Design of an Engineered Probiotic for the Treatment of Celiac Disease

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Introduction:

Celiac disease (CD) is an autoimmune condition that affects 1 in every 133 healthy, average Americans[1] and is triggered during ingestion of gluten in the small intestine[2]. Celiac patients predominantly express mutations in *HLA-DQ*, a gene that encodes for cell surface receptor proteins on antigen-presenting cells[2]. Among these variants, 90% of all Celiac patients express variants in *HLA-DQ2* , and an additional 5% present variants in *HLA-DQ8*, suggesting these variants are necessary for a patient to be at risk[2]. The importance of these mutations will be discussed later during pathogenesis. Key elements of Celiac disease include these particular genetic variants, gliadin, tissue transglutaminase (TTG), and the CXCR3 chemokine receptor [2,3].

Pathogenesis

Gluten, a substance commonly found in wheat, barley, and rye, is composed of two different proteins, glutenin and gliadin. Glutenin tends to form a long, elongated rope-like structure, while gliadin wraps upon itself during formation, folding into a compact, spherical shape[4]. Gliadins can be differentiated primarily into γ-,α-,δ- and ω-gliadins, each with their own respective sequence and structure[5]. Sollid et al. (2012) describes certain epitopes of the different gliadins that may be responsible for causing immune responses in CD patients[6]. Shan et al. (2002) discovered a 33-mer byproduct of α2-gliadin metabolism that caused T-cell induction in 14 patients with Celiac disease[7].

Common theory on immunopathogenesis of Celiac disease begins with binding of gliadin or one of its undigested fragments to chemokine receptor CXCR3 on intestinal mucosa, resulting in release of zonulin and increased intestinal permeability, allowing gliadin fragments to pass into the intestinal wall [3,4,8]. Tissue transglutaminase (TTG), a known autoantigen, can also modify these gliadin peptides to increase their overall negative charge as well as binding affinity for HLA-DQ2 [3]. Once antigen-presenting cells bind gliadin, peptides are presented to $CD4^+$ T-cell receptors, causing an autoimmune response, production of gluten and TTG antibodies, increased intraepithelial T-lymphocytes, decreased enterocyte height, and villous atrophy [9].

Currently, the only available treatment for Celiac disease includes a strictly gluten-free diet [10]. One promising strategy to combat CD includes usage of the zonulin antagonist larazotide acetate, a synthetic octapeptide capable of restoring tight junction assembly (intestinal permeability) *in vitro* [11]. Other strategies include inhibiting TTG, blocking HLA-DQ2, or antagonizing IL-15, all of which have yielded promising results, but also pose severe side-effects such as autoimmunity and immunosuppression [12].

Probiotics

The usage of probiotics to degrade and metabolize gluten peptides has also been lightly explored. The probiotic preparation of VSL #3, a group of various wild-type bacteria, has been shown to metabolize gluten peptides *in vitro*, however any single bacterium does not work on its own [13].

Work in this field is scarce and remains to be explored in depth. The utilization of synthetic biology to engineer novel probiotics to maintain gut health has been widely explored, though exploration into Celiac disease remains scarce [14]. Usage of E. coli Nissle may serve to be a reliable probiotic to carry small molecules in the gut [15].

Fur Operon

Gluten-containing products such as wheat flour are generally enriched or fortified with ferrous sulfate to compensate for loss of iron during the milling process[16]. Non-heme iron sources such as ferrous sulfate are almost exclusively absorbed in the duodenum section of the small intestine[17]. Given that iron absorption and symptoms of Celiac disease are both localized to the small intestine, a probiotic approach localized within the gut microbiome may be helpful.

