Engineering the TetO System to Test the Contribution of *FKS1* to Yeast Cell Wall Strength

by
Steffany Jenkins

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Thesis Committee:

Dr. James Robertson, Thesis Director

Dr. Matthew Elrod-Erickson, Second Reader

Dr. Stephen Wright, Thesis Committee Chair

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APPROVED:

_____
Dr. James Robertson, Thesis Director
Associate Professor, Biology

_____
Dr. Matthew Elrod-Erickson, Second Reader
Associate Professor, Biology

_____
Dr. Philip E. Phillips, Thesis Committee Chair
Associate Dean, University Honors College

Abstract

Society has a demand for manufactured proteins such as insulin, which can be produced by the yeast *Saccharomyces cerevisiae*, but such proteins are not easily accessible due to this yeast's rigid cell wall. I hypothesize that if *S. cerevisiae* cells were not able to make as much *FKS1* protein (a protein involved in cell wall synthesis) they would exhibit reduced growth rates and weaker cell walls. The approach is to genetically reprogram the yeast to reduce production of *FKS1* when exposed to doxycycline. To accomplish this, the native *FKS1* and *GSC2* genes were knocked out, leaving only the doxycycline regulated *FKS1* gene. Compared to the wildtype yeast, the TetO regulated yeast exhibited a reduced growth rate when exposed to doxycycline. In pursuit of heterologous proteins, further experimentation of the TetO system may be considered. Different production platforms may prove more appropriate in future studies due to greater efficacy in reducing cell wall strength.

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Section 1: Introduction

1.1 Biotechnology Industry Utilizes Microbes

The pharmaceutical biotechnology industry has become well versed in engineering various mechanisms for heterologous protein expression in microbes. The global market for proteins such as insulin, growth factors, and hepatitis B virus surface antigen is increasing while current production rates are insufficient to meet the rising demand (Lesage and Bussey 2006). A variety of production platforms provide possible solutions, utilizing different cell types, expression systems, and methods of genetic regulation (Stanbury et al. 2017). Using microbes to produce proteins offers a multitude of protein production opportunities as each cell type is genetically unique. Common microbes utilized for protein production include, but are not limited to, various bacteria and yeast strains due to their rapid growth, inexpensive media requirements, and genetic tractability (Jeandet et al. 2013). Bacteria like, *Escherichia coli (E. coli)*, are often used for industrial protein production because they grow quickly, can be maintained inexpensively, and are easily manipulated for protein production and recovery (Nemecek et al. 2006). However, bacteria are insufficient protein producers when those industrial proteins are large or require complex modifications (Lotti and Pollegioni 2014). To produce large or complex proteins at an industrial scale, people often use the yeast *Saccharomyces cerevisiae* (Zhang et al., 2015).

1.2 Yeast Cell Wall as a Barrier

In pursuit of heterologous protein production, the bakers/brewer's yeast *Saccharomyces cerevisiae* is among the many commonly used microbes. The utilization of *S. cerevisiae* as a common microbe is often due to its fermentative

capacity and tolerance to stresses (Regis et al., 2012). Additionally, *S. cerevisiae* is utilized in the industry as a safe, low-cost alternative for producing proteins such as human insulin or compounds such as 1,3-Propanedial (Huang et al., 2018; Rao et al., 2008). Production platforms unique to *S. cerevisiae* have been devised in an effort to improve protein production capacity, such as a method, described by Zhang et al., involving a mutant strain that would co-produce bioethanol and ergosterol. However, it has been found that extraction of desired proteins can be an extensive process because most proteins are retained within the cell which is surrounded by the cell wall. As such, the cell wall creates a barrier in obtaining the proteins within the cell. In order to extract the proteins, the cell wall must be cracked open. As a result, new methods are needed to more efficiently obtain the proteins produced by *S. cerevisiae* (Huang et al. 2018).

1.3 Yeast Cell Wall Components

S. cerevisiae is of interest because it can be genetically engineered to modify the structural components of its cell wall (James et al., 1986). The cell wall forms a unique structure with linkages between polysaccharide-protein complexes that create an inner layer of glucans and chitins and an outer layer of mannoproteins. Of the polysaccharides, glucose forms β-1,3 and β-1,6 linkages to other glucose molecules. In addition to these, glucose also forms β-1,4 linkages to N-acetylglucosamine (GlcNAc). The structural polysaccharide called β-1,3-Glucan forms when the β-1,3 linkages are branched by additional β-1,6 linkages. β-1,3-Glucan provides tensile-like strength and elasticity to the cell wall by forming linkages to other cell wall components such as chitin. An additional component of the cell wall, mannoproteins, are then linked to β-1,6-glucose chains through

a glycosylphosphatidylinositol (GPI) anchor or directly linked to β-1,3-glucan through alkali-sensitive bonds (Lesage and Bussey, 2006).

1.4 FKS1/GSC2

Being that β-1,3-Glucan provides a source of tensile-like strength and elasticity, the β-1,3-Glucan synthase subunits ultimately contribute to assembling the structured cell wall and thereby making the cell difficult to lyse. The FKS family of yeast proteins has been associated with the catalytic subunits of β-1,3-Glucan synthase (Lesage and Bussey, 2006). Specifically, two paralogous genes in the FKS family, *FKS1* and *GSC2*, have been identified to encode the catalytic subunits of β-1,3-Glucan synthase (Mio et al., 1997). Even though genes *FKS1* and *GSC2* are functionally redundant, they exhibit different patterns of expression (Lesage and Bussey, 2006). *FKS1* is expressed during mitotic growth, while *GSC2* is not detected during mitotic growth but expression begins to increase during low levels of glucose and decreasing levels of *FKS1* (Lesage and Bussey, 2006). As such, *GSC2* seems to be expressed under catabolite repression.

In addition to patterns of expression, *FKS1* and *GSC2* mutant strains result in differing cell wall effects. Being an essential pair of genes, a strain of yeast with both *FKS1* and *GSC2* deleted (*fks1Δ gsc2Δ*) would be inviable. A mutant strain with only *FKS1* deleted is associated with reduced activity of β-1,3-Glucan synthase as well as an altered cell wall composition but is still viable as long as the strain has a normally functioning *GSC2* gene (Mio et al., 1997). A mutant strain with only *GSC2* deleted has no associated cell wall defects (Lesage and Bussey, 2006).

1.5 TetO System

The TetO system, described by Cuperus et al., alters expression of a target gene in the presence of the chemical tetracycline, or its more stable derivative doxycycline **(Figure 1)**. For the TetO system to be implemented, a few working parts must be introduced to the yeast individually. The way this system works is comparable to a hand flipping a light switch off. To turn the light off, an activating source is needed (a hand) and a switch that has the potential to be flipped is needed to turn the lights off. The actual light is synonymous with the gene of interest. Once the hand and the light switch are "installed", the light (gene of interest) can be tested to see if it will turn off. If so, this implies that the system is working correctly. Specifically, the system is integrated into *S. cerevisiae* by first introducing the tet-operator site of *E. coli,* which serves as the binding site for a transcriptional tet-activator **(Figure 2)**. As the tet-activator is expressed under the control of a constitutive promoter, a tet-off promoter is also introduced **(Figure 3)**. Once all the working parts are introduced and the cells are in the presence of doxycycline, the tet-activator can no longer interact with RNA polymerase. This prevents transcription of the gene of interest essentially "turning off" the gene (Cuperus et al. 2015).

1.6 Project Outline

The catalytic subunit of 1,3-beta-D-glucan synthase (*FKS1*) is an enzyme that plays a major role in assembling the cell wall, making the cell difficult to lyse. If *S. cerevisiae* cells were not able to make as much *FKS1* protein (pFKS1) as they naturally do, they would exhibit a reduced growth rate as well as weaker cell walls. This work tests that hypothesis by controlling the *FKS1* gene using the TetO system. The approach for this restriction was to genetically reprogram the yeast to reduce production of *FKS1* when

exposed to the chemical doxycycline. To accomplish this, the native *FKS1* and *GSC2* genes were knocked out, leaving only the tetO regulated *FKS1* gene that can be turned off by the introduction of doxycycline **(Figure 4)**.

