouts were also designed. Forward primers on the strand opposite to the overwrite were placed approximately 1000 bp upstream of the overwrite, and reverse primers on the same strand were designed to overlap with the overwrite.

When running a PCR experiment using these primers, mutant colonies that have successfully incorporated the overwrite would therefore result in a band at ~1000 bp, while wild type colonies without the overwrite would not display a band. Primers that perfectly overlap with the overwrite at the 3’ end worked the best and while also reducing non-specific PCR products. The optimal annealing temperature for the PCR protocol was determined by running a gradient PCR and choosing the temperature value that led to the strongest band.

Shewanella Screening Procedure and Growth Curve

One way to analyze the result of knocking out certain metabolism pathways is to grow out *Shewanella* in *Shewanella* Basal Media (SBM) supplemented with the metabolite of choice, and then plot the growth curves. For example, when grown in SBM and formate, wildtype *Shewanella* should grow faster than *S. oneidensis* Δfdh. Using this method, the growth curve for *Shewanella* in SBM and acetate could be used to analyze the effect of knocking out *SO_AceA* and *SO_pflB*. SBM can be prepared as follows.

Solid Components

Name	Chemical Formula	Molecular Weight (g/Mol)	Concentration (M)	Mass for 1 L (g)
Ammonium Chloride	NH_4Cl	53.491	0.0086	0.46
Dibasic Potassium Phosphate	K_2HPO_4	174.17605	0.0013	0.225
Monobasic Potassium Phosphate	KH_2PO_4	136.086	0.0017	0.225
Magnesium Sulfate	$MgSO_4.7H_2O$	246.47	0.0005	0.117
Ammonium Sulfate	$(NH_4)_2SO_4$	132.14	0.0017	0.225
Sodium HEPES	HEPES	260.29	0.1000	26.0290

Liquid Components

Name	Volume for 1 L (mL)	Comment
Mineral Mix	5	Add before autoclaving
Vitamin Mix	5	Add aseptically after autoclaving

1. Dissolve all solid components into approximately ¾ of the final total volume (750 mL for 1 L).
2. Add mineral mix aseptically to the solution.
3. Adjust the volume of solution to approximately 95% of the final volume and adjust the pH of the solution to 7.2 using HCl and NaOH.
4. Adjust the volume of the solution to the final volume.
5. Autoclave the solution.
6. Add vitamin mix to the solution aseptically.

Once prepared, SBM stock solution should be stored at 4 °C. The procedure for generating the growth curves is as follows.

1. Grow *Shewanella* overnight in LB media from stock. For best results, start the culture late in the evening.
2. Back dilute to OD 0.02 in LB media early the next day.
3. Grow to OD 0.4-0.5, then back dilute to OD 0.02 in SBM.
4. Measure OD every couple of hours for 24 hours in plate reader, then plot growth curves.

RESULTS

pX2C9pLacW3Beta Plasmid Verification

The pX2C9pLacW3Beta editing plasmid was verified using culture PCR with primers 803/775 and 2037/2038. At the beginning of this project, the plasmid was successfully verified using PCR in the *S. oneidensis* +PX2 strain. As seen in the image below (Fig. 8), bands were present for both primer pairs.

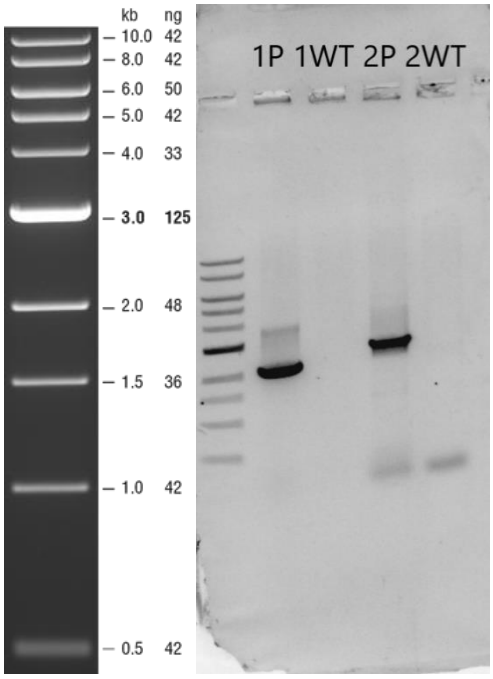


Figure 8: A fragment of about 1997 bp was created from the 803/775 primer pair (1P), and a fragment of about 2497 bp was created from the 2037/2038 primer air (2P). As expected, no bands are present in the wildtype control samples (1WT, 2WT).

After a couple experiments, bands for the 2037/2038 primer set no longer showed up on the gel (Fig. 9). Many attempts were made to troubleshoot this issue, including adjusting the annealing temperature and extension time, growing the cells on different antibiotics, making new primer dilutions from stock, and testing out other primer combinations such as 2036/2037.

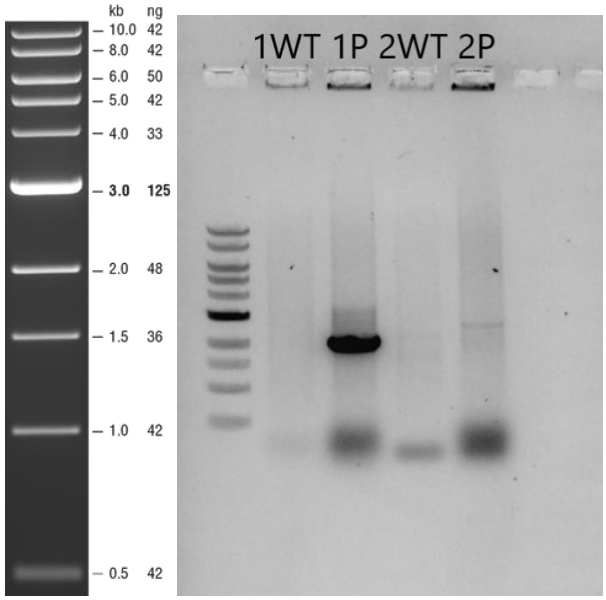


Figure 9: Around one month after the start of this project, the band for the 2037/2038 primer set no longer showed up on the gel.

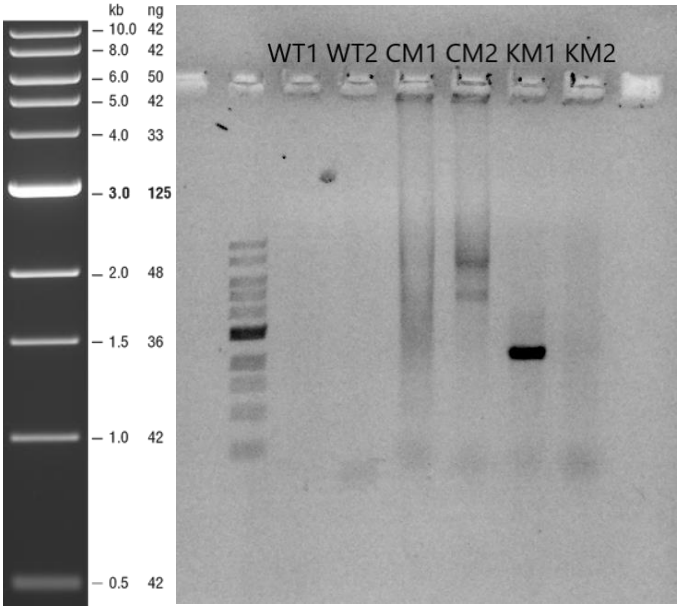


Figure 10*: S. oneidensis* +PX2 was grown on chloramphenicol (Cm20 µM) and kanamycin (Km50 µM), however, a band was only present when grown on kanamycin and using primer pair 803/775.

None of the above methods were successful, so the next option was to try using miniprepped plasmid DNA from *S. oneidensis* +PX2 culture (Fig. 11). For this experiment, a PCR experiment was run using wild type *Shewanella* culture, *S. oneidensis* +PX2 culture, and plasmid DNA from the +PX2 culture.

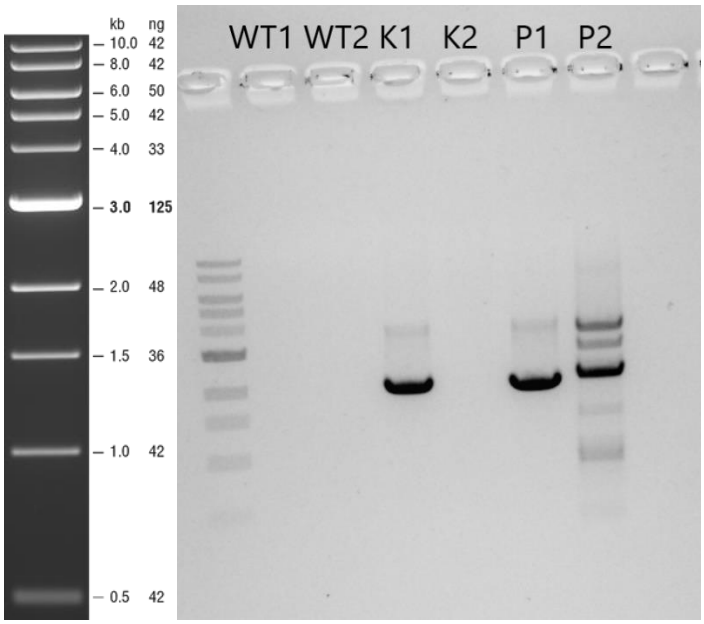


Figure 11: No bands were present in the wild type control lanes (WT1, WT2), a band for only 803/775 was obtained for the *S. oneidensis* +PX2 culture, and bands for both 803/775 and 2037/2038 were present for the plasmid DNA obtained from the miniprep procedure.