Gluten Sensing

Although no genetic sequences with the ability to detect gluten presence have been discovered, elevated presence of iron in the small intestine may serve as a useful indicator to detect whether a patient has consumed a gluten-containing product. In bacteria, uptake of iron must be tightly regulated to prevent overproduction of reactive oxidative species due to Fenton chemistry[18]. Production of siderophores, or peptides produced by bacteria capable of binding to iron, is regulated by the Ferric Uptake Regulator protein (Fur) encoded by the *fur* gene natively expressed in *E. coli*[19]. Fur can bind ferrous iron (Fe^{2+}). Once bound, the Fur-Fe complex may form a dimer with another identical complex, and bind to genetic sequences containing a "fur box". Fur box sequences located within the promoter region of prokaryotic transcriptional units are capable of being transcriptionally repressed by the Fur dimer[19].Utilization of this classical repression system to engineer a transcriptional unit activated by the elevated presence of iron may serve as a promising system to detect and neutralize the effects of gluten peptide presence.

Signal Peptides

As discussed before, larazotide is a synthetic peptide potentially capable of combatting CD. A probiotic approach to therapeutic delivery would require the survival of the host during peptide expression, directing the focus towards extracellular secretion of the mature protein to reduce cytotoxicity and formation of inclusion bodies. One potential route for secretion includes the Sec secretory system, a two-step pathway which requires the addition of a signal sequence N-terminal to the target sequence[20].

Though many signal peptides with affinity for the Sec pathway have been identified, the relationship between signal sequences and their respective peptide sequences has not been elucidated[21]. However, it is possible to utilize screening techniques to choose which signal peptide may be best for the secretion of the target protein[21].
In this study, we designed a genetic construct capable of detecting the presence of iron and secreting larazotide within *E. coli* Nissile and engineered portions of the construct as proof of concept within *E. coli* BL21 DE3.

Materials and Methods:

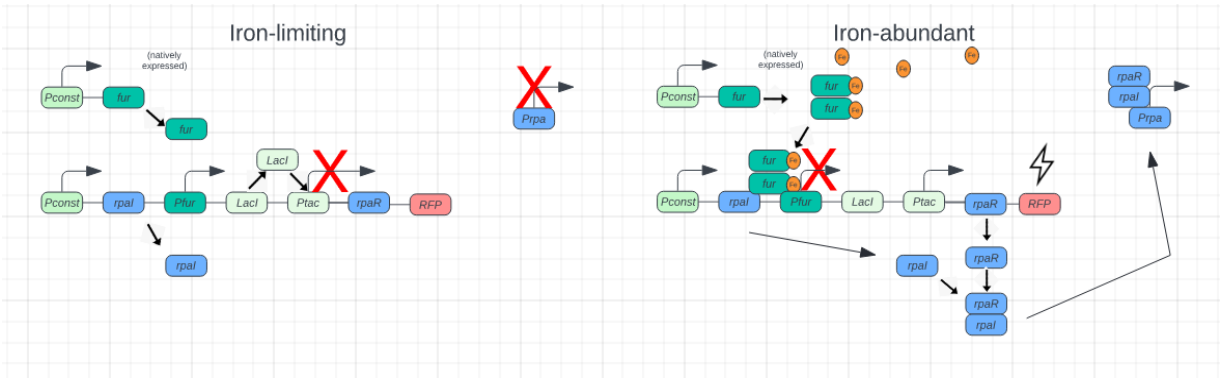
Iron-sensing construct design

To construct a system that inputs ferrous concentration and outputs regulation of transcription, promoters utilizing the Fur box were researched and the sequence for a tightly regulated promoter, Pfhua1, was found from the iGEM20_HZAU_China group wiki (http://parts.igem.org/Part:BBa_K3378002:Design). This promoter reportedly transcribed low fluorescence under regular and iron-rich conditions within LB media, and elevated fluorescence under iron-poor conditions in M9 minimal
media.

A double repression system utilizing the Pfhua1 promoter was constructed using the CIDAR Moclo Extension Part Kit, Vol. 1 (https://media.addgene.org/cms/filer_public/e3/64/e364349e-eb5a-4ee7-a3a7-d615aa88f817/180529_cidar_vol_i_guide.pdf). LacI was placed downstream of the Pfhua1 promoter, and the Lac-repressible promoter Ptac was placed further downstream. We also incorporated a rpaI/R system for interaction with other genetic circuits in response to iron influx. Thus, under iron rich conditions, hypothetically, the Fur-Fe dimer would repress transcription of a repressor, which in turn would induce transcription of a downstream gene. The final circuit designs for all experiments are shown below in **Figure 1**.