If such a system were installed in yeast to control the *FKS1* gene, then successfully altering the expression of the *FKS1* gene would presumably decrease the amount of β-glucans in the cell wall; therefore, weakening the cell wall enough to lyse the cells with more gentle treatments (Klis et al 2002). If successful, the effect of the TetO system will be confirmed by the ease at which the *S. cerevisiae* cells lyse. The effectiveness of the TetO system to reduce cell wall strength will be confirmed through analysis of cell concentrations **(Figure 5)**, the presence of a candidate reporter protein (luciferase enzyme) in the supernatant **(Figure 6 and 7)**, and the engineered cells susceptibility to lysis **(Figure 8)**. Any data that indicate the cell wall integrity is compromised will support the hypothesis.

Section 2: Materials and Methods

2.1 Media and Culture Conditions

The yeast strain, yBR-*ura*3ΔCEN.PK113-7D, was cultured and used for transformation with the TetO regulated bioluminescent gene and the TetO regulated *FKS1* gene. The yeast strains were cultured in yeast extract peptone dextrose (YPD) medium. This medium is composed of 1% yeast extract (Becton Dickinson Co.), 2% peptone (Becton Dickinson Co.), and 2% glucose (Fisher) in distilled water. Agar (Acros Organics) (2%) was also added in the case of preparing solid YPD medium. Antibiotic selection media for yeast were prepared using either 0.2 mg/mL of hygromycin (Corning), 0.2 mg/ml of G418 (RPI Corporation), 0.1 mg/ml of zeocin (Invitrogen), or 50 µg/ml nourseothricin (RPI Corporation) added to YPD media. Uracil auxotrophic selection media was used for stable selection of pRS306-CYC-tTA and was comprise of 0.67% Yeast Nitrogen Base without amino acids (Becton Dickinson Co.), 750 mg/L CSM-Uracil amino acid supplement (Formedium), 2% glucose, and 2% agar. Luciferin (Gold Bio.) (100 µM) was added to the YPD media to observe bioluminescence in modified yeast. Doxycycline (RPI Corporation) (Dox) was diluted to a concentration of 10 µg/mL and added to the media when testing the TetO system. Top 10 transformation *E. coli* cells were grown in luria broth (LB) medium which contained 100 µg/mL of ampicillin (Sigma Aldrich) (amp). Agar (1.5%) was also added in the case of preparing a solid LB medium.

2.2 PCR Amplification

PCR amplifications followed The GoTaq Flexi System from Promega. The reactions included 4mM $MgCl_2$ (Fisher), 1µM of each primer, 0.2 µM of deoxynucleotide triphosphates (dNTPS) (Promega), and 1.25 units of GoTaq DNA Polymerase (Promega).

The PCR solution was placed in a thermocycler that cycled the solution through conditions as described by The GoTaq Flexi System from Promega. The first phase of a cycle held the solution at 94°C for 2 minutes, followed by the second phase of another 0.5 minutes at 94°C. The third phase of the cycle lasted for 0.5 minutes, and the temperature of this phase ranged anywhere from 50-70°C depending on which primers were used to prepare the PCR solution. The fourth phase of the cycle raised the temperature to 72°C for 1 minute/kb. The fifth and final phase of a cycle held the solution at 72°C for another 3 minutes. Phases 2-4 were repeated 30 times before the temperature was lowered to 4°C where it was held until the product was removed.

2.3 Restriction Digest

A 20 µL solution containing 2 µL of buffer (New England Biolabs), 1000 ng of the desired deoxyribonucleic acid (DNA), and 0.5 µL of restriction enzyme (New England Biolabs) was prepared. The solution was placed in a 37°C water bath for 1 hour. If digesting a vector, 0.5 µl of rSAP (recombinant Shrimp Alkaline Phosphatase) from New England Biolabs was added and placed back in the water bath for another hour. After the second hour, the product was then extracted and loaded onto a gel.

2.4 Gel Electrophoresis

Gels were made with 40 mL of a 1xTris-Acetate-Ethylenediaminetetraacetic acid (TAE), 0.32-0.4 g of agarose, and 2 µL of SYBR Safe DNA gel stain (Invitrogen). The TAE solution was composed of 40 mM of Tris (Fisher), 20 mM of acetic acid (Fisher), and 1 mM of Ethylenediaminetetraacetic acid (EDTA) (Fisher). 1kb DNA Ladder from New England Biolabs was used to compare band lengths. The gel was run at 80-100 V until the dye line was about 75% down the gel, typically about 1-1.5 hours.

2.5 DNA Purification

A GeneJET Gel Extraction Kit from Fermentas Life Sciences was used to carry out DNA purification of bands from agarose gels. The protocol outlined in the kit was followed as is. Appropriate volume of the provided binding buffer was added to the gel slice to form a 1:1 volume: weight solution (100 µL of binding buffer:100 mg of agarose gel). The solution was incubated at 56°C for 10 minutes and mixed every few minutes until the gel slice completely dissolved. The solution was added to the GeneJET purification column and was centrifuged for 1 minute. The flow-through was then discarded and the column was placed back into the same collection tube. After this, 700 µL of wash buffer from the kit was added to the purification column and the solution was centrifuged again for 1 minute. The flow-through was discarded and the column was placed back into the same collection tube. The now empty purification column was centrifuged for 1 minute in order to remove any residual wash buffer. The purification column was then transferred into a clean microcentrifuge tube and 30 µL of nuclease-free water was added to the center of the column's membrane. The column was centrifuged for 1 minute and the purified DNA was extracted and stored at -20°C.

The GeneJET Plasmid Miniprep Kit from ThermoScientific was used to purify plasmid DNA from bacteria. A colony of E. *coli* cells was taken from a freshly streaked plate and inoculated with 5 mL of LB medium at 37°C for 12-16 hours while shaking. The bacterial culture was harvested by centrifugation at 8,000 rpm for 2 minutes at room temperature. The supernatant was removed, and the pelleted cells were resuspended in 250 µL of the resuspension solution provided in the kit. 250 µL of the kit's lysis solution was added, and the tube was mixed using inversion. 350 µL of the provided neutralization

solution was added, and the tube was immediately mixed thoroughly by inverting it several times and centrifuged for 5 minutes. The supernatant was carefully transferred to the provided GeneJET spin column ensuring that the white precipitate was not disturbed. The spin column was centrifuged for 1 minute. The flow-through was discarded and the column was placed back into the same collection tube. 500 µL of the wash solution from the kit was added to the spin column, and the solution was centrifuged for 30-60 seconds. The flow-through was discarded and the column was placed back into the same collection tube. This washing procedure was repeated using a fresh 500 µL of wash solution. The flow-through was discarded and the column was centrifuged for another minute to remove any remaining wash solution. The spin column was transferred into a fresh microcentrifuge tube and 50 µL of nuclease-free water was added to the center of the column's membrane to facilitate the elution of the plasmid DNA. The column was incubated at room temperature for 2 minutes and then was centrifuged for 2 minutes. The purified plasmid DNA was collected and stored at -20°C.

The "smash 'n grab" protocol was used to purify DNA from yeast. In order to do so, a pellet of yeast was formed by centrifuging 1.5 mL of cells at 3000 rpm for 1 minute. The supernatant was discarded, and the pellet was resuspended in 0.2 mL of lysis buffer (Fisher) (10 mM Tris pH 8, 1 mM EDTA, 100 Mm NaCl, 1% SDS, and 2% Triton X-100), 0.3 g of glass beads, and 0.2 ml of a 1:1 phenol/chloroform mix (Acros Organics). The solution was then vortexed at top speed for 4x 30 seconds with ice in between for a total of 2 minutes being vortexed. Afterword, 0.2 mL of TE (10 mM Tris, 1 mM EDTA, pH 8.0) was then added to the tube and vortexed again for a few seconds. The tube was centrifuged for 5 minutes at 3000 rpm. Next the aqueous phase of the tube was transferred to a

new Eppendorf tube and then two volumes of 100% ethanol (Fisher) were added to the new tube. This was centrifuged for 15 minutes at 3000 rpm. Using the aspirator, the supernatant was discarded, and the remaining pellet was rinsed with 0.5 mL of cold 70% ethanol (Fisher) and placed back into the centrifuge for 5 minutes. The supernatant was again discarded, and the tube was vacuum dried. Remaining pellet was resuspended in 20 µL of TE.