From the results of this experiment, it was confirmed that the *S. oneidensis* +PX2 culture did in fact have the entire pX2C9pLacW3Beta plasmid, though for unknown reasons, only the reaction with primer pair 803/775 resulted in a band. In all future experiments, if there was a band for at least one of the primer pairs, it was assumed the entire plasmid was present and the recombineering protocol would work.

During the second semester of the M.Eng. program, the focus of this project was shifted to knocking out *SO_AceA* and *SO_pflB*. Before the recombineering protocol could be used to knock out these genes, the PX2 plasmid was inserted into *S. oneidensis* Δfdh using the transformation protocol above, and the plasmid was verified using PCR and gel electrophoresis (Fig. 12).

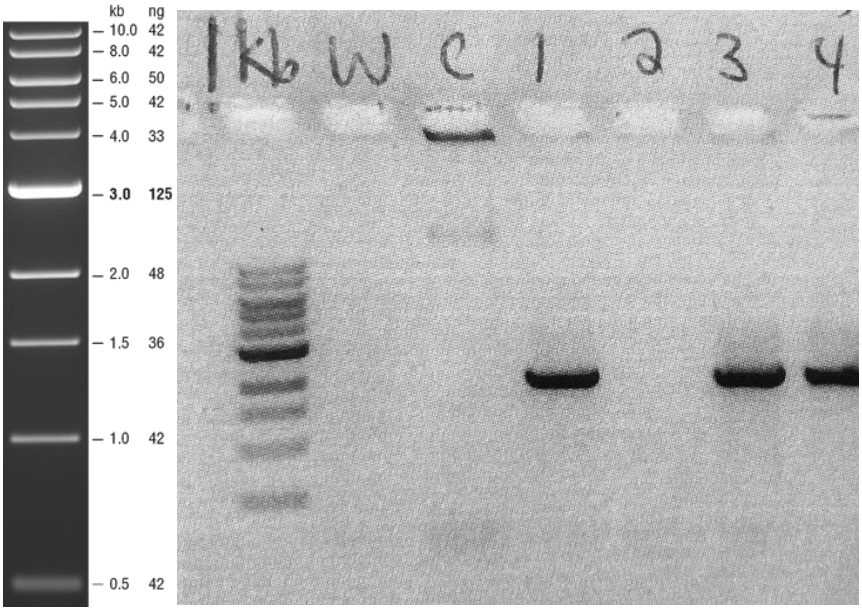


Figure 12: Using primer set 803/775, no bands were present for the wild type (W) and the *S. oneidensis* Δfdh (C) control lanes. Samples 1, 2, and 4 were all grown in LB+Km50 after electroporation, and the plasmid was present in all three. Sample 3 was grown in plain LB after electroporation, and the plasmid was not present as a result.

SO_0181 Knockout

The *SO_0181* gene was knocked out using the recombineering protocol as described above. Two unique ssDNAs were tested: ssDNA_0181_2 (2039) and ssDNA_0181_3 (1265). For 2039, main culture tubes containing transformants were obtained and verified using primer set 1260/1264. Subcultures were grown from the main transformation culture, and individual mutant colonies were identified using PCR and gel electrophoresis. As seen in Figure 13, one mutant colony was discovered from this first recombineering experiment.

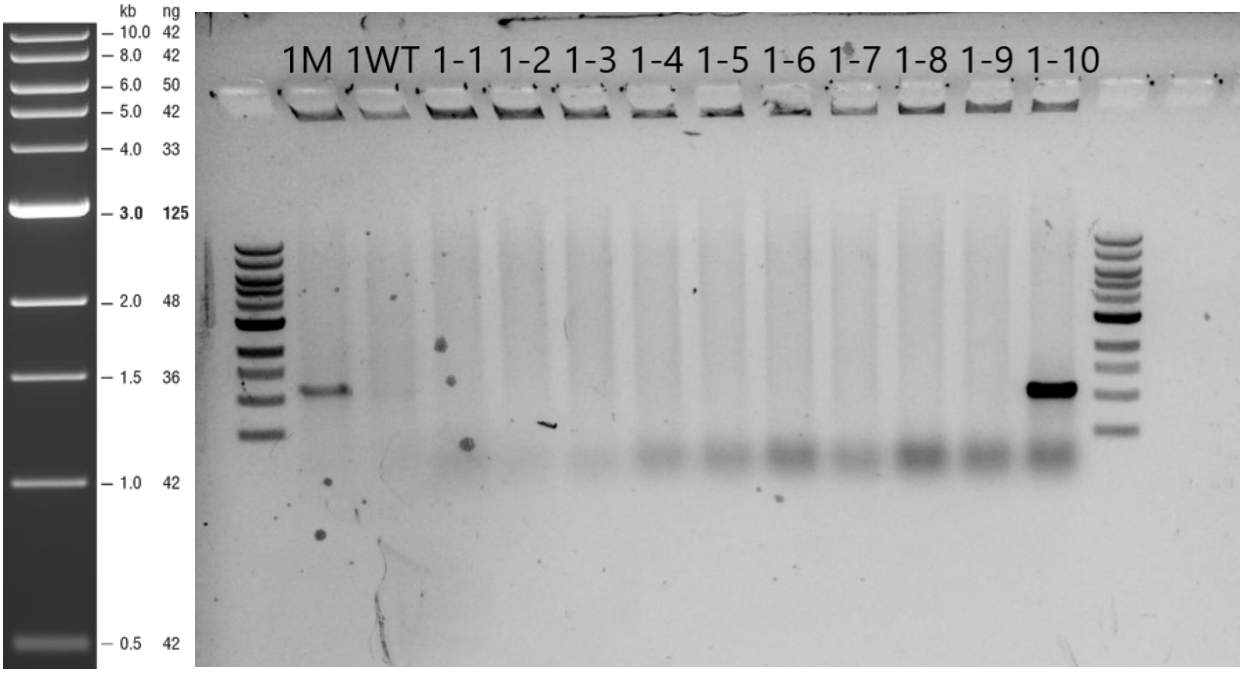


Figure 13: A fragment of about 1011 bp was obtained for the main transformation culture using ssDNA 2039 (1M). No bands were obtained for the wild type control (1WT), as well as the first nine subcultures (1-1 through 1-9). A band was obtained for the tenth subculture (1-10), indicating that this subculture contains mutants.

For 1265, main culture tubes containing transformants were obtained and verified using primer set 1260/1266. Like before, subcultures were grown from the main transformation culture, and individual mutant colonies were identified using PCR and gel electrophoresis. As seen in Figure 14, no mutant colonies were identified from the subcultures.