A.

B.



C.

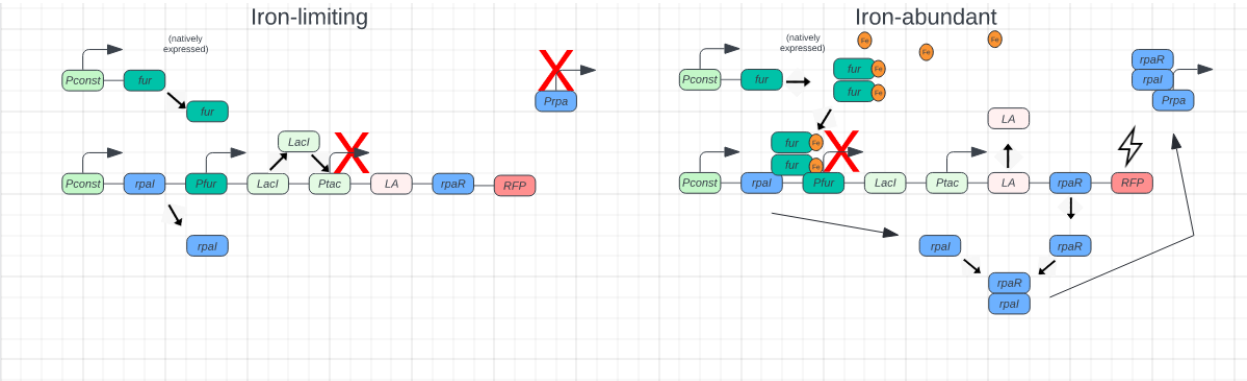


Figure 1. Genetic circuit designs for Experiment 1 (A), Experiment 2 (B), and Experiment 3 (C), respectively. **A.** In Experiment 1, basic promoter functionality is tested using mScarlet (RFP) as a reporter. Under iron-deficient conditions, RFP is expressed, and under iron-sufficient conditions, RFP transcription is repressed. **B.** In Experiment 2, double repression is utilized via the LacI-Ptac system, RFP is utilized as a reporter, and the rpaI-rpaR complex is included for interaction with other circuits. Under iron-deficient conditions, RFP transcription is repressed and rpaR transcription is repressed. Under iron-sufficient conditions, RFP is expressed and the rpaI/R complex is formed, serving as an inducer for the Prpa promoter (located on another genetic circuit). **C.** In Experiment 3, the genetic construct from Experiment 2 was modified to allow for transcription and secretion of the synthetic Larazotide gene (LA). Transcription of LA is activated by elevated levels of iron.

Gene part and construct design: serine protease and release system

Gene sequences for Subtilisin A (subA) (GenBank Accession: AB920398.1) and Subtilisin Carlsberg (subC) (GenBank Accession: X03341.1) were obtained from NCBI GenBank and the gene for KumaMax (kmax) was obtained from the iGEM parts library.

When not natively from *E. coli*, gene sequences were codon optimized using the IDT codon optimization tool. Benchling was used to conduct a virtual restriction enzyme digest on each gene part to check if there were any native BsaI cut sites. BsaI is the restriction enzyme around which 3G Assembly operates, so native BsaI cut sites were removed so that the gene would stay intact during Transcriptional Unit (TU) construction and thus the protein of interest would be constructed as intended. BsaI cut sites were removed by replacing the codons of the cut site with codons for equivalent amino acids based upon the *E. coli* codon table used in the IDT codon optimization tool.

Geneious Prime was used to conduct virtual Golden Gate and Gibson reactions to test the viability of designed gene parts and TUs.

3G Assembly

All transcriptional units and plasmids were constructed following the 3G Assembly protocol[23], which utilizes both Golden Gate and Gibson assembly to allow for the construction of a genetic circuit from modular parts in a single-day workflow.