In the case where products of PCR required restriction digestion, the PCR product was purified in the following way. The appropriate amount of binding buffer from the GeneJET PCR purification kit was added to completed PCR mixture to form a 1:1 volume (100 µL of binding buffer:100 µL PCR mixture). The solution was added to a GeneJET purification column and centrifuged for 30-60 seconds. The flow-through was discarded and 700 µL of the provided wash buffer was added to the purification column. The solution was centrifuged again for 30-60 seconds. The flow-through was discarded and the column was placed back into the same collection tube. The empty purification column was centrifuged for 1 minute in order to remove any remaining wash buffer. The purification column was transferred to a clean microcentrifuge tube, and 50 µL of the kit's elution buffer was added to the center of the column's membrane. The column was centrifuged for 1 minute and the purified DNA was then collected and stored at -20°C.

2.6 Ligation Reaction

Ligation reactions consisted of a 20 µL solution containing 1x Ligase buffer (New England Biolabs), 50 ng of vector, an amount of insert to yield a 3:1 molar ratio of insert to vector, and 200 units of T4 DNA ligase from New England Biolabs. Ligation reactions were incubated at 4°C overnight.

2.7 Transformation Protocols

The Top 10 *E. coli* transformation process began by thawing *E. coli* cells on ice. Pre-chilled tubes were labeled and 100 µL of cells was added to each of the tubes. Then, 5 µL of ligation solution was added to the tubes and they were incubated on ice for 30 minutes. The next step was to heat shock the cells at 42°C for 45 seconds. The tubes were then placed back on ice for 2 minutes to recover before 400 µL of Luria broth (LB) was added to the tubes. The cells were incubated at 37°C while shaking for 1 hour. The final step of the transformation was to plate about 50 µL on an LB/Ampicillin (AMP) plate.

The small yeast transformation process began by adding 1 mL of a 5 mL culture in log phase growth to an Eppendorf tube and spinning it down at 3,000 rpm for 5 minutes. The culture was washed with 500 µL of a 1X Tris-EDTA/0.1 M Lithium acetate (Fisher) (TE/LiOAc) solution and spun down again. The Eppendorf tube was placed on a roller inside a 30°C incubator for 1 hour. Following the 1-hour incubation period, 3-5 µg of plasmid was added directly to the cells along with 15 µL of Herring sperm DNA (Promega) and 700 µL of PEG/TE/LiOAc solution (40% Polyethylene glycol (Promega), 1X TE, 0.1M Lithium Acetate). The combined cell mixture was incubated on a roller at 30°C for 30 minutes and then placed in a 42°C water bath for 15 minutes. In order to pellet the cells, the tube was centrifuged for a few seconds. The supernatant was then discarded and the cells were resuspended in 100 µL of YPD and immediately plated for auxotrophic selections. However, the cells were resuspended in 3 mL of the YPD and outgrown for 3-4 hours before being plated for antibiotic selections.

2.8 Cloning Procedure

Plasmids were constructed using a basic cloning approach whereby a piece of DNA "the insert" was ligated into another piece of DNA "the vector". Inserts were created using PCR involving primers that added specific restriction sites on each end of the product. PCR products were purified as described previously and treated with appropriate restriction enzymes to create the necessary sticky ends for the vector to receive the insert. Simultaneously, creating the vector portion of the cloning procedure began with a plasmid that was digested with a pair of restriction enzymes necessary to produce the required sticky ends to receive the insert. Following the digestion, the cut vector was treated with rSAP and incubated at 37°C for 1 hour to remove 5' phosphates from the ends of the vector. After digesting insert and vector (and rSAP treating the vector), these cut products were run in a 1% agarose gel to isolate the appropriate digestion products. Bands containing the appropriately sized pieces were excised from the gel with a razer blade and purified from the gel as describe earlier. Concentrations of purified insert and vector were quantified with a nanodrop. Insert and vector were ligated together in a ligation reaction as described previously. Ligation products were transformed into Top10 *E. coli* as described previously and selected on LB plates containing 100 μM ampicillin. Colonies from the transformation were PCR-screened for evidence that they contained the insert. A colony showing evidence of successful transformation of intended plasmid was cultured overnight in 5 ml of LB containing ampicillin. From this culture, 0.5 ml of it were used to create a freezer stock (0.5 ml culture plus 0.25 ml of 50% glycerol (Fisher) frozen at -80°C) and the remaining 4.5 ml were used to miniprep (purify) the plasmid as described

earlier. After the plasmid was purified, its construction was validated by restriction analysis and the plasmid was frozen at -20°C until further use.

2.9 Cell Lysis Assays

The cells were grown in a 30°C shaking incubator for two days in YPD. To test cell concentration, 250 µl of cells from 3 tubes of each condition (Dox and No Dox) were suspended in 1750 µl water (2 ml total vol) for a 1/8 dilution each. Absorbances from these were measured in the spectrophotometer (600 nm) **(Figure 5)**. To test for luciferase enzymes in the supernatant, 1 ml from each of 3 tubes that had been treated with dox and 3 tubes that had not been treated was transferred to a clean microcentrifuge tube and centrifuged at 8000 rpm for 5 min. The top 750 µl of supernatant was then moved to a new tube (being careful not to disrupt any cells that had pelleted), and centrifuged again. The top 500 µl of supernatant was then transferred to a new tube and 1 µl of substrate (50 mM luciferin, 250mM Tris-HCl, 50mM MgCl2, 5 mM ATP, and 50mM DTT) (Fisher) was added to each new tube. These tubes were incubated at room temperature for 5 min, and then luminescence was measured in a luminometer **(Figure 7)**. To test the engineered cells susceptibility to lysis, 1 ml from each of 3 tubes that had been treated with dox and 3 tubes that had not been treated was transferred to a clean microcentrifuge tube and centrifuged at 8000 rpm for 5 min. The supernatant was then discarded, and the pellet was resuspended in 500 µl of TBS (Tris buffered saline, pH 7.6) (Fisher). Following resuspension, 300 µl of glass beads were added, and the tube was vortexed at top speed for 2 min. Next, 500 µl of TBS was added and the tube was inverted several times to mix. The tube was centrifuged again at 8000 rpm for 5 min to pellet beads, unlysed cells, and cell debris. The top 750 µl of supernatant was transferred to a new tube (being careful not to disrupt any cells that had

pelleted) and centrifuged again as before. The top 500 µl of supernatant was transferred to a new tube and 1 µl of substrate (50 mM luciferin, 250mM Tris-HCl, 50mM MgCl2, 5 mM ATP, and 50mM DTT) was added to each tube which were then incubated at room temp for 5 min. Luminescence was then measured in a luminometer **(Figure 8)**.

Section 3: Results

It was hypothesized that *S. cerevisiae* cells would presumably exhibit a reduced growth rate as well as weaker cell walls if they were not able to make as much *FKS1* protein (pFKS1) as they naturally do. The approach for this restriction was to genetically reprogram the yeast to reduce production of the *FKS1* protein (pFKS1) when exposed to the chemical doxycycline. The first step was to demonstrate that genetically programmed yeast can turn off the production of a bioluminescent reporter gene (a stand-in for *FKS1*) when exposed to doxycycline. This approach showed the genetic manipulations were behaving as expected. Having observed a significant (p-value=8.313x10^-05) reduced bioluminescence as influenced by doxycycline, the next objective compared the natural *FKS1* promoter and tet-off promoter activity levels in the presence and absence of doxycycline. Results of a t-test showed a p-value of 0.5472 which indicates both promoters drive similar levels of gene expression **(Figure 9)**. Following these results, the tet-off promoter was used to target the *FKS1* gene with the expectation that doxycycline restricted levels of *FKS1* in the cell wall, similar to what was seen with the bioluminescent reporter gene **(Figure 4)**.