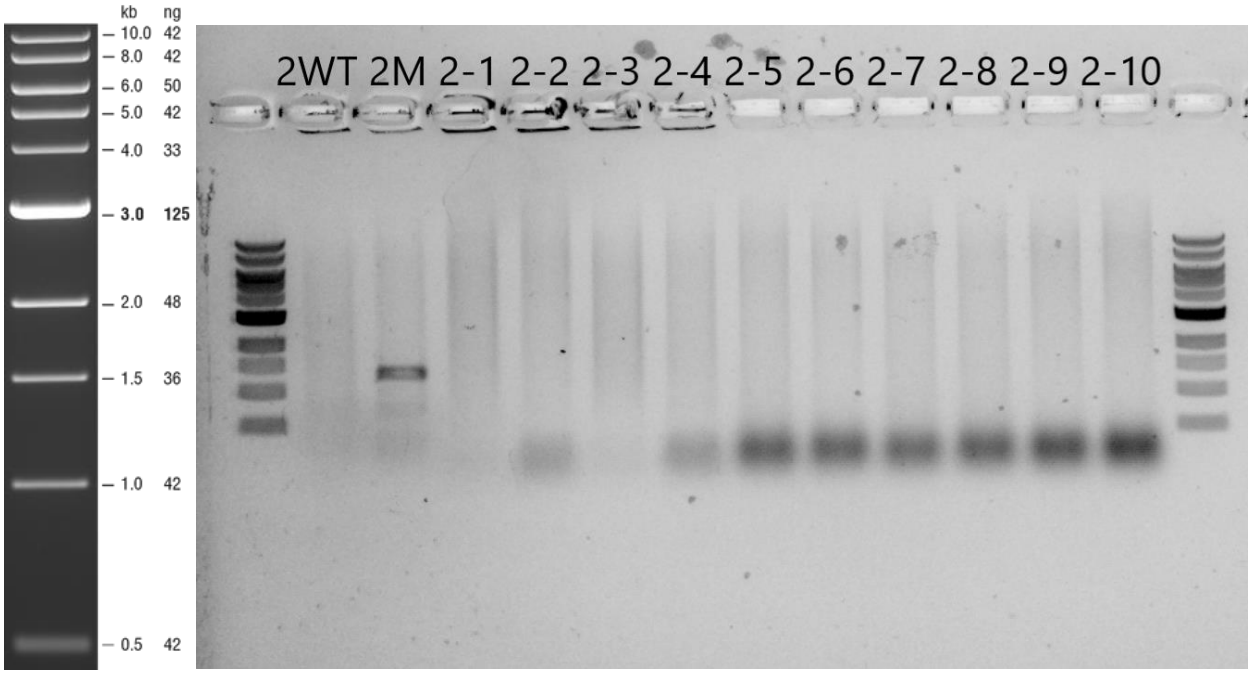


Figure 14: A fragment of about 1004 bp was obtained for the main transformation culture (2M). No bands were obtained for the wild type control (2WT), as well as all ten subcultures (2-1 through 2-10).

From these first experiments, it was determined that the recombineering protocol worked, albeit with an extremely low success rate. In following experiments, to increase the throughput of the recombineering process, colonies grown from the main transformation culture were picked using the automated colony picker and plated on 96-well plates with LB+Km50. Copies of all plates were made and cryopreserved by mixing an equal amount of culture and 20% glycerol solution, then frozen at -80 °C. As seen in Figures 15 and 16, no mutants were found when using ssDNA 1265, and one mutant colony was found when using ssDNA 2039 in well 12B.

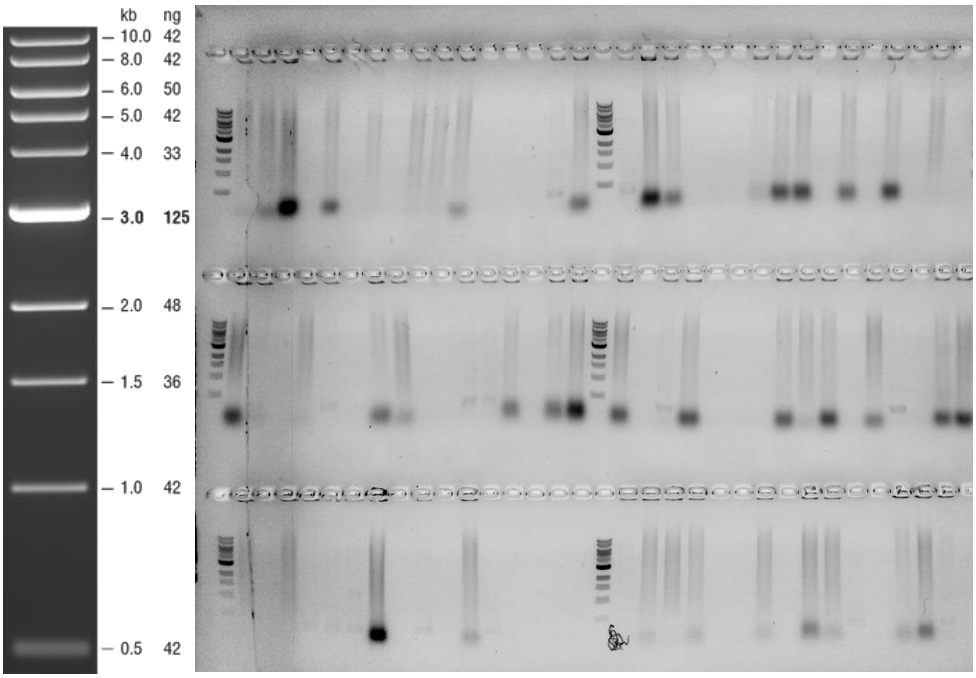


Figure 15: None of the subcultures from the main transformation culture using ssDNA 1265 contained mutants.

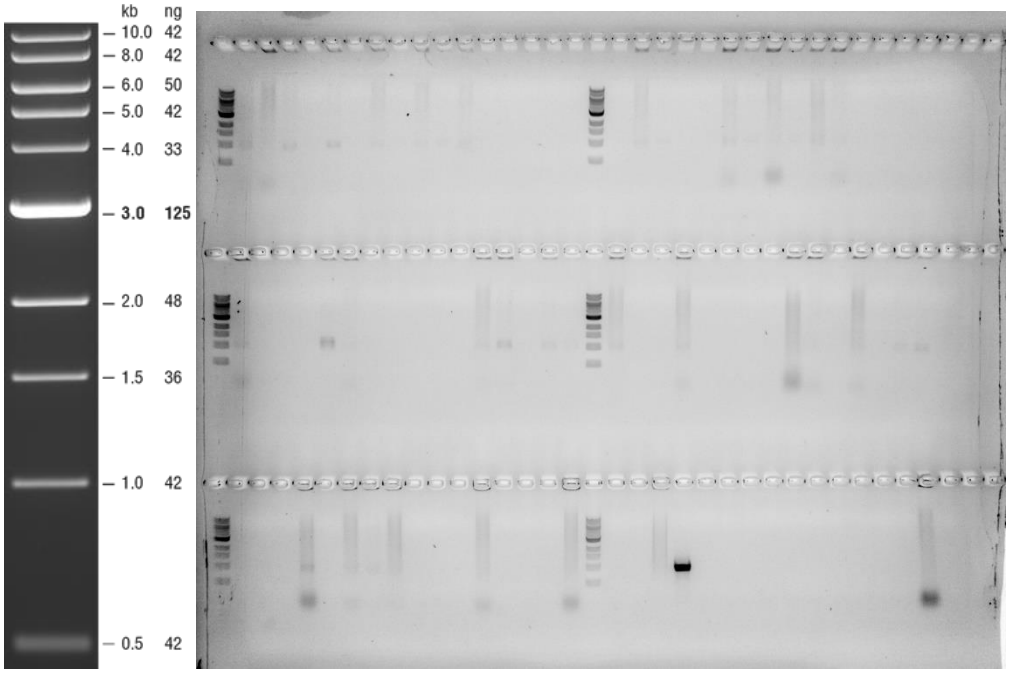


Figure 16: One subculture (12B) from the main transformation culture using ssDNA 2039 contained mutants.

To verify that the transformant found in Figure 16 retained the mutation, the transformant culture was recovered from glycerol stock and struck out on LB+Km50 plates. However, none of the resulting colonies contained the mutation. It is possible that the original colony containing the transformant was not pure, and the glycerol stock contains a mixture of wild type and mutant cells, making it difficult to isolate a purely mutant colony.

SO_AceA Knockout

After inserting the editing plasmid into *S. oneidensis* Δfdh, the *SO_AceA* gene was knocked out using ssDNA_AceA_mut1 (1769). Initially, the mutation was verified using the primer set 1767/1768, however, this led to non-specific binding in both the wild type and mutant cultures even when varying the annealing temperature.

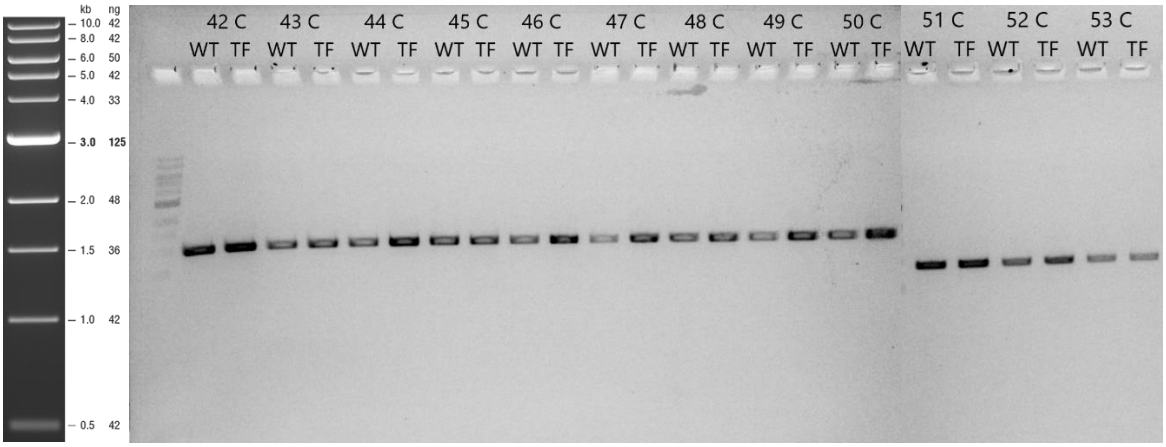


Figure 17: Primer set 1767/1768 showed non-specific binding even with different annealing temperatures.

