Engineering *Synechococcus CB0101* to Improve Iron Uptake and Processing

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***Abstract*- In 1/3 of the world's oceans, the iron concentration limits phytoplankton growth. Iron is required for photosynthesis and is a critical micronutrient for the base of the marine food web. A better ability to capture iron could increase phytoplankton populations which would have benefits such as reducing atmospheric carbon dioxide. Experiments have been conducted to supplement iron in the ocean as a way of improving phytoplankton populations which then act as a carbon sink. Although continuous iron supplementation is possible, improving the organisms' ability to capture iron is a more sustainable way of addressing the issue. In choosing a phytoplankton to engineer, we decided upon *Synechococcus* (cyanobacteria) because it consumes high levels of CO2, has a high replication rate, and has been used by many iGEM teams in the past. Our project will engineer cyanobacteria to transport iron into cells and reduce it to the bioavailable Fe(II) form. The increased iron utilization will increase photosynthesis and growth of phytoplankton. To prevent harmful phytoplankton blooms, a kill switch will also be added to the cells to prevent overgrowth of cells if iron concentration were to increase significantly. This modification will stabilize the food supply for the marine food chain and absorb CO2 from the atmosphere.**

***Index Terms*-** Carbon sequestration, Cyanobacteria, Global warming, Phytoplankton

I. INTRODUCTION

Phytoplankton populations are declining worldwide due to increased surface temperature and limited iron availability. This absence resonates up the food chain, from tiny krill to the massive whales that feed on them (Ryabov, 2017). Iron is a critical micronutrient for the base of the marine food web since it is required for photosynthesis (Schoffman, 2016).

Phytoplankton have evolved to consume the Fe(II) form of iron. However, the Fe (II) concentration in the ocean is low and most iron is in the Fe(III) form. Phytoplankton have evolved a variety of ways to acquire iron in the Fe(II) form they require (Schoffman, 2016). One such way is through the use of siderophores and reductases. Siderophores are proteins secreted by phytoplankton which capture Fe(III) from the water (Ahlgren, 2019) These ligands have an extremely strong affinity for Fe(III), so they are effective in capturing the ion from the water. Once inside the cell, phytoplankton must separate the iron from the siderophore using reductases. Reductases separate Fe(III) from the siderophore by converting it to Fe(II) which has a low affinity for siderophores. Through this process, organisms can capture the iron they need and convert it to a usable form.

As a global trend, the biomass of phytoplankton is decreasing by ~1% per year (Boyce, 2010). However, in iron deficient regions of the ocean, phytoplankton have evolved a variety of ways to better use iron. Phytoplankton in these regions exhibit higher expression rates of genes coding for proteins such as ferritin, flavodoxin, iron uptake proteins, and siderophores (Ahlgren, 2019). Our project will modify cyanobacteria, *Synechococcus CB0101*, taking inspiration from the naturally evolved isolates described in Ahlgren et. al. We reason that bringing together the mutations that have evolved separately into one strain will enhance the ability of phytoplankton to grow in varying ocean conditions that are low in iron. The increase in available iron will increase photosynthesis in phytoplankton. This growth of phytoplankton will stabilize the marine food chain as well as absorb CO_2 from the atmosphere.

II. MATERIALS AND METHODS

Characterization of Cyanobacteria Growth in Varying Concentrations of Iron

In order to test the importance of iron to the phytoplankton population, *Synechococcus CB0101* at an initial OD of 0.05 was put into 5 conical tubes containing SN growth media with different iron concentrations: No iron, 0.01X, 0.1X, 1X (0.023 mM), and 10X normal iron concentration. Stock concentrations were based on the UTex Culture Collection of Algae. Growth was measured by optical density (O.D.) after 2-3 weeks. *CB0101* was grown under light intensity measuring 1000-1700 Lux and either at room temperature or at 30℃.

Improving Iron Consumption Efficiency in Cyanobacteria

Ahlgren *et al.* describes several genes related to iron consumption that have evolved to be expressed at a higher level in iron-deficient environments. Several of these coding regions were synthesized with promoter, RBS, and terminator and cloned into pSB1C3. Separate samples of *E. coli* cells were modified with each of these genes. These genes and their functions can be found in Table 1.