3.1 Build a Stable, Integrating Version of the TetO System

The Cuperus et al TetO system as constructed by the authors required underlying conditions that had to be met in the organisms it was used in. One of these conditions included continuously culturing the TetO transformed cells in media that lacked uracil. Because their TetO system didn't integrate into the chromosome, a limitation was apparent. If the yeast were ever removed from the -ura condition, the cells could potentially divide in a way that loses the tet-activator. A stable version of this Cuperus et al TetO system was

constructed to integrate into the yeast genome in order to overcome this limitation. The first step to integrating the TetO system into the chromosome was to introduce an activating source, tet-activator plasmid. In order to build the integrable tet-activator plasmid, the tet-activator element from p416 CYC-tTA plasmid was amplified using polymerase chain reaction (PCR) with primers, Pcyc(Not)5 and Tcyc(Nhe)3, which add a NotI restriction site to the 5' end and a NheI restriction site to the 3' end. The tet-activator fragment was then cloned and inserted into the NotI and SpeI sites of the pRS306 plasmid. **Figure 2** shows this cloning operation that created an integrable and selectable plasmid with the tet-activator incorporated. A PCR screen was set up using primers, Pcyc(Not)5 and Tcyc(Nhe)3, that would amplify the tet-activator element (1.6kb) of the resulting colonies along with a positive control, Pcyc-tTA-Tcyc element to identify successful construction and *E. coil* transformants. **Figure 10** shows the successful results of this PCR screen. To further confirm the product was successfully constructed, the miniprep DNA was digested using the restriction enzyme, EcoRI. **Figure 11** shows that the 4.6 kb and 1.3kb digest pattern on the gel in lanes 4 and 5 matched the pattern that was expected if the plasmid was constructed correctly.

3.2 Add Integrable TetO System to Yeast

The linearized plasmid, pRS306 CYC-tTA, was then transformed into a robust wildtype yeast strain, CenPK113-7D-ura, which is useful in producing proteins of interest. In order to confirm the transformation was successful, the DNA was PCR amplified from the candidate yeast's genomic DNA using primers, Pcyc(Not)5 and Tcyc(Nhe)3, that amplified the tet-activator element. In relation to the chromosome, **Figure 12** illustrates how the tet-activator element (1.6kb) was PCR amplified using those

primers. **Figure 13** confirms successful integration of pRS306 CYC-tTA into CenPK113-7D-ura with positive bands at 1.6kb in lanes 1 and 2 that correlate with the tet-activator element.

3.3 Remove Endogenous GSC2 gene

In a *GSC2* deletion strain, *FKS1* plays an essential role in the formation of *S. cerevisiae* cell wall. As such, removing the endogenous *GSC2* gene would isolate *FKS1*'s role in forming the cell wall when under the control of the TetO system. To build this deletion strain, the KanMX deletion element (2.2kb) was PCR amplified from a previously existing *GSC2* deletion strain (created by Winzeler et al., 1999) using primers Aconf5 and Dconf3. The first lane in **Figure 11** confirmed successful amplification. The KanMX deletion element was then transformed into the yeast and confirmed in **Figure 14**. Primers, KanC and GSC2DS3, were used to amplify part of the KanMX deletion and the end of the *GSC2* gene. **Figure 14** confirms success of construction with positive bands of 1.0kb in lanes 1, 2 and 3.

3.4 Test Response to Doxycycline Using Bioluminescent Reporter

The effects of the TetO system can now be observed by linking the tet-off promoter to CBG reporter gene and observing the amount of bioluminescence produced. In order to build this construct, the tet-off promoter (200bp) was PCR amplified from pCM181-Luc1 using primers, ptOFF(Xma)5 and ptOFF(Bgl)3, that added XmaI and BglII restriction sites. The second lane in **Figure 11** shows successful amplification of the tet-off promoter with a positive band at 200bp. The TEF1 promoter from pRS305-hph-Ptef-CBG99 was switched with the tet-off promoter creating a plasmid that could be regulated by doxycycline. **Figure 3** shows this construction design to make PtOFF-CBG99-Hph305. To

confirm the plasmid was correct, the candidate DNA was PCR amplified using primers, ptOFF(Xma)5 and ptOFF(Bgl)3, shown in **Figure 15** lanes 1 and 2. To further confirm, the candidate plasmid was also digested using the restriction enzyme SalI, shown in **Figure 15** lanes 3 and 4. A negative control, pRS305-hph-Ptef-CBG99, was placed in lane 5. **Figure 15** confirms a successful ligation as the PCR amplified DNA correlated with the 200bp tet-off promoter in lanes 1 and 2. **Figure 15** also confirmed a successful ligation as lanes 3 and 4 showed the digest pattern, 7kb and 2kb which corresponds to the predicted pattern if successful. The negative control, pRS305-hph-Ptef-CBG99, showed the digest band pattern of 9kb which is what was expected if no promoter modifications occurred. The dox regulated plasmid was then transformed into the yeast. **Figure 16** shows the transformed yeast which were selected on media that allowed visualization of bioluminescence. Measurements in Table 3 show stunted bioluminescence in the colonies with doxycycline compared to the colonies without doxycycline.

Construction of a plasmid for which the tet-off promoter was switched with the *FKS1* promoter was then performed to compare activity levels between the untreated tet-off promoter and the native FKS1 promoter. This comparison was important to determine whether expression levels from the TetO system (in the absence of doxycycline) were similar to the natural expression levels from the *FKS1* promoter. If the expression levels were not similar, potential complications, such as cell growth, could arise in a cell that only had *FKS1* produced through the TetO system. **Figure 17** confirms successful PCR amplification of the *FKS1* promoter (1.0kb) using primers, pFKS(Xma)5 and pFKS(Bgl)3. **Figure 18** shows the construction design for pFKS1-CBG-Hph305, and it was confirmed in **Figure 19** by PCR amplifying the *FKS1* promoter using

primers pFKS(Xma)5 and pFKS(Bgl)3. **Figure 20** shows digest of construct using ApaI restriction enzyme for further confirmation. The predicted digest pattern of 7.5kb, 1.8kb, and 0.7 kb was seen in lane 3, confirming a successful construction.

The two strains of yeast were alternately patched in a grid format onto an agar medium that contained doxycycline **(Figure 21)** and another medium without doxycycline **(Figure 22).** The two plates were imaged by a ChemiDoc camera that takes sequential pictures of plate bioluminescence for a programed exposure time. **Figure 9** quantifies the difference in bioluminescence of the two strains of yeast in the presence and absence of doxycycline. Columns 1 and 2 in **Figure 9** show similar native activity levels of the *FKS1* promoter and the tet-off promoter. Column 2 illustrates the TetO system will drive expression of the targeted gene (CBG reporter gene) in the absence of doxycycline. Column 3 illustrates that even though doxycycline is present, the targeted gene (CBG reporter gene controlled by the *FKS1* promoter rather than tet-off promoter) does not alter the promoter's ability to drive gene expression. Column 4 shows a reduced bioluminescence in the tet-off regulated CBG reporter compared to column 2.

3.5 Replace Bioluminescent Gene with FKS1 Gene

Following the bioluminescent reporter results, the tet-off promoter was used to regulate the *FKS1* gene with the expectation that doxycycline will restrict levels of *FKS1* transcription similar to what was seen with the reporter gene and bioluminescence. The native *FKS1* coding sequence is 5.6 kb which was too large to efficiently manipulate through conventional PCR cloning methods, so the *FKS1* coding sequence was assembled in two parts (the left half and then the right half). The left half of the *FKS1* gene was amplified using primers, FKS1(Bam)5 and FKS1mid(Afl)3,

and confirmed in **Figure 23** lanes 2-4 with bands of 2.6kb. The right half of the *FKS1* gene was amplified using primers, FKS1mid(Afl)5 and FKS1(Nhe)3, and confirmed in **Figure 23** lanes 5-7 with bands of 3.0kb. The left half of *FKS1* was inserted into the cloning vector, pAllet. **Figure 24**, lanes 1 and 2, confirmed successful ligation by using primers, FKS1(Bam)5 and FKS1mid(Afl)3, to amplify the left half of the *FKS1* gene (2.6kb). Lane 3 in **Figure 24** shows positive control, left half of *FKS1* gene PCR product. The right half of the *FKS1* gene was inserted into the previously mentioned modified version of pAllet and then the whole *FKS1* gene (5.6kb) was cut out of pAllet and inserted into ptOFF-*CBG*-Hph305 to make ptOFF-*FKS1*-Hph305 as seen in **Figure 4**. **Figure 25** shows successful ligation of ptOFF-CBG-Hph305 and the whole *FKS1* gene through restriction digest of ptOFF-*FKS1*-Hph305 using restriction enzymes, AflII and BamHI. Plasmid, ptOFF-*FKS1*-Hph305, was transformed into yeast. Successful transformation was confirmed using primers, ADHIterm(Not)3 and FKS1mid(Afl)5, for a band pattern of ~3.3 kb as seen in lanes 2 and 3 of **Figure 26**. This was compared with the positive control, ptOFF-*FKS1*-Hph305, in lane 4.