Three more reverse primers were ordered in order to troubleshoot this error: 1770, 1795, and 1796. Ultimately, the primer set 1767/1795 gave the best results, and a gradient PCR was run in order to determine the optimal annealing temperature (Fig. 18). Once the primers were sorted out, they could then be used to verify the *SO_AceA* knockout. Over the course of one semester, two recombineering experiments were conducted, and both successfully yielded transformants (Fig. 19).

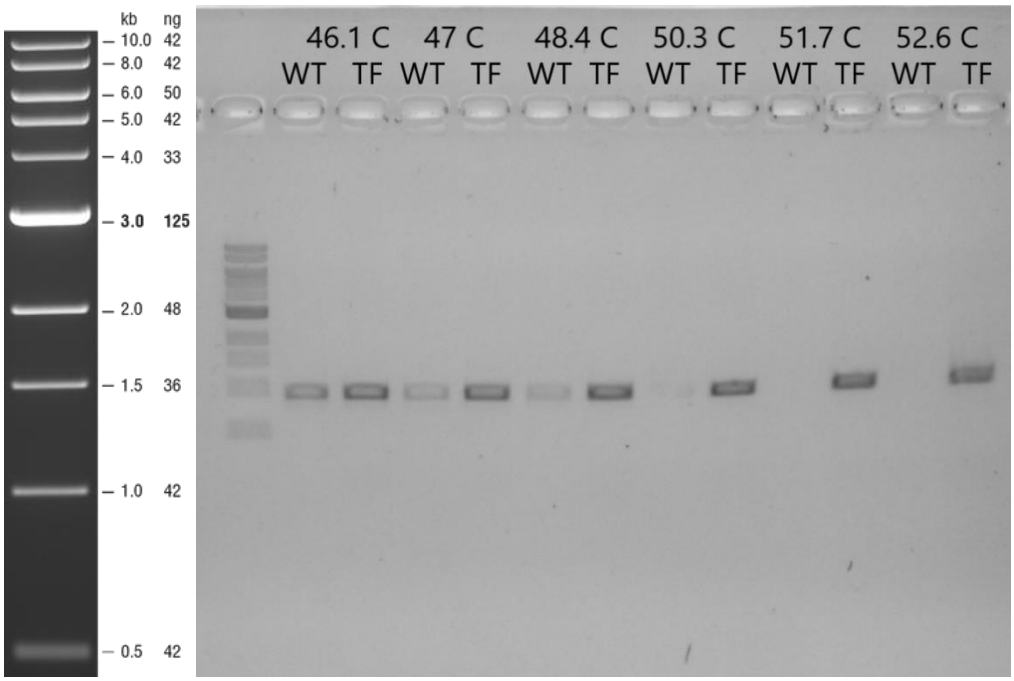


Figure 18: A gradient PCR was run to find the optimal annealing temperature for primer set 1767/1795. Fragments of about 929 bp in length were obtained, and the best temperature was found to be between around 52 °C.

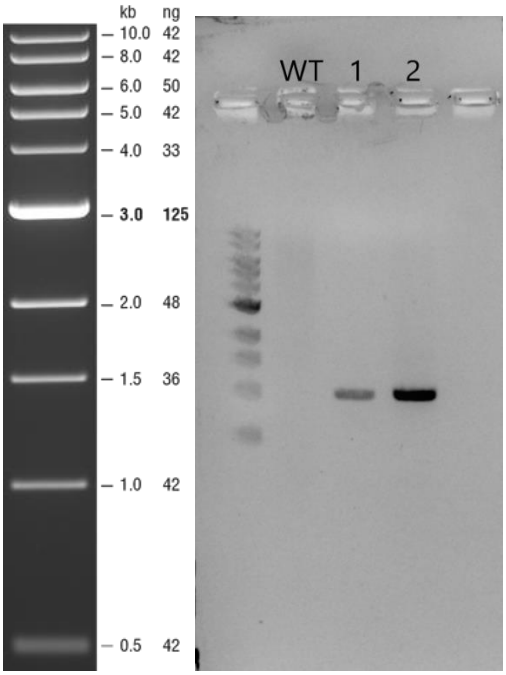


Figure 19: The wild type (WT) showed no band, as expected. The recombineering experiments from 2/18/22 (#1) and 4/13/22 (#2) both showed fragments of about 929 bp in length.

While the first recombineering experiment (2/18/22) yielded a mixture of wild type and mutant cells, no individual mutant colonies were found. For the second recombineering experiment (4/13/22), minor tweaks were made in an attempt to increase the transformation rate, such as allowing the culture to recover in a culture tube rather than Eppendorf tube in order to allow for more oxygen. After verifying the presence of *SO_AceA* knockout using PCR and gel electrophoresis, at least one colony containing mutants was found (Fig. 20).

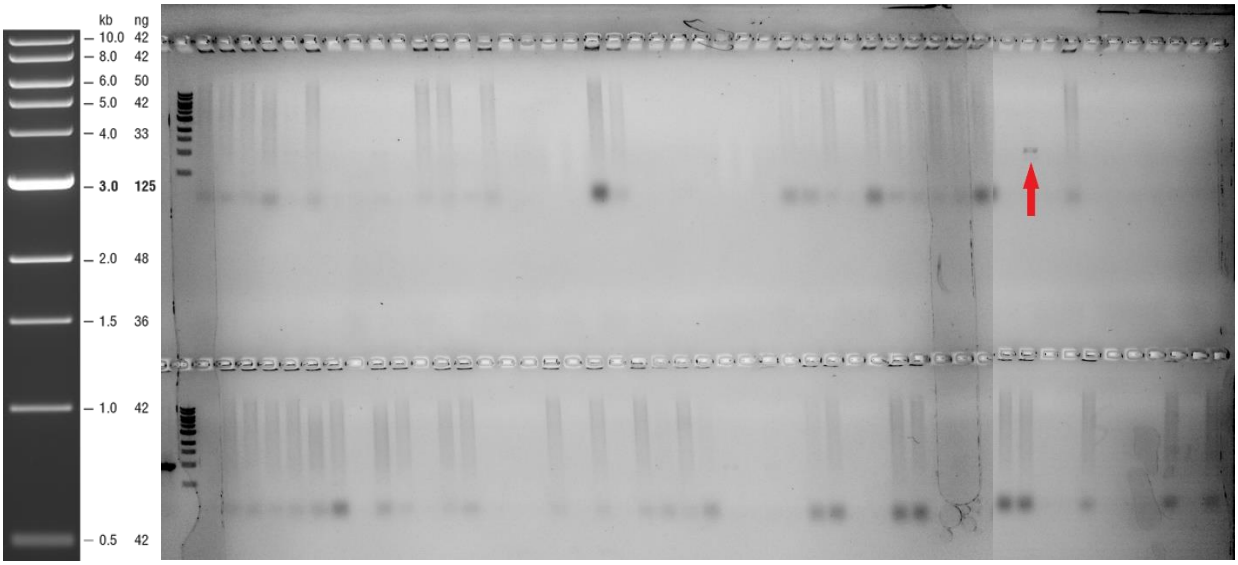


Figure 20: For the second recombineering experiment using ssDNA 1769, several samples in the 96-well plate showed very faint bands, indicating a possibility of containing mutants. The strongest band, marked with the red arrow, is located in well 5D.

Next, cells from well 5D were struck out from the glycerol stock and grown on LB+Km50 plates as well as in LB+Km50 liquid culture tubes. Wells 9E and 12B were also struck out, since they showed extremely faint bands. After multiple trials, no bands were found for any of the three samples. It is possible that the wells contain very minute amounts of mutant cells, and the majority of cells are wild type. Unfortunately, due to the limited amount of time, the *SO_pflB* knockout was not carried out. However, potential primers and ssDNAs were designed in anticipation of future experiments that may expand on the work done during this project.

Growth Curve for wild type and S. oneidensis Δfdh in Shewanella Basal Media

The growth rates for the wild type and Δfdh strains in SBM, SBM + 0.5 M acetate, SBM + 0.5 M formate, and SBM + 0.275 M lactate were plotted using a plate reader. The plate reader was set to shake at a fast, continuous rate while maintaining a temperature of 30 °C, and OD readings were taken at 1-hour intervals for 24 hours (25 reads in total). The 96-well plate was set up according to *Table 1* below.