Results:

PCR Product

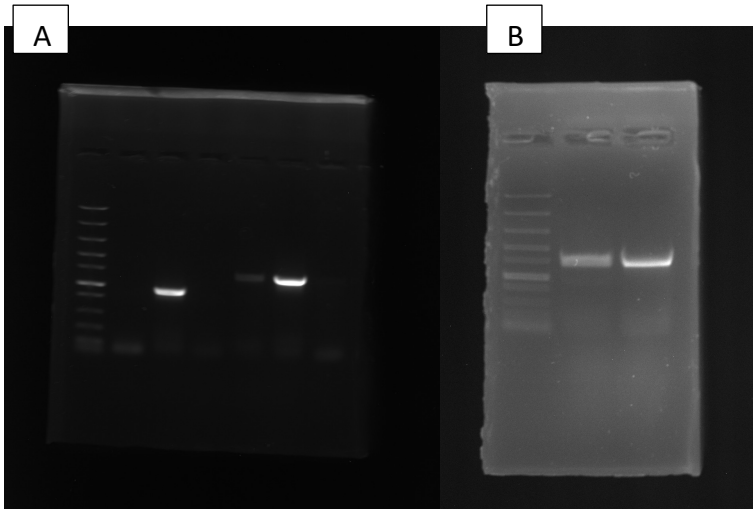


Figure 2. A. Test PCR results. Lane 1 shows the FAST DNA Ladder, Lanes 2-4 show control P5d-mScarlet assemblies, and Lane 4-6 show Pfhua1-mScarlet assemblies. B. 40 uL PCR bands of PCR products corresponding to Lanes 4 and 5 of test PCR results.

Ultraviolet scans on electrophoresis gel containing our test PCR and Golden Gate PCR products are shown in **Figure 2**. In terms of strength of TU construction, U1-U3 adapter usage seemed to yield the strongest assembly results, U1-UX adapter usage showed faint bands, and U3-UX showed no band formation. Final PCR included the U1-UX and U1-U3 transcriptional unit constructs and yielded similar results with faint bands on U1-UX adapter usage and strong bands on U1-U3 adapter usage.

Transformation Product

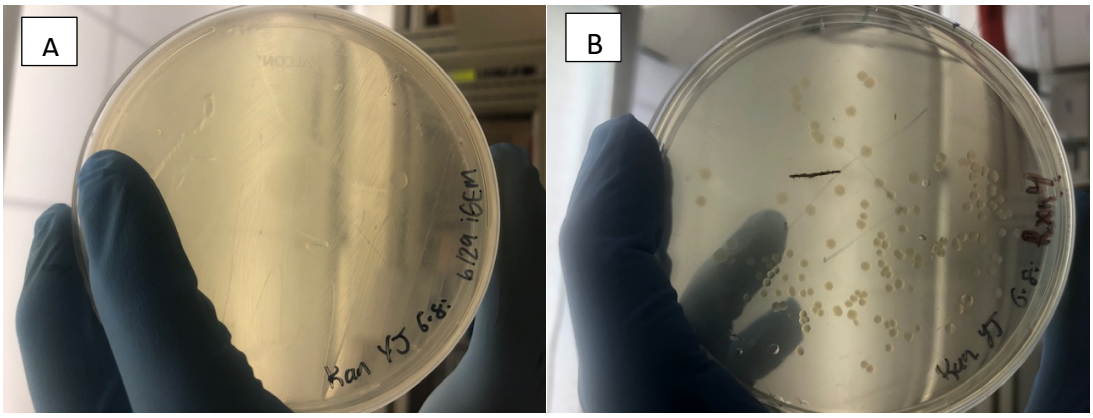


Figure 3. A. Transformation Product of Pfhua-mScarlet TU in Kanamycin-resistant v41m vector backbone on 6/29. B. Transformation product of identical construct on 7/20.

Successful transformations of both constructs on Kanamycin-resistant LB agar plates are shown in **Figure 3**. Transformation of the first genetic construct yielded 2 colonies, and transformation

of the second genetic construct yielded over 50 colonies. No red fluorescence was visible to the naked eye on either plate.