3.6 Knockout Native FKS1 Gene

The construct thus far includes both an introduced tetO regulated *FKS1* gene and the native naturally regulated *FKS1* gene. The native *FKS1* gene can then be knocked out, leaving only the tetO regulated *FKS1* gene that can be turned off by the introduction of doxycycline. The first step to knock out the native *FKS1* gene consisted of PCR amplifying a nourseothricin N-acetyl transferase (NAT) gene which confers resistance to the antibiotic nourseothricin. The NAT gene (1.1kb) was PCR amplified from a cloning vector, pYM-N9, with primers, Nat(FKS1us)5 and Nat(FKS1da)3, that have 40 bases of *FKS1* homology

to permit targeted homologous recombination. Lane 6 in **Figure 26** shows the correct band size for the NAT gene (1.1kb). The amplified NAT gene was transformed into the yeast and selected on plates containing nourseothricin. **Figure 27** confirms the PCR amplified knockout *FKS1* gene using primers, FKS1koscrn5 and FKS1koscrn3. Lanes 2,4, and 5 show the correct band size (~1.7kb) for confirming the native *FKS1* gene knockout.

3.7 Test Doxycycline Regulated FKS1 for Cell Wall Integrity

The final plasmid introduced was pRS304-ShBle-Ptef-CBG99 which provided a constitutively expressed CBG reporter gene as a way to test cell wall integrity. This plasmid was added to both the tetOFF-FKS1 strain and the wildtype strain for eventual comparison. The CBG reporter gene is useful because it is an easily detectable soluble protein that would either be inside the yeast cells if they did not lyse or outside in the supernatant if the cells did lyse. As such, the location of the bioluminescence will be informative of whether the cell wall integrity is compromised or not **(Figure 6)**. The wildtype strain and final engineered construct cell concentrations recorded are seen in Table 4. Because cell numbers differed between these two strains, in order to compare the effect doxycycline had on cell concentration of both strains, the measurements were normalized to the "no dox" condition for each strain as seen in table 5. **Figure 5** illustrates the normalized cell concentration differences. The *FKS1* regulated cell concentration is significantly reduced in the presence of doxycycline (p-value=3.239x10^-05). Table 6 shows the amount of luciferase in the supernatant when the cells are in the presence and absence of doxycycline. **Figure 7** illustrates the data from Table 6 that indicated relatively no difference in the amount of luciferase in the supernatant from the cells lysing by themselves (p-value=0.317). The cells were then lysed using bead beating to test their

susceptibility to lysis through more rigorous treatment. The bioluminescence, recorded in Table 7, of the wildtype strain and *FKS1* regulated strain shows the cells susceptibility to lysis. Table 8 is the normalized data of the cells susceptibility to lysis which **Figure 8** illustrates. The doxycycline regulated *FKS1* cells in **Figure 8** did not exhibit a significant (p-value= 0.09584) increase in susceptibility to traditional lysing methods when exposed to doxycycline.

Section 4: Discussion

4.1 TetO System Response to Doxycycline Using Bioluminescent Reporter

For this research, the effectiveness of the TetO system was determined through reduced bioluminescence in a doxycycline regulated CBG gene. **Figure 16** confirms expression of the doxycycline regulated bioluminescent reporter gene. Initial observations of the TetO system, seen in Table 3, showed stunted bioluminescent measurements in the presence of doxycycline. This indicates the system was successfully altering the genetically programmed yeast to turn off bioluminescent reporter gene production when exposed to doxycycline. Similar results have been determined by Ikushima et al. 2015. Having observed the reduced bioluminescence as influenced by doxycycline, the next objective compared the natural *FKS1* promoter and tet-off promoter activity levels in the presence and absence of doxycycline. **Figures 15 and 16** confirm the expression of the tet-off promoter and *FKS1* promoter regulating the bioluminescent gene. Computer analysis of **Figure 21 and 22** entailed pertinent information for the native activity levels of the *FKS1* promoter and the tet-off promoter. **Figure 17** showed similar activity levels suggesting that both promoters drive similar levels of gene expression. If the native activity levels of both primers had driven different levels of gene expression, tet-off variants could have been constructed in order to drive a similar expression as *FKS1* promoter (Cuperus et al. 2015). In addition, **Figure 9** also confirmed the TetO system was working as expected due to reduced bioluminescence in the tet-off yeast in the presence of doxycycline as compared to in the absence of doxycycline.

4.2 TetO System Regulating FKS1

The effectiveness of the TetO system to reduce cell wall integrity by controlling *FKS1* was then confirmed through analysis of cell concentrations, the presence of a candidate reporter protein (luciferase enzyme) in the supernatant, and the engineered cells susceptibility to lysis. Assay 1 results, seen in **Figure 5**, show fewer cells per unit volume in the *FKS1* regulated strain when exposed to doxycycline. Reduced cell concentrations were not seen in the wildtype strain in either treatment. This suggested that the doxycycline treatment effectively slowed the growth of the *FKS1* regulated strain.

Assay 2 measurements, seen in Table 6, did not suggest that the *FKS1* regulated yeast would lyse automatically when exposed to doxycycline. **Figure 7** illustrates that whether in the absence or presence of doxycycline, there was relatively no difference in the amount of luciferase found in the supernatant of the *FKS1* regulated cells lysing themselves. Although, the results for this assay are reliant upon the protein's ability to remain intact and active in the media until the end. Possible proteases could be present in the media resulting in protein degradation prior to assay maturation. As such, early degradation of desired cell proteins could be a potential vulnerability of assay 2 due to the possibility of undetected cell lysis. Results from assay 1 (**Figure 5**) indicate the regulated FKS1 cells exhibited a reduced growth rate when exposed to doxycycline. The significant p-value (3.239x10^-05) suggests that the doxycycline regulated *FKS1* yeast have a reduced growth rate which may be indicative of a compromised cell wall. Results from assay 2 (**Figure 7**) indicate that the regulated *FKS1* yeast do not automatically lyse when exposed to doxycycline. Results from assay 3 (**Figure 8**) illustrate that the supernatant from regulated *FKS1* cells do not show a higher bioluminescence when in the

presence of doxycycline than when in the absence of doxycycline. These results confirmed that TetO regulated FKS1 yeast did not exhibit an increased susceptibility to traditional lysing methods when exposed to doxycycline.

4.3 Future Directions

The slowed growth seen in assay 1 may be due to *FKS1* and *GSC2* differing patterns of expression. Additionally, *FKS1* and *GSC2* mutant strains are known to result in differing cell wall effects. If a random mutation occurs in the population which bypasses the burden of growth, then those mutant cells will grow faster and therefore become more representative in the population. While the TetO system has successfully altered the growth rate of *S. cerevisiae,* different production platforms may be considered in order to compromise cell wall integrity to the extent needed for obtaining heterologous proteins. Such production platforms may involve hijacking a glucanase that functions to weaken the *S. cerevisiae* cell wall. Increasing productivity of such glucanase would increase its ability to weaken the cell wall.

Section 5: Conclusions

In this study, the TetO system was confirmed to successfully alter gene expression of a bioluminescent gene. The effectiveness of the TetO system in reducing cell wall integrity by controlling *FKS1* was also determined. This found that when *S. cerevisiae* cells were not able to make as much *FKS1* protein (pFKS1) as they naturally do, they exhibited a reduced growth rate which indicates a compromised cell wall. Results from assays 2 and 3 suggest that TetO engineered yeast do not lysis more when exposed to doxycycline. Although, these results are subject to limitations given that possible protein degradation may have occurred during assay 2 maturation. Therefore, in order to compromise cell wall integrity to the extent needed to obtain heterologous proteins, future studies may consider different production platforms such as targeting a glucanase that functions to weaken the *S. cerevisiae* cell wall as opposed to targeting a glucan synthase (*FKS1/GSC2)* that functions to strengthen the cell wall.

References

Cuperus, J.T., Lo, R.S., Shumaker, L., Proctor, J., Fields, S. (2015). A tetO toolkit to alter expression of genes in *Saccharomyces cerevisiae*. ACS Synthetic Biology 4(7):842–852. https://doi:10.1021/sb500363y.

Huang, M., Wang, G., Qin, J., Petranovic, D., Nielsen, J. (2018). Engineering the protein secretory pathway of *Saccharomyces cerevisiae* enables improved protein production. Proceedings of the National Academy of Sciences of the United States of America 115(47). https://doi:10.1073/pnas.1809921115.