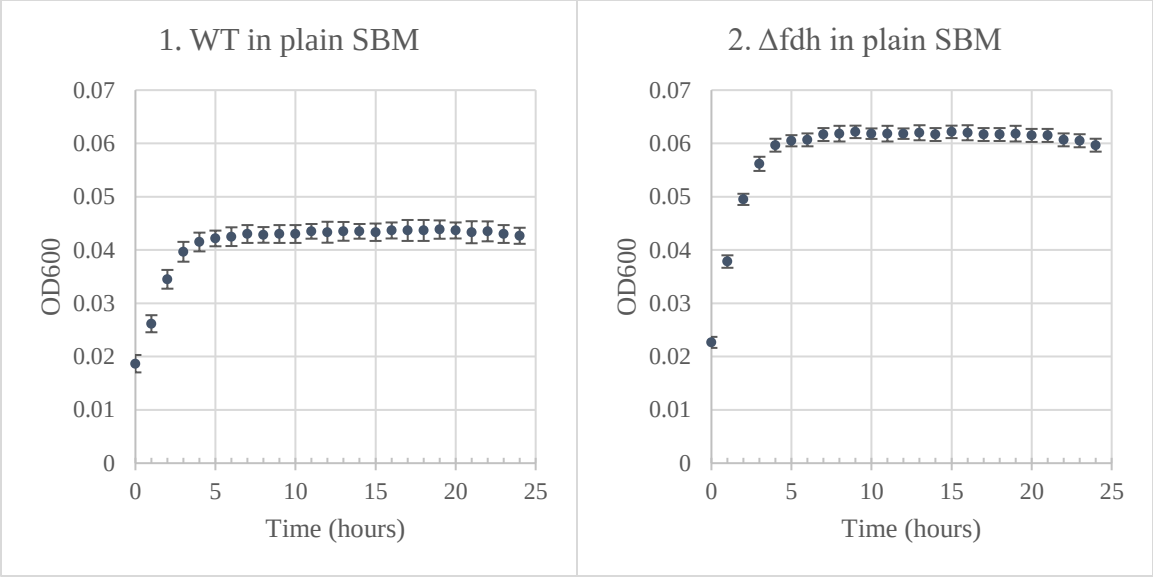
Table 1. 96-well setup to plot growth curves for wild type and *S. oneidensis* Δfdh.

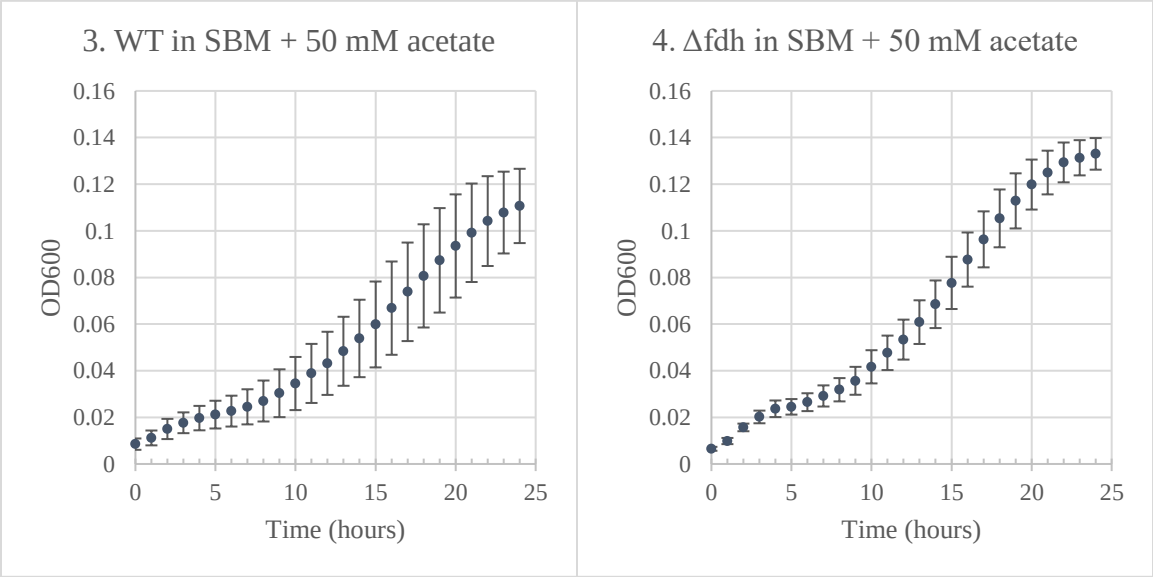
	1	2	3	4	5	6	7	8	9	10	11	12
A	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O
B	H_2O	SBM	1	2	3	4	5	6	7	8	SBM	H_2O
C	H_2O	SBM	1	2	3	4	5	6	7	8	SBM	H_2O
D	H_2O	SBM	1	2	3	4	5	6	7	8	SBM	H_2O
E	H_2O	SBM	1	2	3	4	5	6	7	8	SBM	H_2O
F	H_2O	SBM	1	2	3	4	5	6	7	8	SBM	H_2O
G	H_2O	SBM	1	2	3	4	5	6	7	8	SBM	H_2O
H	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O

The wells containing water were placed around the perimeter of the plate in order to prevent evaporation from the rest of the plate, and the plain SBM wells acted as blanks for calibration. The remaining rows are as follows.

1. *S.O.* wild type in plain SBM (negative control).
2. *S.O.* Δfdh in plain SBM (negative control).
3. *S.O.* wild type in SBM + 50 mM acetate.
4. *S.O.* Δfdh in SBM + 50 mM acetate.
5. *S.O.* wild type in SBM + 50 mM formate.
6. *S.O.* Δfdh in SBM + 50 mM formate.
7. *S.O.* Δfdh in SBM + 50 mM lactate (positive control).
8. *S.O.* wild type in SBM + 50 mM lactate (positive control).

After 24 hours, the data collected were exported to an Excel spreadsheet, and the OD were plotted against the time of each reading (Fig. 21).


1. WT in plain SBM
OD600
Time (hours)
2. Δfdh in plain SBM
OD600
Time (hours)


3. WT in SBM + 50 mM acetate
OD600
Time (hours)
4. Δfdh in SBM + 50 mM acetate
OD600
Time (hours)

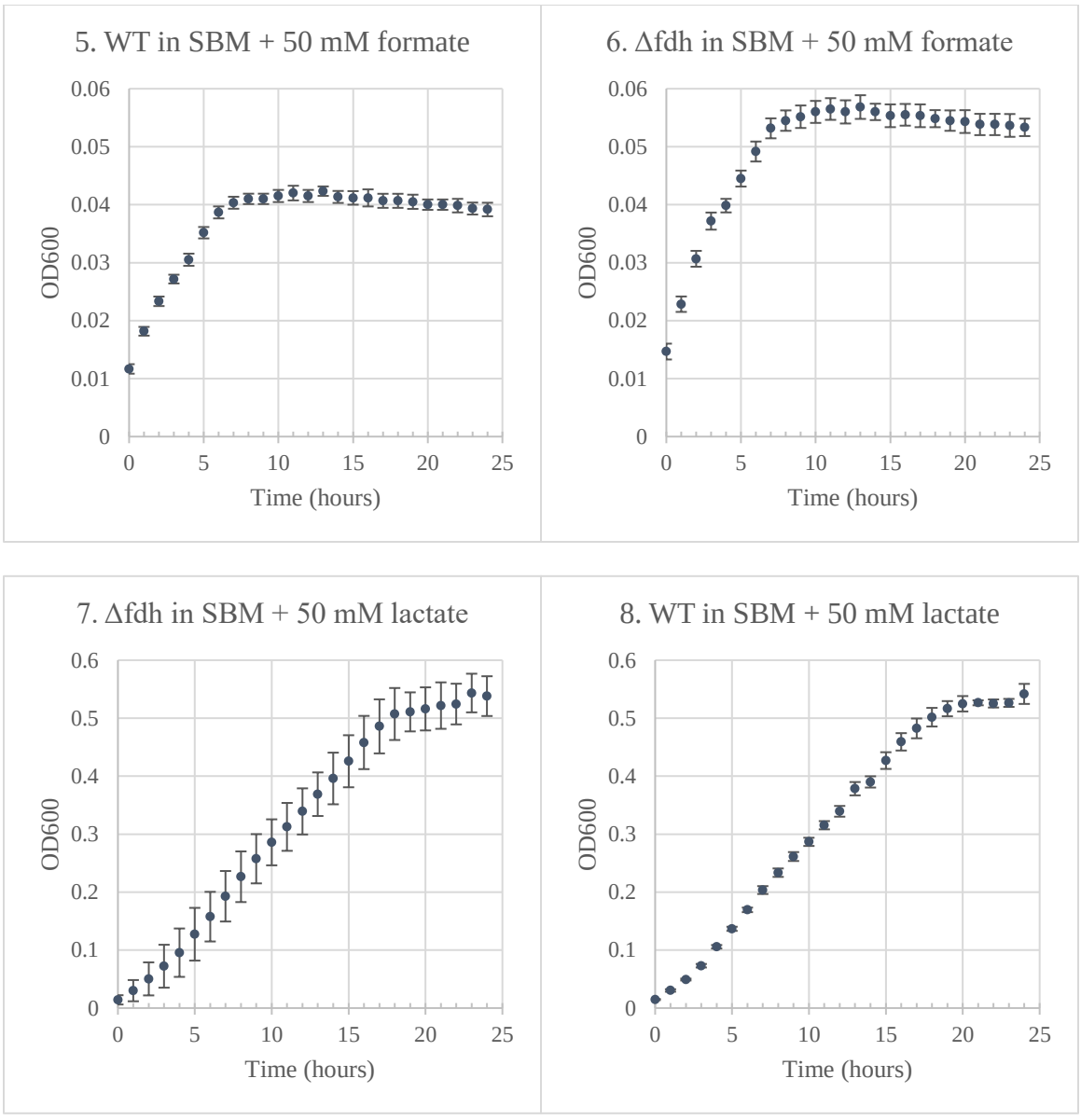


Figure 21: The growth curves for *S.O.* wild type and *S.O.* Δfdh in SBM, SBM + acetate, SBM + formate, and SBM + lactate were created by plotting the OD600 readings against time.

