**Sugaway: Using Synthetic Biology to Treat Diabetes**

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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## Introduction

Diabetes Mellitus are a group of metabolic disorders which all share a common characteristic of inducing elevated blood glucose levels. Blood glucose levels are primarily under the hormonal control of insulin and glucagon. Under hyperglycemic conditions, insulin is secreted by pancreatic β-cells and binds to the insulin receptor of other cells to trigger the uptake of extracellular glucose. Glucose is then used for various metabolic purposes, or stored as glycogen for long-term energy storage (Petersen & Shulman, 2018). Under hypoglycemic conditions, glucagon is secreted from pancreatic α-cells, which bind to the glucagon receptor and initiate the release of glucose through glycogenolysis and gluconeogenesis (Ojha et al., 2019). The most predominant forms of DM are type 1 diabetes (T1D) and type 2 diabetes (T2D), which effect roughly 2 million and 34 million Americans, respectively (CDC, 2022; *Statistics About Diabetes | ADA*, n.d.). T1D arises from an autoimmune response where T-cell and B-cell responses attack and destroy pancreatic β-cells, which leads to the lack of insulin production. T1D is influenced by both genetic and environmental factors, and is characterized by the presence of autoantibodies circulating the plasma (Los & Wilt, 2022). T2D occurs due to a growing insulin resistance in target cells, which prevents them from uptaking extracellular glucose. The mechanism behind insulin resistance is still unknown, but studies have shown that genetic factors and lifestyle choices can play a role in developing insulin resistance (Wilcox, 2005). If left unmanaged, diabetes can lead to several complications such as cardiovascular disease, diabetic neuropathy, and cancer (Wu et al., 2014).

While there are no current cures for diabetes, both T1D and T2D can be managed with a combination of lifestyle changes and medication. Reducing carbohydrate intake and increased exercise has been shown to lower blood glucose levels, while medications such as Metformin or

different Sulfonylureas can be prescribed based on the severity of the diabetes (*Diabetes Management*, n.d.; Rajkumar, 2020). However, the primary and most potent treatment for diabetes is to begin insulin therapy by administering insulin directly into the bloodstream. However, insulin has been the prime target of price gouging by United States pharmaceutical companies, which has led to insulin prices increasing over 1000% in the past 20 years (Rajkumar, 2020). This has left many diabetic patients without access to affordable insulin, and has led to several deaths from individuals unable to use insulin or using expired insulin (Rajkumar, 2020).

Recent advances in the field of synthetic biology have opened up new avenues for the treatment of metabolic disorders. Several studies have shown the usage of synthetic gene circuits to either detect various biomarkers or to trigger particular cascades which lead to the transcription of a specific gene product, which can be fine-tuned to treat specific disorders (Teixeira, Ana P. & Fussenegger, Martin, 2016). For example, Bai et al. showed the usage of a closed-loop genetic circuit to detect liver failure and consequently secrete hepatic growth factor as a therapeutic protein in response to the liver failure (Bai et al., 2016). Similar work has been done with diabetes by both Ye et al. and Xie et al., and their work suggests that a synthetic biology approach may also be used to develop a less expensive insulin supplement which can reduce a diabetic patient's reliance on insulin injections (Xie, Mingqi et al., 2016; Ye et al., 2017). We aim to do this by designing a genetically modified bacterium, which will mimic the effects of insulin by up taking extracellular glucose and converting it into glycogen. This bacterium is a proof-of-concept prototype for a probiotic insulin supplement which could be taken prior to a meal as a way to reduce postprandial glycemic spikes.

Glycogen synthesis in Escherichia coli is well documented and are controlled by multiple genes, many which are present in the glgBXCAP operon. Of particular importance are the genes phosphoglucomutase (PGM), branching enzyme (glgB), ADP-glucose phosphorylase (glgC), and glycogen synthase (glgA). PGM catalyzes the movement of a phosphate group from the 1-carbon to the 6- carbon to produce glucose 6-phosphate. glgC catalyzes the addition of ADP from ATP, converting glucose 6-phosphate to ADP-glucose. The ADP-glucose is then used by glgA to form the backbone chain of α-(1,4) glucan chains. glgB will catalyze the formation of new α-(1,6) linkages to branch off the main α-(1,4) glucan chain (Almagro et al., 2015). We hypothesize that an increase in expression of key glycogen synthesis enzymes will consequently lead to an increase in intracellular glycogen concentration.

## Results

A glycogen synthesis plasmid, named pKID1, was generated by placing genes PGM, glgB, glgC, and glgA under the influence of a rhamnose inducible promoter. This sequence was then cloned into plasmid backbone pD861 and transformed into JM109 cells for further analysis.

### *pKID1 produces a protein product under rhamnose induction*

Protein expression from the plasmid was verified by inoculating pKID1-transformed bacteria in 0% rhamnose (control) or 0.2% rhamnose (rhamnose-induced samples) for 8 hours to determine the inducibility of pKID1. Protein extracts were separated and probed with an anti6xHis antibody to show stable protein expression after 4 hours of rhamnose induction (Figure 1). Equal protein loading was ensured by probing with GAPDH, which showed consistent protein loading across all samples. These results were verified across two separate biological replicates to ensure that pKID1 was able to express protein only in the presence of rhamnose.