Ikushima, S., Zhao, Y., Boeke, J.D. (2015). Development of a tightly controlled off switch for *Saccharomyces cerevisiae* regulated by camphor, a low-cost natural product. G3 Genes, Genomes, Genetics 5(10). https://doi:10.1534/g3.114.012765.

Jamas, S., Rha, C.K., Sinskey, A.J. (1986). Morphology of yeast cell wall as affected by genetic manipulation of β(1 → 6) glycosidic linkage. Biotechnology Bioengineering 28(6). https://doi:10.1002/bit.260280602.

Janke, C., Magiera, M.M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart, A., Doenges, G., Schwob, E., Schiebel, E., Knop, M. (2004). A versatile toolbox for PCR-based tagging of yeast genes: New fluorescent proteins, more markers and promoter substitution cassettes. Yeast 21(11). https://doi:10.1002/yea.1142.

Jeandet, P., Vasserot, Y., Chastang, T., Courot, E. (2013). Engineering microbial cells for the biosynthesis of natural compounds of pharmaceutical significance. Biomed Research International 2013. https://doi:10.1155/2013/780145.

Klis, F.M., Mol, P., Hellingwerf, K., Brul, S. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. In FEMS Microbiology Review 26(3). https://doi:10.1016/S0168-6445(02)00087-6.

Lesage, G., Bussey, H. (2006). Cell wall assembly in *Saccharomyces cerevisiae*. Microbiology and Molecular Biology Review 70(2). https://doi:10.1128/mmbr.00038-05.

Lotti, M., Pollegioni, L. (2014). Aggregation of recombinant proteins: Understanding basic issues to overcome production bottlenecks. In: Uversky, V., series editor. Protein aggregation in bacteria: Functional and structural properties of inclusion bodies in bacterial cells. 1st ed. Hoboken (NJ): Wiley; 221-246.

Mio, T., Adachi-Shimizu, M., Tachibana, Y., Tabuchi, H., Inoue, S.B., Yabe, T., Yamada-Okabe, T., Arisawa, M., Watanabe, T., Yamada-Okabe, H. (1997). Cloning of the *Candida albicans* homolog of *Saccharomyces cerevisiae* GSC1/FKS1 and its involvement in β-1,3-glucan synthesis. Journal of Bacteriology 179(13). https://doi:10.1128/jb.179.13.4096-4105.1997.

Nemecek, S., Marisch, K., Juric, R., Bayer, K. (2006). Design of transcriptional fusions of stress sensitive promoters and GFP to monitor the overburden of *E.coli* hosts during recombinant protein production. Bioprocess and Biosystems Engineering 5(1):47-53. https://doi:10.1186/1475-2859-5-S1-S24.

Rao, Z., Ma, Z., Shen, W., Fang, H., Zhuge, J., Wang, X. (2008). Engineered *Saccharomyces cerevisiae* that produces 1,3-propanediol from D-glucose. Journal of Applied Microbiology 105(6). https://doi:10.1111/j.1365-2672.2008.03868.x.

Reis, V.C.B., Nicola, A.M., De Souza Oliveira Neto, O., Batista, V.D.F., De Moraes, L.M.P., Torres, F.A.G. (2012). Genetic characterization and construction of an auxotrophic strain of *Saccharomyces cerevisiae* JP1, a Brazilian industrial yeast strain for bioethanol production. Journal of Industrial Microbiology and Biotechnology 39(11). https://doi:10.1007/s10295-012-1170-5.

Robertson, J.B., Davis, C.R., Johnson, C.H. (2013). Visible light alters yeast metabolic rhythms by inhibiting respiration. Proceedings of the National Academy of Sciences of the United States of America 110(52). https://doi:10.1073/pnas.1313369110.

Stanbury, P.F., Whitaker, A., Hall, S.J. (2017). The production of heterologous proteins. In: Principles of Fermentation Technology. 3rd ed. Oxford (United Kingdom): Butterworth-Heinemann; 725-775.

Winzeler, E., Shoemaker, D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J., Bussey, H., Chu, A., Connelly, C., Davis, K., Dietrich, F., Dow, S., El Bakkoury, M., Foury, F., Friend, S., Gentalen, E., Giaever, G., Hegemann, J., Jones, T., Laub, M., Liao, H., Liebundguth, N., Lockhart, D., Lucau-Danila, A., Lussier, M., M'Rabet, N., Menard, P., Mittmann, M., Pai, C., Rebischung, C., Revuelta, J., Riles, L., Roberts, C., Ross-MacDonald, P., Scherens, B., Snyder, M., Sookhai-Mahadeo, S., Storms, R., Véronneau, S., Voet, M., Volckaert, G., Ward, T., Wysocki, R., Yen, G., Yu, K., Zimmermann, K., Philippsen, P., Johnston, M., Davis, R. (1999). Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science 285(5429):901-906. https://doi:10.1126/science.285.5429.901.

Zhang, K., Tong, M., Gao, K., Di, Y., Wang, P., Zhang, C., Wu, X., Zheng, D. (2015). Genomic reconstruction to improve bioethanol and ergosterol production of industrial yeast Saccharomyces cerevisiae. Journal of Industrial Microbiology and Biotechnology 42(2). https://doi:10.1007/s10295-014-1556-7.

Appendix A-1: Figures

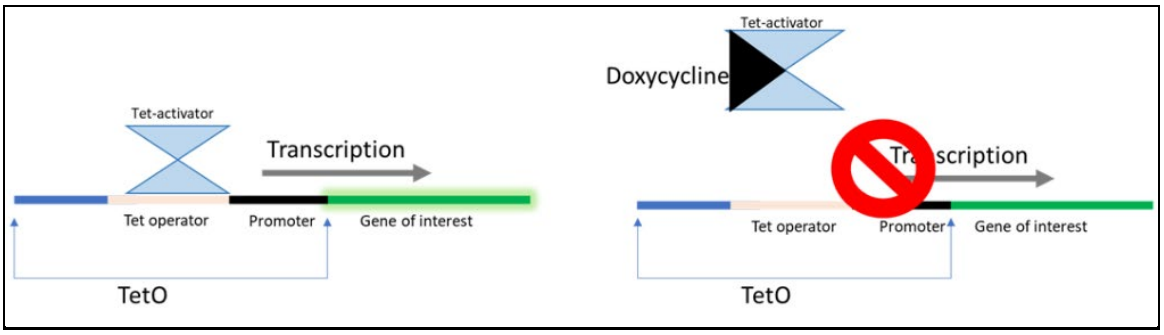

Figure 1: Representation of TetO system: Regulation of Tet-off system. In absence of Doxycycline, the Tet-activator binds the Tet-operator, and transcription of the downstream gene proceeds. In presence of Doxycycline, the Tet-activator is recruited away from the operator, and transcription is repressed.