For plain SBM, both the wild type and Δfdh strains showed initial growth but rapidly tapered off due to the lack of nutrients in the solution. For SBM + acetate, both strains showed more growth than in plain SBM, and both reached exponential phase at around 10–20 hours. For SBM + formate, both strains showed rapid initial growth but tapered off quickly. In the Δfdh strain, the lack of growth is likely due to the formate dehydrogenase gene knockouts. The wild-type strain

was expected to grow well in SBM + formate, but the lack of growth could be because *S. oneidensis* uses formic acid only as an electron carrier, and there is no other carbon source that allows for further growth [Serre2006a]. Finally, for SBM + lactate, both strains reach exponential phase quickly and grew rapidly compared to the other growth conditions.

DISCUSSION

ssDNA recombineering is a simple and efficient method for knocking out genes in *Shewanella oneidensis* MR-1. The editing plasmid and ssDNA can be transformed into *S. oneidensis* using the transformation and recombineering protocols, respectively, and the ssDNA oligos and primers for verification are easy to design and inexpensive. In total, the transformation and recombineering protocols each take about 3 to 4 days to implement. This timeframe includes about 1 to 2 days for setup and preparation, 1 day for the electroporation and recovery steps, and 1 day to allow for growth after electroporation. The effort and time needed to carry out this recombineering process is much lower in comparison to traditional methods, such as transposon mutagenesis and targeted knockouts using suicide vectors [Corts2019a, Corts2019b].

However, the transformation efficiency for this process is extremely low. Over the course of this project, only a handful of transformants were recovered. No transformants were found when using ssDNA 1265 to knock out *SO_0181*, 2 transformants were found when using ssDNA 2039 to knock out *SO_0181*, and at least 1 transformant was found when using ssDNA 1769 to knock out *SO_AceA*. Out of a total a few hundred colonies, the success rate for experiments done in this project was well below the estimated 5% that Corts *et al.* reported [Corts2019a]. To increase recombination rate in future experiments, some minor tweaks to the recombineering protocol could be tested out. For example, growing the overnight cultures to a higher OD (~0.6-0.7) may lead to a higher number of transformants, and washing the cells in glycerol instead of sorbitol could increase the recombination rate. In addition, during the electroporation step, it was found that the electroporator would consistently undershoot the target voltage (12 kV), and increasing the voltage to accommodate for this discrepancy could help boost the recombination rate.

Finally, the recovery step could also have an effect on the recombination rate–Corts *et al.* suggests a recovery period of about 2 hours, though increasing the recovery period could potentially allow for more cells to take up and retain the ssDNA oligos.

The ssDNA oligo design could possibly affect the recombination rate as well. In a study done by Wang *et al.*, the researchers used multiplex automated genome engineering (MAGE), in which the ssDNA recombineering process is automated in order to simultaneously modify many genomic locations at once. As seen in Figure 22, they measured the recombination efficiency for oligos up to 30 bp in length, and they found that oligos of about 5 bp or less in length had the highest efficiency [Wang2009a].

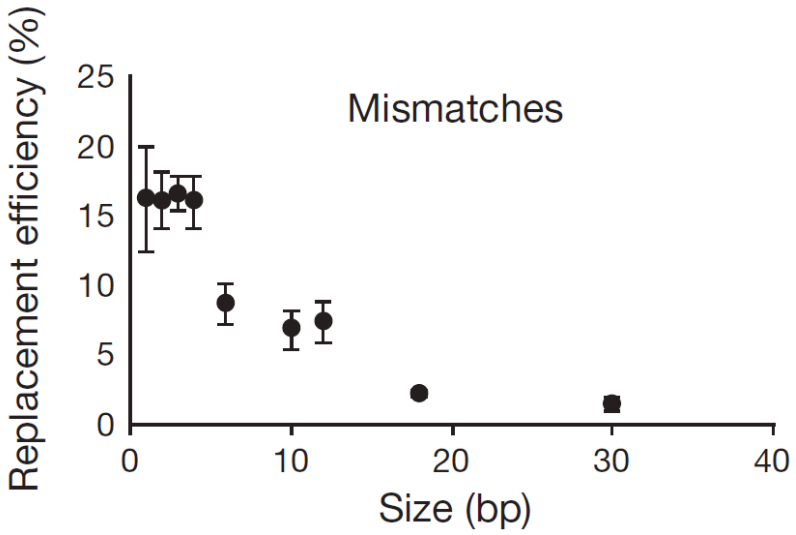


Figure 22: Increasing the size of the ssDNA oligos that result in point mutations can lead to a decrease in recombination efficiency [Wang2009a].

It is not clear if this correlation found by Wang *et al.* is consistent with the results obtained in this project. Comparing the two ssDNA oligos used for the *SO_0181* knockout, the overwrite is 15-bp long in ssDNA 1265 and 9-bp long in ssDNA 2039. ssDNA 2039 yielded more transformants than ssDNA 1265, and the difference in recombination rate could be explained by

the correlation described by Wang *et al.* Taking this into account, the ssDNA oligo used for the *SO_AceA* knockout was designed to have a 6-bp overwrite, and the ssDNA oligo for the *SO_pflB* knockout was designed to have a 5-bp overwrite. After carrying out multiple recombineering experiments to knock out *SO_0181* and *SO_AceA*, the recombination rate when using the oligo with a 6-bp mismatch was not noticeably higher than the rate when using the oligo with a 9-bp mismatch. To optimize the recombination rate for recombineering in *S. oneidensis*, further experimentation should be conducted in order to determine the optimal mismatch length.

Next Steps: SO_pflB Knockout

Due to the low recombination rate and difficulty faced when isolating a colony with the *SO_AceA* knockout, there was not enough time remaining in the semester to work on the *SO_pflB* knockout experiment. Once an *SO_AceA* knockout is successfully acquired, the next step would be to implement the recombineering protocol using an ssDNA oligo designed to knock out *SO_pflB.* The ssDNA oligo and primers for this phase of the project were designed using the same method as the *SO_AceA* knockout experiment, and they are listed in *Table 2* in the Appendix. As seen in Figure 23, 5 base pairs were overwritten, leading to an ssDNA sequence of 105 bp. After executing the recombineering protocol, the presence of both the *SO_AceA* and *SO_pflB* knockout mutations can then be verified using PCR and gel electrophoresis.

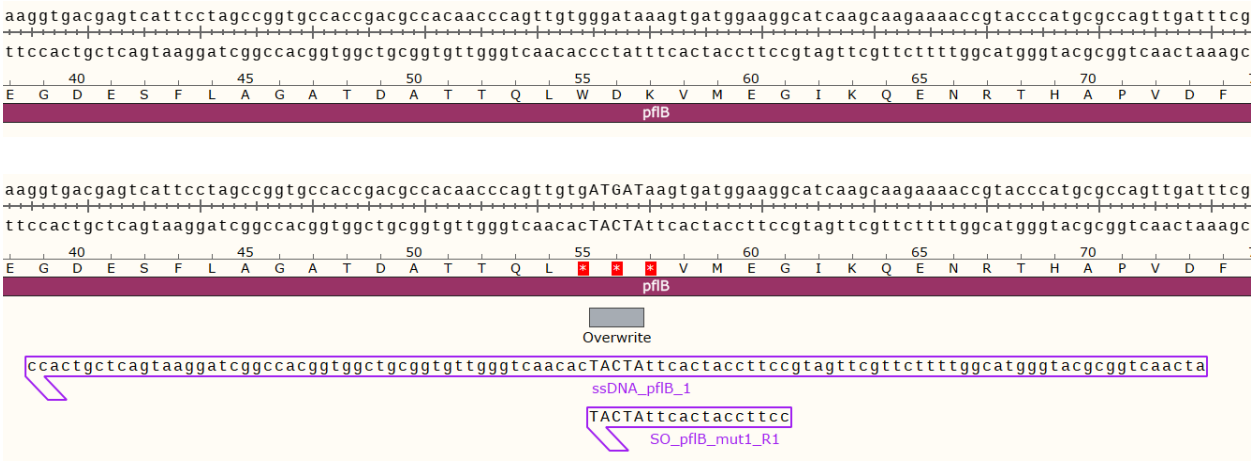


Figure 23: Using the original sequence (above), an ssDNA oligo consisting of a 5-bp overwrite flanked by 50-bp homologous arms was created (below). A reverse primer was designed to overlap with the overwrite.