TABLE 1: *List of low Fe^{2+} adaptation genes and function (modified from Ahlgren et al.)*

Gene Names	Function
feoA	Transition metal binding ion, works in complex with *feoB*
feoB	Transmembrane transporter of a GTP-driven Fe^{2+} uptake system
isiB	encodes for Flavodoxin, which functions as an electron donor in redox reactions
idiA	Metal binding ion, protects against oxidative damage
pcopM	Encodes for Ferritin, an Iron storage protein
tonB	Siderophore uptake across the membrane
zupT	Mediates uptake of divalent cations and Fe^{2+}

Designing an Iron Sensitive Kill Switch

In order to prevent phytoplankton blooms, a kill switch was engineered using the pAceB promoter that will be implemented as a regulator upstream of the previously mentioned iron consumption genes. The pAceB promoter contains a binding site for FUR, a ligand that binds iron at concentrations of 10^{-7}-10^{-4} $Mol.L^{-1}$. However, as those values are above oceanic iron levels we wanted to modify the promoter to be sensitive at iron concentrations found in the ocean.

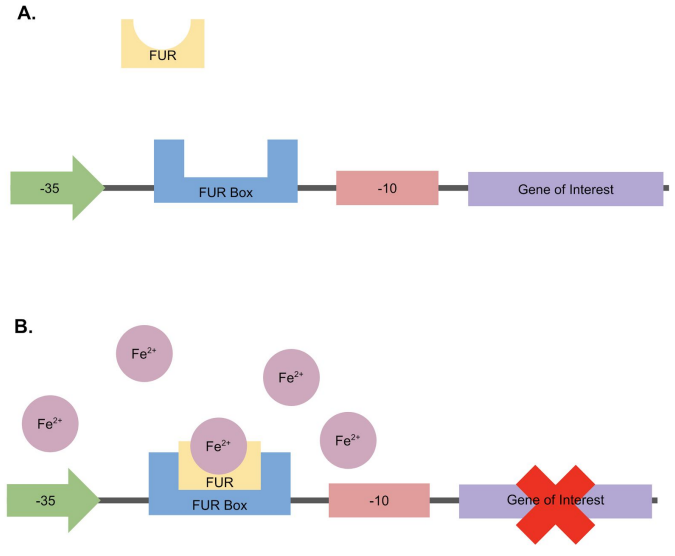

FIGURE 1: *Diagram showing the inhibition of the pAceB promoter. (A) With the absence of Fe(II), FUR cannot bind to the FUR Box, and gene expression is uninhibited. (B) The Fe(II) molecule binds to the FUR ligand, which binds to the FUR Box, inhibiting the promoter system.*

When bound to FUR, pAceB downregulates gene expression. Seven promoter constructs, PFur-1, PFur-2, PFur-3, PFur-1-2, PFur-1-3, PFur-2-3, and PFur-1-2-3, were designed and titled for the respective positions of their fur boxes. To determine the sensitivity of the promoters, separate plasmid constructs containing mCherry fluorescent protein in place of the iron uptake genes will be generated. Fluorescence will first be measured in E. coli cells and then the promoter that expresses the least mCherry will be used in subsequent studies with *CB0101*.

III. RESULTS AND FINDINGS

In this part of the investigation, we aimed to characterize the growth of Cyanobacteria in the presence of iron and transform *E. coli* with our iron uptake genes and kill switch constructs to test their viability in a living system.

From measuring the O.D. of the growth of *Synechococcus CB0101* in varying concentrations of Fe(II) (*Figure 2)* it can be observed that as the concentration of Fe(II) increases in the media there is an increased growth of *Synechococcus CB0101*.

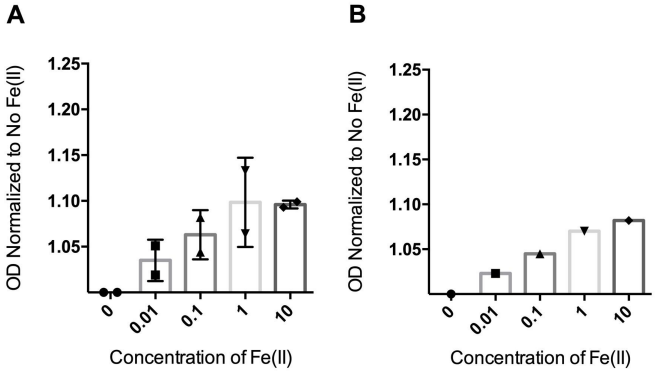

FIGURE 2: *Optical density of Synechococcus CB0101 at varying concentrations of Fe(II). All values are normalized to no Fe(II) control. (A) O.D. for samples 1 and 2 were measured following 3 weeks of growth at 30°C. (B) O.D. for sample 3 was collected after 1 week of growth at 20°C.*