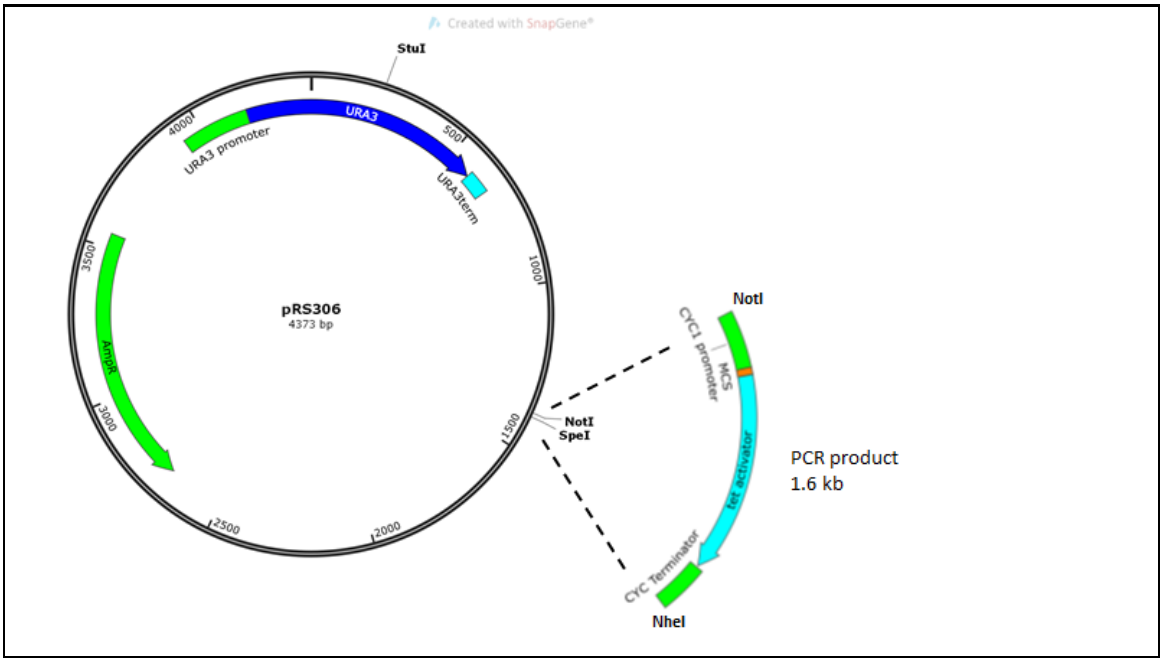

Figure 2: Construction of pRS306-CYC-tTA, an Integrating Plasmid bearing the Tet Activator. The plasmid pRS306-CYC-tTA was built by adding a 1.6 kb PCR product containing the Tet-activator sequence to the plasmid pRS306 using the restriction sites NotI at the 5' end and the compatible sticky ends produced by the restriction enzymes SpeI and NheI at the 3' end.

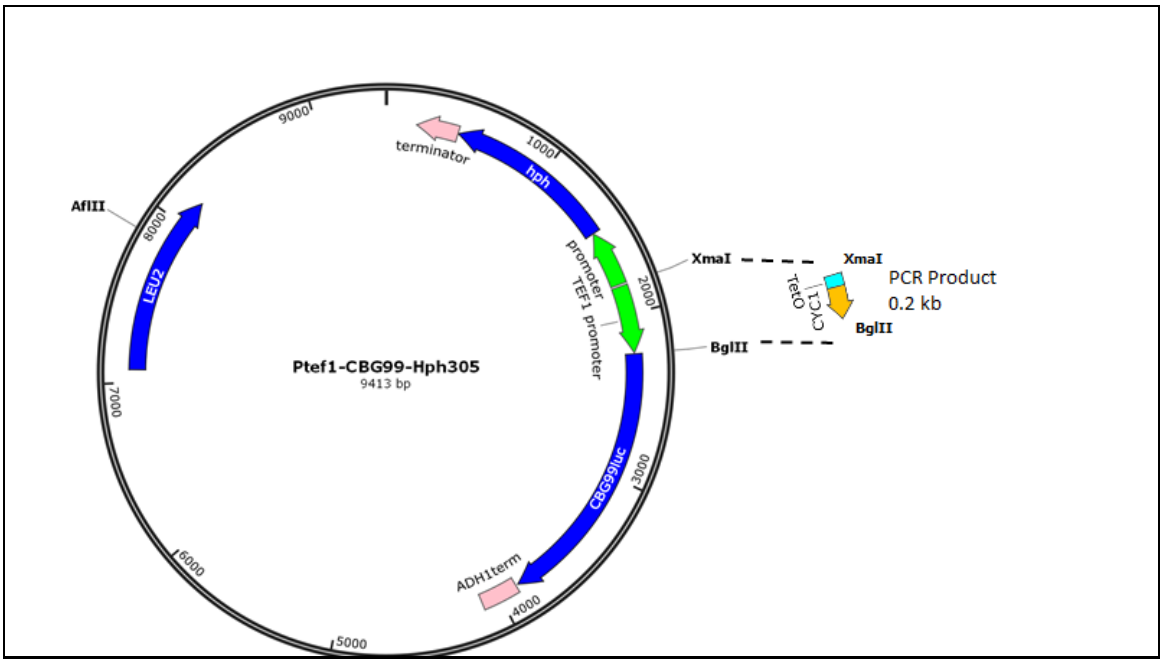

Figure 3: Construction of PtOFF-CBG99-hph305, a Doxycycline Regulated Bioluminescent Reporter. The plasmid PtOFF-CBG99-hph305 was built by adding a 0.2 kb PCR product containing the tet-off promoter, which consisted of the tet-operator upstream of the basal CYC1 promoter, to the plasmid pRS305-hph-Ptef-CBG99 using the restriction sites XmaI at the 5' end and BglII at the 3' end. This PCR product replaced the *TEF1* promoter that was previously in the plasmid.

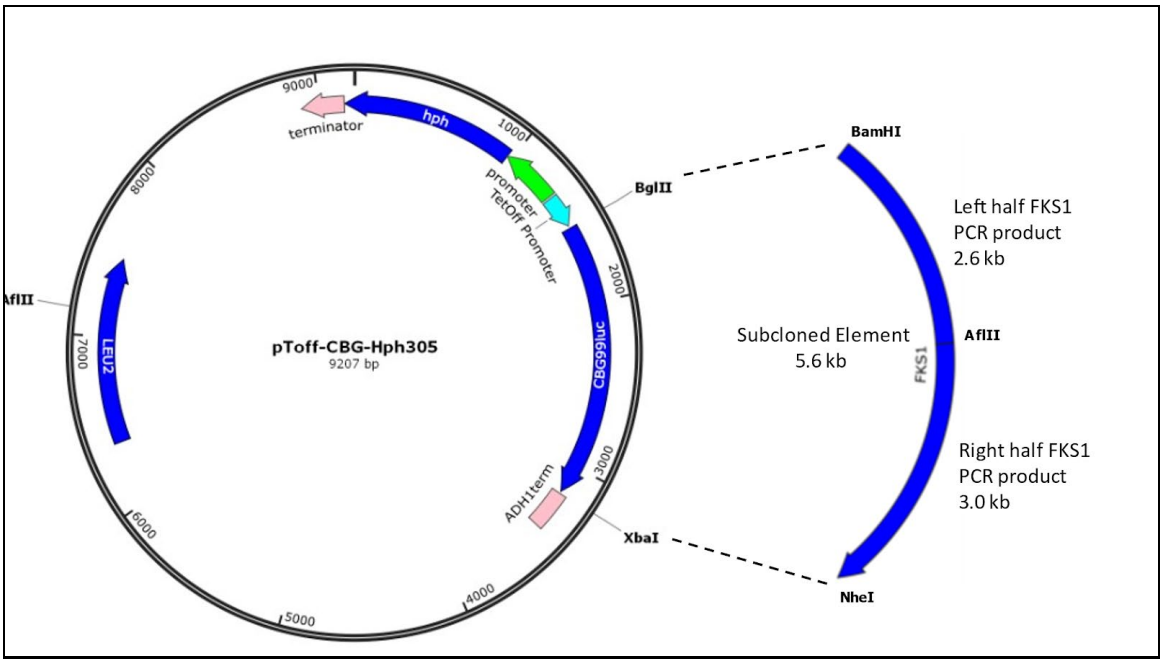

Figure 4: Construction of pToff-*FKS1*-Hph305, a Doxycycline Regulated FKS1 gene. The plasmid pToff-*FKS1*-Hph305 was built by adding a 5.6 kb element containing the subcloned FKS1 gene to the plasmid pToff-CBG99-Hph305 using the restriction sites BamHI at the 5' end and NheI at the 3' end. The AflII site within the subcloned FKS1 gene was used to piece the left and right halves together when cloned into pAllet. This 5.7 kb *FKS1* coding sequence replaced the 2.6 kb *CBG99* coding sequence within the original plasmid.