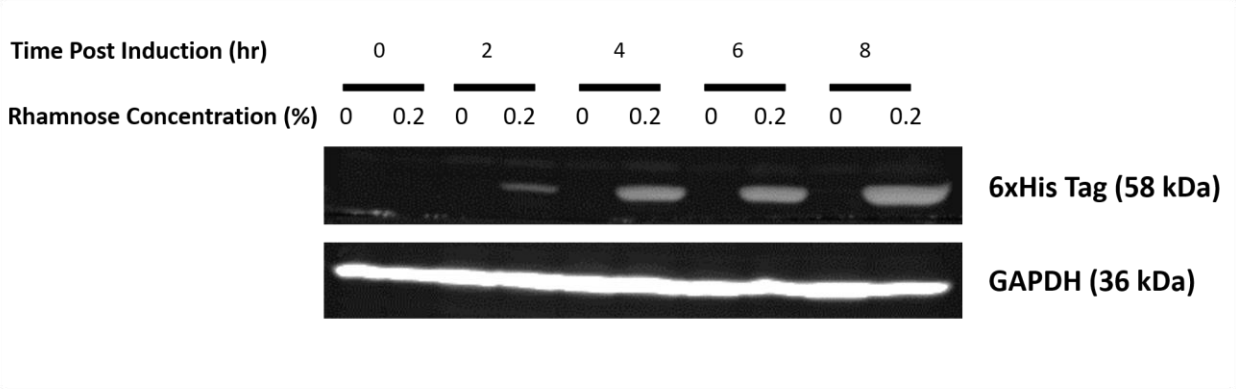
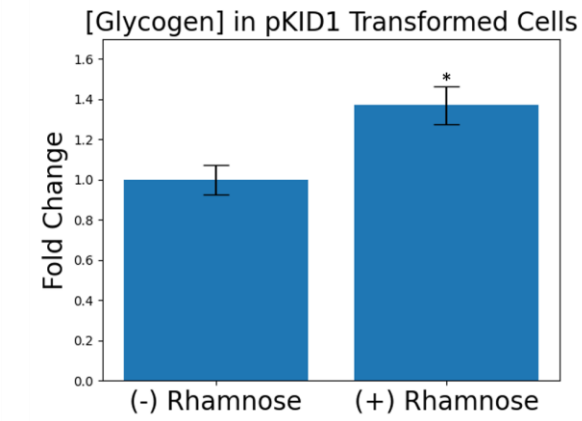



**Fig. 1. Rhamnose induction leads to increased 6xHis expression on first open reading frame.** Membrane was probed with an anti-6xHis antibody to determine expression of first open reading frame on pKID1. GAPDH was probed as a loading control to ensure equal protein loading in each well.

*pKID1 increases intracellular glycogen concentration under rhamnose induction*

pKID1-transformed cells were incubated in M9 media supplemented with 0.8% glucose and tryptone to validate the functionality of the protein product produced from the plasmid. Cell extracts were assayed for glycogen content, which showed that rhamnose-induced cells had a 37% increase in intracellular glycogen content compared to uninduced cells (Figure 2). These results




| | [Glycogen]/cell (μM/cell) |
|---|---|
| (-) Rhamnose | $7.74 * 10^{-8}$ |
| (+) Rhamnose | $1.06 * 10^{-7}$ |

**Fig. 2. Rhamnose induction leads to an increased intracellular glycogen concentration. (Left)** Intracellular glycogen concentration increased by 37% in the presence of rhamnose compared to uninduced cells (* $p$ = 0.01 with a Student's $t$-test at α = 0.05). **(Right)** Intracellular glycogen concentration per cell shows a 37% increase in induced cells compared to uninduced cells.

were statistically significant with a Student's t-test (p = 0.01) at an α = 0.05. To further quantify the effects of pKID1, glycogen concentration of an individual bacterium was calculated, which showed a 37% increase in intracellular content compared to uninduced cells. These results were

verified with two independent technical replicates and cells transformed with the plasmid backbone (pD861) were used as a negative control.

## Discussion

The growing inaccessibility to insulin treatment in the United States due to the rising cost of insulin and growing prevalence of diabetes has indicated the need for an alternative supplemental treatment. Our work here shows the use of a genetically modified bacterium as a biological device that can increase the intracellular concentration of glycogen. This biological device serves as a prototype for a less expensive probiotic treatment which can reduce the reliance on insulin treatment.