Fluorescence Assay

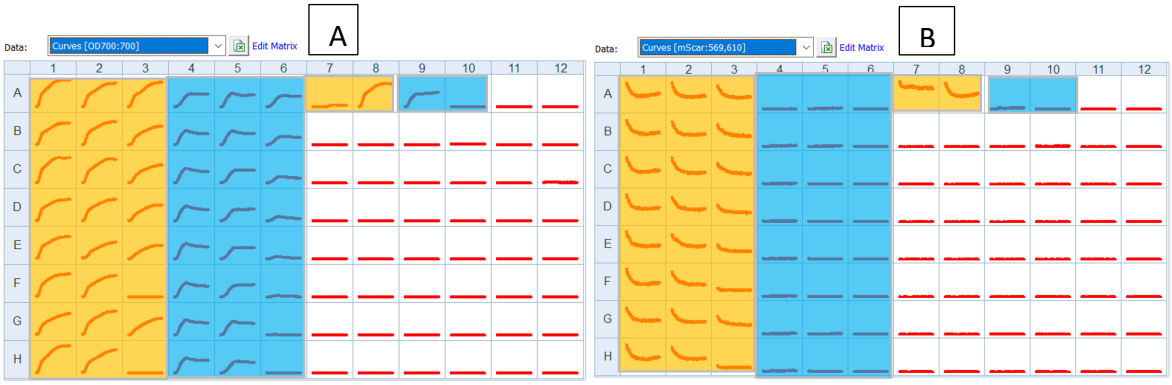
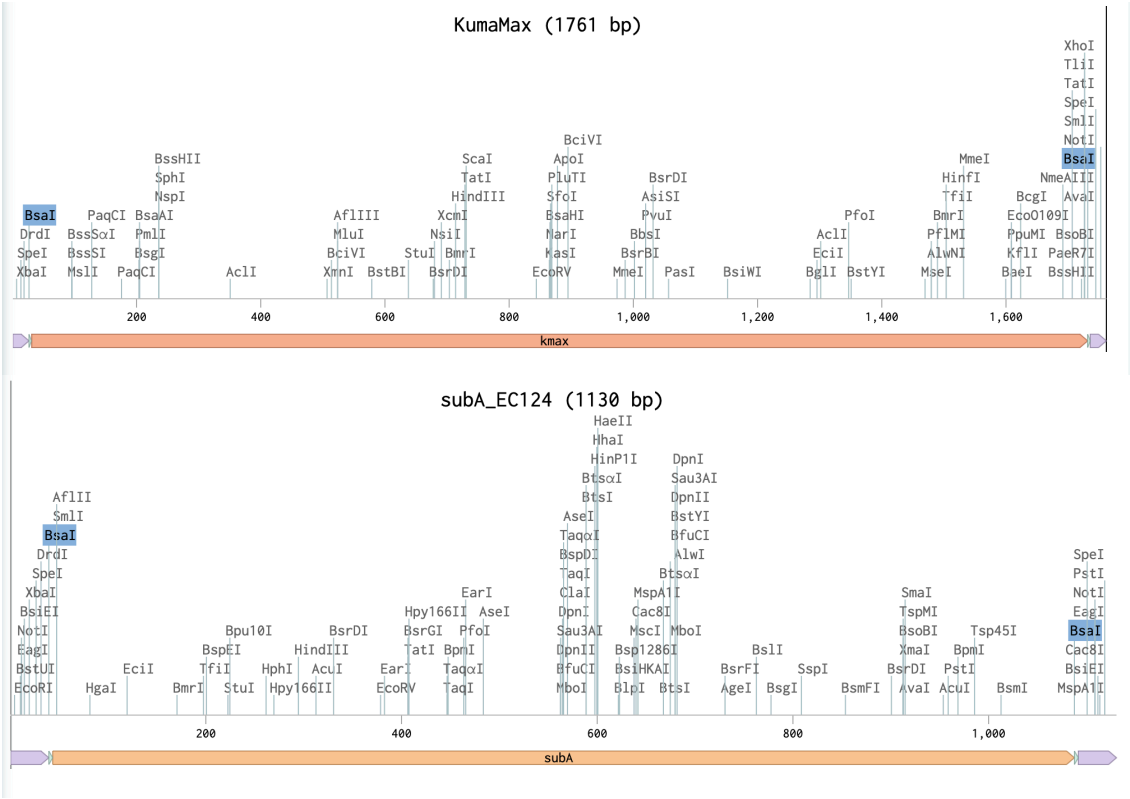