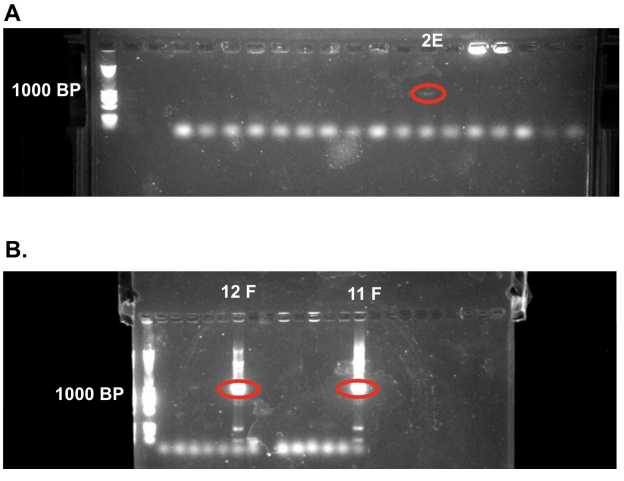

FIGURE 3: *Results of gel electrophoresis for E. coli transformations. (A) Gel containing PCR product from E. coli samples transformed with Kill Switch constructs. Amplification was observed for sample 2E, a colony transformed with construct PFur1-3. This construct contained two separate FUR binding sites. (B) Gel containing PCR product from E. coli samples transformed with Iron Consumption genes. Amplification was observed for samples 11F and 12F, both samples that were transformed with the undigested vector backbone. No amplification was observed for experimental plates*

It can be seen from *Figure 3A* that the construction of pFur1-3-mCherry was successful. The amplification of the undigested vector in *Figure 3B* validates the transformation and PCR, but the lack of amplification in experimental samples implies further testing of additional bacterial colonies is required.

IV. DISCUSSION

In our experiments, we are using a relatively new strain of Synechococcus, CB0101 (Reference), so we wanted to verify that iron is a limiting nutrient for them. The strain grew relatively slowly during the first round of growth characterization at 30℃ so growth conditions were optimized to 20℃ and will be used throughout the remainder of our project.

From the results observed in *Figure 2*, it can be seen that the growth of *Synechococcus CB0101* is limited by iron concentration. This validates our hypothesis that by increasing the amount of Fe(II) available to phytoplanktons it should lead to increased growth. Future work will consist of transforming *Synechococcus CB0101* with each iron consumption gene and testing whether this enhances the iron uptake ability of the newly transformed strain of *Synechococcus*.

As observed in the gel electrophoresis depicted in *Figure 3A,* pFur1-3 appears to be the most viable construct for testing changes to the promoter since it was the only construct that yielded amplification, however, it is necessary to repeat the transformation so that additional promoter systems can be prepared. Once this step has been repeated, each promoter system's ability to downregulate the expression of mCherry in the presence of iron can be compared. The construct that yields the least expression of mCherry will be used in the final construct containing the modified pAceB promoter, a Lac Operon acting as an inverter, and a CCDB suicide gene. The Lac operon will block transcription of CCDB under normal conditions. With the presence of iron, expression of Lac will be downregulated, leading to expression of CCDB. Once the viability of this construct has been proven, it will be adapted for *Synechococcus CB0101.* This will allow for iron mediated apoptosis to avoid the possibility of a phytoplankton bloom.

The autofluorescence of cyanobacteria can lead to difficulties when genetically modifying them with fluorescent proteins. Therefore, different reporter proteins will be characterized in *CB0101* to see what can be best detected even with autofluorescence.

V. CONCLUSION

The results of the preliminary tests in this study prove the validity of improving phytoplankton growth in iron deficient environments through enhanced consumption of iron. It was shown that iron is vital to the growth of phytoplankton, and our literature review has revealed that consumption can be improved in low iron environments. To make such an improvement safe, a kill switch was engineered to address the possibility of overgrowth. The enhancements described in this study will have positive implications on the aquatic ecosystem as phytoplankton sit at the bottom of the marine food web.

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