Next Steps: Recombineering with CRISPR-Cas9 and Multiplex Automated Genetic Engineering

From the results of this study, it was found that recombineering without a selection factor is ineffective. Even in a highly optimized system as developed by Corts *et al.*, recombineering alone can only achieve a recombination rate of about 5%. In a more recent study by Corts *et al.*, the researchers found that coupling ssDNA recombineering with CRISPR/Cas9-mediated counter-selection can improve the recombination rate to over 90% regardless of the target gene. This system uses two plasmids: an sgRNA targeting vector and an editing vector that contains Cas9 and W3 Beta, such as pX2C9pLacW3Beta [Corts2019b].

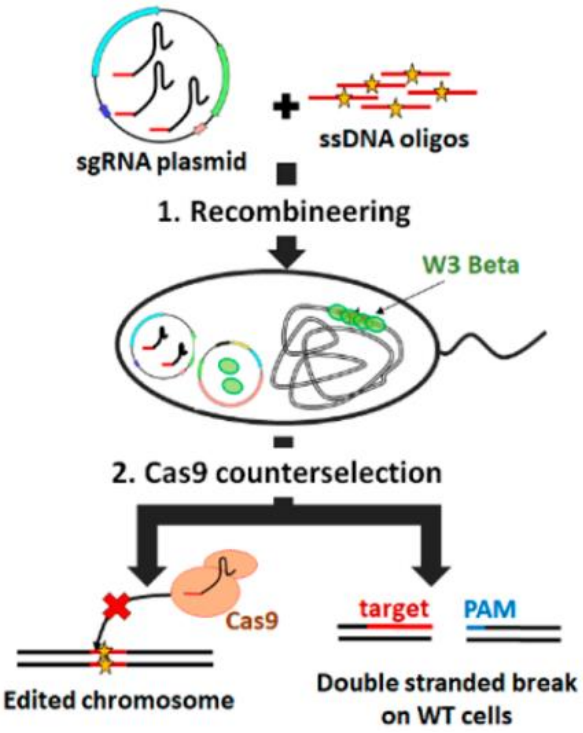


Figure 24: Coupling ssDNA recombineering with CRISPR/Cas9-mediated counter-selection can drastically improve the recombination rate [Corts2019b].

In bacteria, clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR associated) systems are used as part of their adaptive immune system. The CRISPR loci is an array that is made up of short repetitive sequences and spacers, which match regions of DNA present by viruses. This allows the bacterium to keep a record of previous foreign invasions and guard against future encounters. Transcription of this array leads to the formation of CRISPR RNAs, or crRNAs, which then guide the Cas machinery to cleave a specific region of the target DNA that is complementary to the spacer sequence known as the protospacer. In addition, many CRISPR/Cas systems require the presence of a protospacer-adjacent motif (PAM), which prevent autoimmunity against the spacers from the main CRISPR array. Cas9 nucleases cleave foreign DNA through the combined activity of their RuvC and HNH domains, which each nick one strand of DNA to create a blunt-ended DSB. Most bacteria lack nonhomologous end joining

(NHEJ) activity, therefore this cut is usually lethal. Using this system developed by Corts *et al.* (Fig. 25), the Cas9 protein is able to eliminate chromosomes carrying the wild-type DNA sequence and therefore enhance selection of genetic alterations introduced by recombineering [Corts2019b].

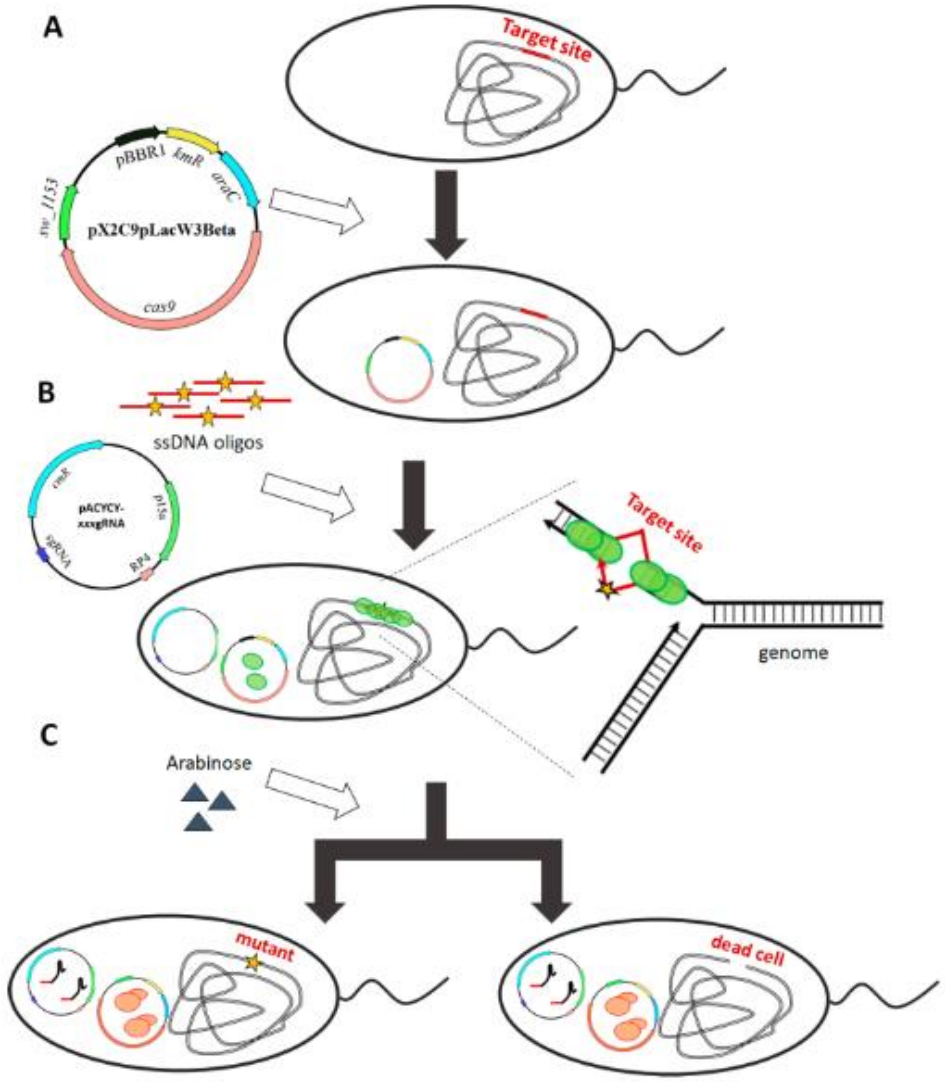


Figure 25: A) pX2C9pLacW3Bet is an editing plasmid that encodes the Cas9 protein as well as the W3 Beta recombinase, and this is introduced into host cells first by electrotransformation. B) pACYC-xxxgRNA is a plasmid that contains the gRNA to target the gene of interest, and this plasmid is co-transformed with ssDNA oligos to generate the desired target mutations. After a period of recovery, cells are plated on LB solid agar medium with kanamycin and chloramphenicol, then supplemented with arabinose to induce Cas9-mediated counter-selection [Corts2019b].

Even though the efficiency rate with this CRISPR/Cas9 is much improved, it can still be relatively a relatively slow process if many genes need to be knocked out. Multiplex automated genome engineering (MAGE) can be used for large-scale programming and evolution of cells, and using this technique, multiple locations on the chromosome can be targeted simultaneously. Wang *et al.* constructed an automated MAGE system that allows for a cyclical and scalable process to produce combinatorial genomic diversity in *E. coli*, and they tested this process using 24 genes in the lycopene pathway (Fig. 26) [Wang2009a].