Figure 4. A. OD700 and B. mScarlet Fluorescence measurement with *E. coli* grown in LB media (Yellow) and M9 minimal media (Cyan) over increasing levels of EDTA concentration down the wells. Controls include LB media and LB media with transformed Bacteria, and M9 media with transformed bacteria and M9 media. (top right).

Results for the fluorescence assay on the 96-well plate are shown in **Figure 4**. No significant fluorescent activity is observed between medium as well as EDTA concentration.

Serine Protease and KR2_HlyAt Linker 3G Part Design



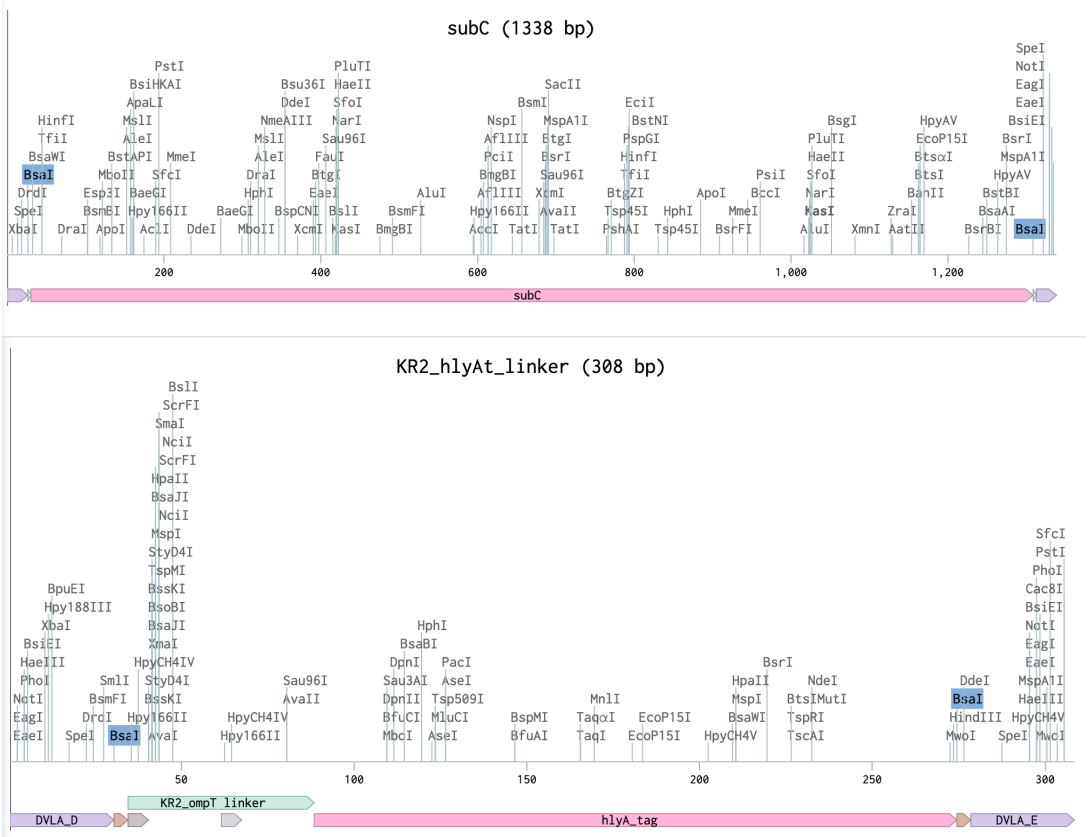


Figure 5: Annotated 3G compatible gene part designs from Benchling.

Genetic Circuits for the Release of Serine Protease

The primary approach for protein release was secretion using an HlyA system. Two separate sets circuit designs were created. The first utilized an IL22-HlyA system that had been previously designed into a 3G-compatible part.