The first step in the creation of the biological device was the design of the glycogen synthesis plasmid pKID1. The plasmid was designed using the primary enzymes involved in glycogen synthesis: PGM, glgB, glgC, and glgA. The three genes glgB, glgC, and glgA were placed in their native sequential order as seen in the glgBXCAP operon. PGM was placed in front of these three genes and as the first open reading frame of the construct. These enzymes were all placed under a rhamnose inducible promotor to allow for selective transcription of gene products. This allowed for selective expression of construct, which would be intended as the bacterium should only activate in hyperglycemic conditions. This plasmid serves as one component of a larger synthetic gene network which would be used to generate the most therapeutic bacterium. Due to its modularity and presence of an inducible promoter, this plasmid design serves as a template for future constructs which can be added to the synthetic gene network. This includes constructs which can increase the glucose uptake into the bacterium, or a kill-switch plasmid which will limit the growth of the bacterium.

Once pKID1 had been fully designed, it had to be ensured that it was able to produce a protein product and that the resulting proteins were functional. This was validated and supported by the results shown in Figure 1 and Figure 2, which showed that there is a protein product formed and that it leads to a statistically significant increase in intracellular glycogen concentration. This result suggests that the addition and activation of pKID1 by rhamnose induction increases the rate of glycogen synthesis through the increased expression of each enzyme involved in the glycogen synthesis pathway.

To further support these results, additional experiments need to be done to further characterize the effects of pKID1 on the glycogen synthesis pathway. Modeling flux through the glycogen synthesis pathway can reveal regulatory points and rate-limiting steps in the synthesis, which would be points of improvement to the pKID1 design to better optimize glycogen formation. This can be determined through computational modeling, and then verified *ex vivo* by measuring both protein expression and protein functionality. A current limitation to the pKID1 design is that only the first open reading frame is tagged with an epitope tag, which meant that the results from Figure 1 are limited to a protein product being produced only from the first reading frame. In future iterations of the pKID1 design, multiple epitope tags would be placed on all open reading frames to provide an easy way to check protein expression.

The results presented herein support the design of a novel biological device which has implications to be used as a diabetic therapeutic. As a therapeutic, the genetically modified bacteria will be packaged into a probiotic pill which a diabetic patient could take prior to a meal. This would consequently replace their typical insulin dosage. As the probiotic pill is ingested, the bacteria in the probiotic will colonize the small intestine where other microorganisms are present as a part of the microbiota. Here, the bacteria will be able to mimic the effects of insulin by

uptaking excess glucose from a digested meal and converting it into glycogen. A cheaper probiotic option for diabetic patients is more financially accessible and alternative option to give to diabetic patients who may not be comfortable with using needles for an insulin injection. This decreases their current reliance on expensive insulin therapies and would have large beneficial implications on the current diabetic therapeutic market.

## Materials and Methods:

### *Design and Generation of pKID1*

Construct pKID1 was generated by sequentially placing genes PGM, glgB, glgC, and glgA under the influence of a rhamnose inducible promoter. This sequence was then cloned into plasmid backbone pD861 (provided by Dr. Steven Zeichner's laboratory at the University of Virginia) between restriction sites XbaI and NotI. Plasmid was amplified in electrocompetent DH5α cells and extracted with a Maxiprep kit (Qiagen).

### *Cells and Reagents*

All further cell-based experiments after the initial pKID1 amplification process were done in chemically competent JM109 cells. Chemical transformations involved adding 100ng of plasmid to ice-cold JM109 cells for 5 minutes prior to a 1-minute pulse in 42°C water bath. Cells were returned to ice and incubated for 1 hour with S.O.C. media (ThermoFisher) prior to streaking on LB Broth + Kanamycin (50 μg/mL) plates. Successful transformants were verified by gel electrophoresis, where plasmids were extracted by a Miniprep kit (Qiagen) and separated on a 0.8% agarose gel.

### *Rhamnose Induction Assay*

Cells were inoculated overnight in LB + Kanamycin (50 μg/mL) prior to rhamnose induction. Cells were consequently inoculated the next day at OD600 = 0.05 in either the presence

of 0.2% L-Rhamnose (Sigma) or absence of L-Rhamnose for 8 hours. Samples were collected every two hours to determine the ideal induction period for protein expression.

*Western Blotting*

Protein was extracted from each sample from the rhamnose induction assay and electrophoretically separated by SDS-PAGE. Proteins were transferred to a 0.2 μm nitrocellulose membrane and blocked with a 5% BSA solution. The membrane was then probed with a primary mouse anti6xHis antibody and secondary goat anti-mouse conjugated antibody prior to being developed. Equal protein loading was ensured by probing the membrane for GAPDH as a loading control. Glycogen Concentration Assay Cells were inoculated overnight in LB + Kanamycin (50 μg/mL) prior to measuring intracellular glycogen. Cells were inoculated the next day at OD600 = 0.05 in media (M9 media + 0.8% glucose + 1% tryptone) in either the presence or absence of 0.2% rhamnose for 6 hours. Samples were then collected and prepared by resuspending bacterial pellets in PBS and heating to 95°C for 15 minutes. Silica beads were then added to the resuspended bacterial pellets and vortexed for 1 minute to disrupt the bacterial membrane. Cells were then spun at 10,000 RPM for 30 minutes at 4°C and the supernatants were collected for glycogen measurement. Glycogen measurement was done with Cell Biolabs, Inc. colorimetric Glycogen Assay kit (MET-5022) as per manufacturer's protocol.

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