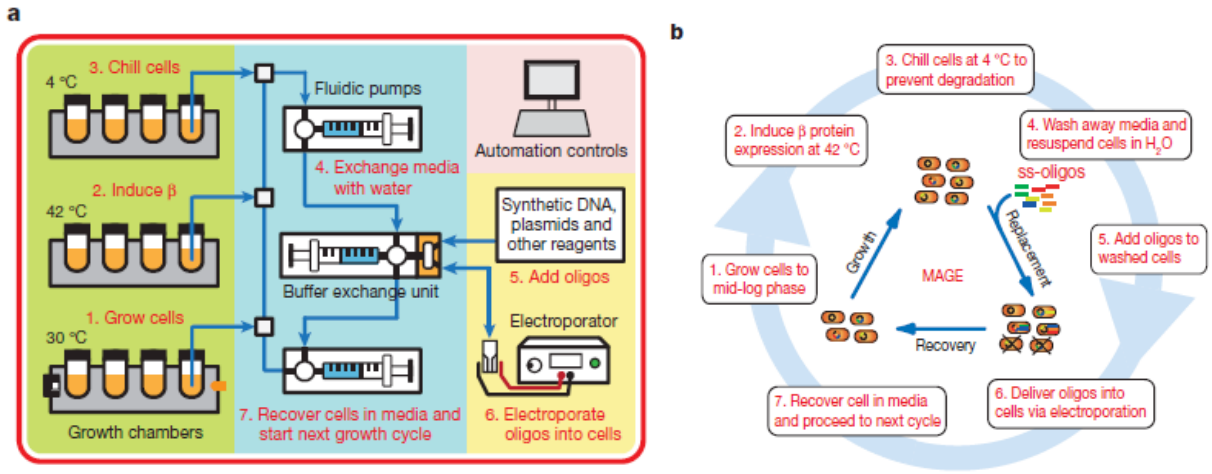


Figure 26: A) Overall schematic of the MAGE process used for editing genes in *E. coli*. B) MAGE automation has a total run time of about 2–4 hours per cycle. In each cycle, ssDNA oligos are added using electroporation at 18 kV cm^{-1}, and each electroporation event also serves to dilute the cell population, which is grown to mid-log phase again to begin the next cycle [Wang2009a].

In more recent studies, researchers have developed a CRISPR optimized MAGE recombineering (CRMAGE) system, which combines CRISPR/Cas9 and λ Red recombineering based MAGE technology to allow for efficient and fast genome engineering in *E. coli.* CRMAGE can be multiplexed and enables introduction of at least two mutations in a single round of recombineering. Using CRMAGE, Ronda *et al.*, were able to achieve a recombineering efficiency of between 96.5% and 99.7% for a group of three target genes [Ronda2015a]. The

CRMAGE platform also utilizes CRISPR/Cas9 for plasmid curing since the presence of too many plasmids in a cell creates a metabolic burden. CRMAGE allows for highly efficient genome editing, and further research in this topic may open up promising opportunities for automation of genome-scale engineering. Extending the MAGE and CRMAGE platforms to *S. oneidensis* would help enable the understanding of the extracellular electron uptake mechanism as well as boost research in engineering microbial systems for electrical energy storage.

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APPENDIX

Table 2. List of Primers and ssDNA Oligos

Name	Sequence (‘5 – ‘3 Orientation)	Function
pACYC-BB-R1 (775)	CCACACAACATACGAGCCG	Reverse primer for verification of *pX2C9pLacW3Beta*
cas9_ (803)	ATTAAAAAAGGTATTTTAC AGACT GTAAAAGTTGTTGA	Forward primer for verification of *pX2C9pLacW3Beta*
pX2_F (2036)	AAGGGCCTCGTGATACGC	Forward primer for verification of *pX2C9pLacW3Beta*
pX2_R (2037)	TCTTAAAGTTCGTATCTCCGCCTAT	Reverse primer for verification of *pX2C9pLacW3Beta*
repA_pBBR1 (2038)	GCTGTTGGGGCATGGC	Forward primer for verification of *pX2C9pLacW3Beta*
ssDNA_0181_3 (1265)	ATTGGCAAAAGTTGGTGAGAGTCA TAACCTTGCCAAGCTCCCTCAGGGC GGTTGGATTATCCGGCTATCATGTT CAGCGTTGCTGCTGTTCATGAATAT CAATCATTAGCTGGCCATTGGGTA GAAGTTAATGTA	ssDNA for *SO_0181* knockout
ssDNA_0181_2 (2039)	GAGAGTCATAACCTTGCCAAGCTC CCTCAGGGCGGTTGGATTATGAAC TATTACTTATTAGCGTTGCTGCTGT TCATGAATATCAATCATTAGCTGGC CATTGGGTAGAAG	ssDNA for *SO_0181* knockout
Recom_0181_F (1260)	AACGTCTATGGCAAAAATG	Forward primer for verification of *SO_0181* knockout using ssDNAs 1265 and 2039
Recom_0181_R3 (1264)	GTTGGATTATGAACTATTACTTATT	Reverse primer for verification of *SO_0181* knockout using ssDNA 2039

Recom_0181_R4 (1266)	ATTGGCAAAAGTTGGTGAGAGTCA TAACCTTGCCAAGCTCCCTCAGGGC GGTTGGATTATCCGGCTATCATGTT CAGCGTTGCTGCTGTTCATGAATAT CAATCATTAGCTGGCCATTGGGTA GAAGTTAATGTA	Reverse primer for verification of *SO_0181* knockout using ssDNA 1265
SO_AceA_F1 (1767)	CTCCAGACTGTTGATCAA	Forward primer for verification of *SO_AceA* knockout using ssDNA 1769
SO_AceA_mut1_R1 (1768)	CTTACTATTATGGACGACG	Reverse primer for verification of *SO_AceA* knockout using ssDNA 1769
ssDNA_AceA_mut1 (1769)	CAATGGTGTTTTCGGGTACGATTGA ACCACGAAGTGCCACAACTTCTTCT TACTATTATGGACGACGGACGTTTT TCCAACGTGGATTCTCTGCCCAATC TTTTTT	ssDNA for *SO_AceA* knockout
SO_AceA_R1 (1770)	GAGCCACCGTTAACG	Reverse primer for verification of *SO_AceA* knockout using ssDNA 1769
SO_AceA_mut1_R2 (1795)	GCCACAACTTCTTCTTACTAT	Reverse primer for verification of *SO_AceA* knockout using ssDNA 1769
SO_AceA_mut1_R3 (1796)	GCCACAACTTCTTCTTACTATTATG G	Reverse primer for verification of *SO_AceA* knockout using ssDNA 1769
ssDNA_pflB_1	ATCAACTGGCGCATGGGTACGGTT TTCTTGCTTGATGCCTTCCATCACT TATCATCACAACTGGGTTGTGGCGT CGGTGGCACCGGCTAGGAATGACT CGTCACC	Potential ssDNA for *SO_pflB* knockout
SO_pflB_mut1_R1	CCTTCCATCACTTATCAT	Potential reverse primer for verification of *SO_pflB* knockout

SO_pflB_F1	GCCTGTTAAAATATCGTC	Potential forward primer for verification of *SO_pflB* knockout

Table 3. List of Organisms

*All organisms are *Shewanella oneidensis* MR-1.

Location	Name/Description	Plasmid/Genetic Background
BOX 1 #11	*S. oneidensis* wild type	Wild type
BOX 1 #44	*S. oneidensis* +PX2	pX2C9pLacW3Beta editing plasmid
BOX 1 #82	*S. oneidensis* Gralnick (JG2255) Δfdh	Clean deletion of *SO_Δfdh*
S.O. BOX II #1	*S. oneidensis* TF1	Mixture of wild type and transformants with deletion of *SO_0181* using ssDNA 2039
S.O. BOX II #2	*S. oneidensis* TF1 (Backup)	Mixture of wild type and transformants with deletion of *SO_0181* using ssDNA 2039
S.O. BOX II #3	*S. oneidensis* TF2	Mixture of wild type and transformants with deletion of *SO_0181* using ssDNA 1265
S.O. BOX II #4	*S. oneidensis* TF2 (Backup)	Mixture of wild type and transformants with deletion of *SO_0181* using ssDNA 1265
S.O. BOX II #5	*S. oneidensis* Δfdh +PX2	Clean deletion of *SO_Δfdh* and pX2C9pLacW3Beta editing plasmid
S.O. BOX II #6	*S. oneidensis* Δfdh +PX2 (Backup)	Clean deletion of *SO_Δfdh* and pX2C9pLacW3Beta editing plasmid
S.O. BOX II #7	*S. oneidensis* Δfdh ΔAceA +PX2	Mixture of *S. oneidensis* Δfdh +PX2 and transformants with deletion of *SO_AceA* using ssDNA 1769

S.O. BOX II #8	*S. oneidensis* ΔfdhΔAceA +PX2 (Backup)	Mixture of *S. oneidensis* Δfdh +PX2 and transformants with deletion of *SO_AceA* using ssDNA 1769
S.O. BOX II #9	*S. oneidensis* ΔfdhΔAceA +PX2 5D	Mixture of *S. oneidensis* Δfdh +PX2 and transformants with deletion of *SO_AceA*
S.O. BOX II #10	*S. oneidensis* ΔfdhΔAceA +PX2 5D (Backup)	Mixture of *S. oneidensis* Δfdh +PX2 and transformants with deletion of *SO_AceA*
S.O. BOX II #11	*S. oneidensis* ΔfdhΔAceA +PX2 9E	Mixture of *S. oneidensis* Δfdh +PX2 and transformants with deletion of *SO_AceA*
S.O. BOX II #12	*S. oneidensis* ΔfdhΔAceA +PX2 12B	Mixture of *S. oneidensis* Δfdh +PX2 and transformants with deletion of *SO_AceA*