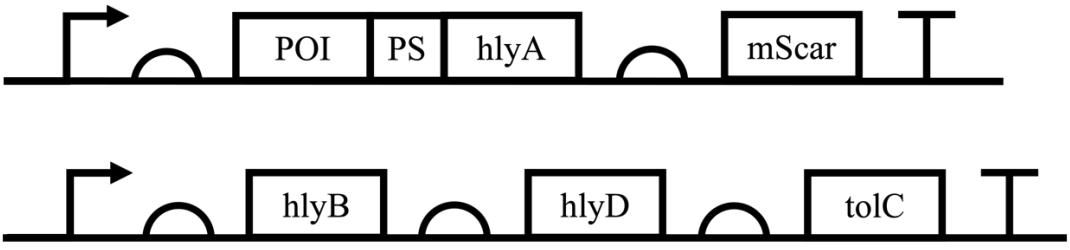


Figure 6: Secretion using IL22-HlyA system (from Murray lab courtesy of Dr. Green)

The second utilized an HlyA system with a linker sequence adapted from a paper by Hanke et. al. The differences between these two systems is the length of HlyA included, the enzyme used to cut the linker sequence and remove the HlyA tag, and the post-processing that would be required to isolate the serine protease protein of interest (POI).

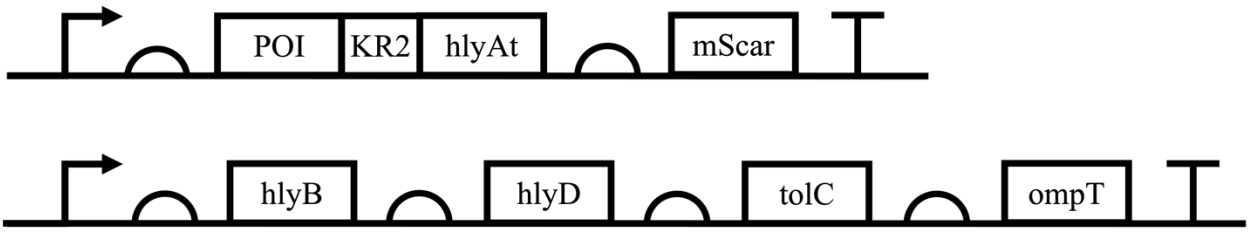


Figure 7: Secretion using HlyA system with OmpT linker adapted from paper by Hanke et. al.

The secondary approach for protein release was cell lysis using the bacteriophage protein Phi-X174E. Two circuits were once again designed, one with constitutive expression of Phi-X174E and the other with quorum sensing.

Figure 8: Phi-X174E lysis (non-quorum sensing)

Figure 9: Phi-X174E lysis (quorum sensing)

Conclusions:

Given the lack of fluorescent activity both qualitatively on the agar plates and quantitatively during the fluorescence assay, troubleshooting was undertaken to better understand why the results differed from expectations. It was determined that the results observed could be attributed to one or more of three distinct possibilities: First, the genetic transcriptional unit could have been incorrectly constructed by use of incorrect adapters and/or primers, as well as an incorrect selection of parts. Second, the v41m backbone used as the vector fragment in the plasmid construction may not have been fully linearized, leading to a transformation of a dummy insert, and thus an incorrect phenotype was observed. Third, the validity of the Pfhua1 promoter sequence in demonstration differing levels of transcription based on ferrous concentration may not be replicable. As of now, the root cause of error in the experimental design is unknown and has yet to be discovered

The results of the study show that although a successful transformation occurred, no corresponding phenotype was visible within the resulting colonies, suggesting a flaw in the experimental design and/or the experimental method. Once the root cause of the flaw is determined, further experimentation must also be performed to successfully construct and confirm the validity of the proof-of-concept plasmids. Looking into the future, we aim to construct a finalized design within a single plasmid, assess the validity of the construct through mass spectrometry analysis of larazotide secretion as well as comparison to a computational model. Thus, the successful construction of the full genetic construct remains a long-term task.

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