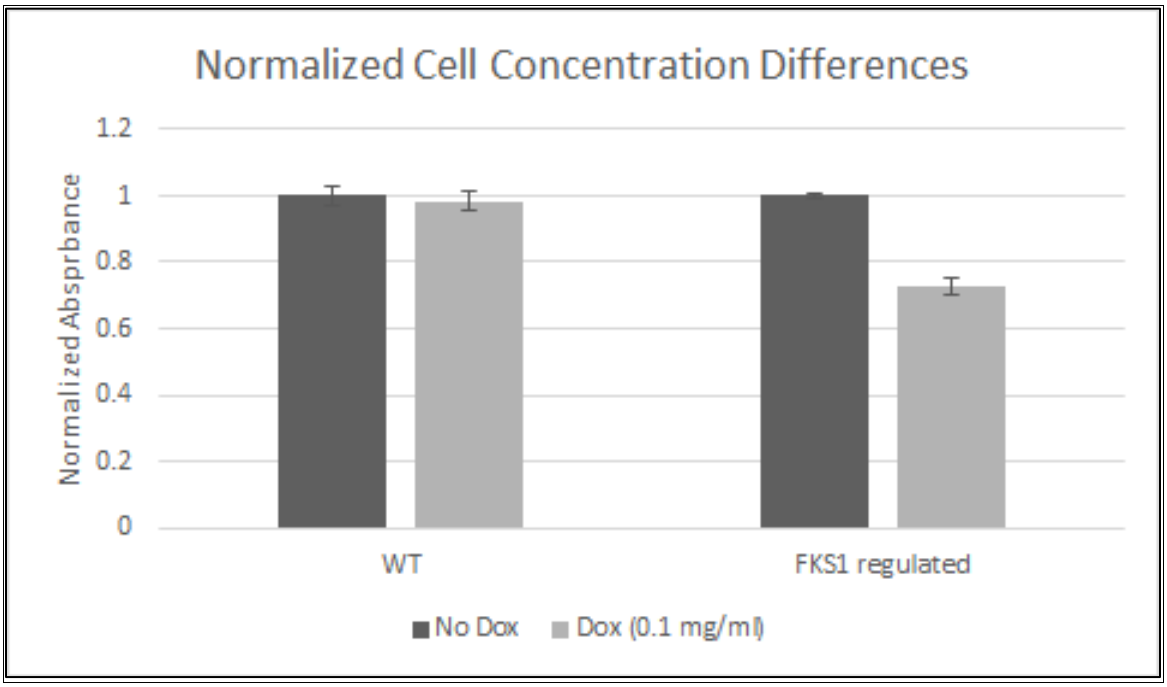

Figure 5: Assay 1 Cell Concentration Differences. The wildtype yeast measurements show similar absorbance levels in the absence of doxycycline and the presence of doxycycline. This shows similar cell concentration levels in the wildtype yeast in both environments (p-value=0.5591). As compared to the *FKS1* regulated yeast in the absence of doxycycline, the *FKS1* regulated cell concentration is reduced in the presence of doxycycline. The p-value was determined to be 3.239x10^-05 which is <0.05 leading to rejecting the null hypothesis that there is no difference in cell concentrations between the FKS1 regulated yeast in the presence and absence of doxycycline. The doxycycline concentration for both wildtype and FKS1 regulated yeast was 0.1mg/ml. Error bars are plus and minus one standard deviation, n=3.

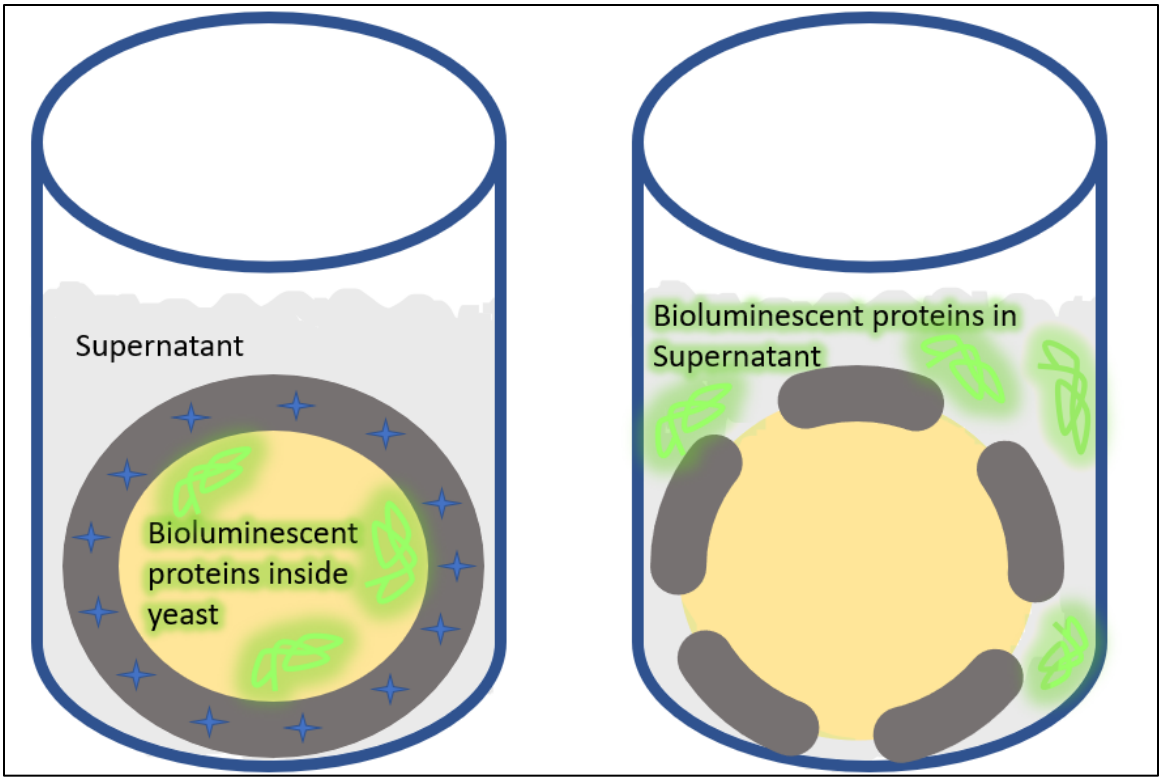

Figure 6: Representation of Bioluminescent Reporter Location. Left: The non-compromised yeast cell wall keeps the bioluminescent proteins inside the yeast and therefore the supernatant does not have any luminescence. **Right:** The bioluminescent proteins are detected in the supernatant when the yeast cell wall is compromised.

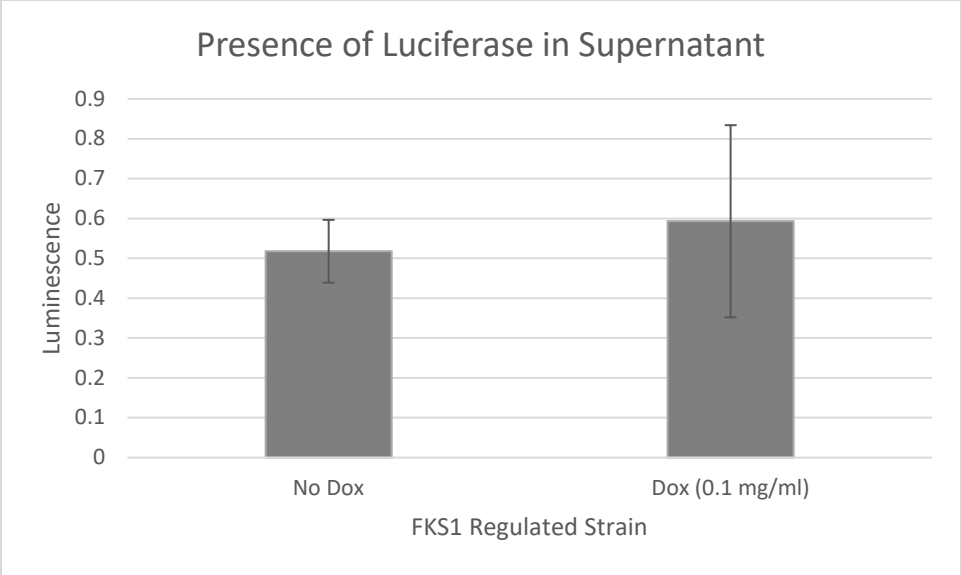

Figure 7: Assay 2 Presence of Luciferase Enzyme in Supernatant. The amount of luminescence in the supernatant was measured with 3 replicates of the FKS1 regulated yeast strain in the presence (0.1mg/ml) and absence of doxycycline. The standard error bars suggest low confidence that there is a difference in luminescence of the FKS1 regulated strain in the presence and absence of doxycycline. The p-value was determined to be 0.317 which is >0.05 leading to not rejecting the null hypothesis that there is no difference in supernatant luminescence between the FKS1 regulated yeast in the presence and absence of doxycycline. Whether in the absence or presence of doxycycline, there is relatively no difference in the amount of luciferase found in the supernatant of the cells lysing themselves.

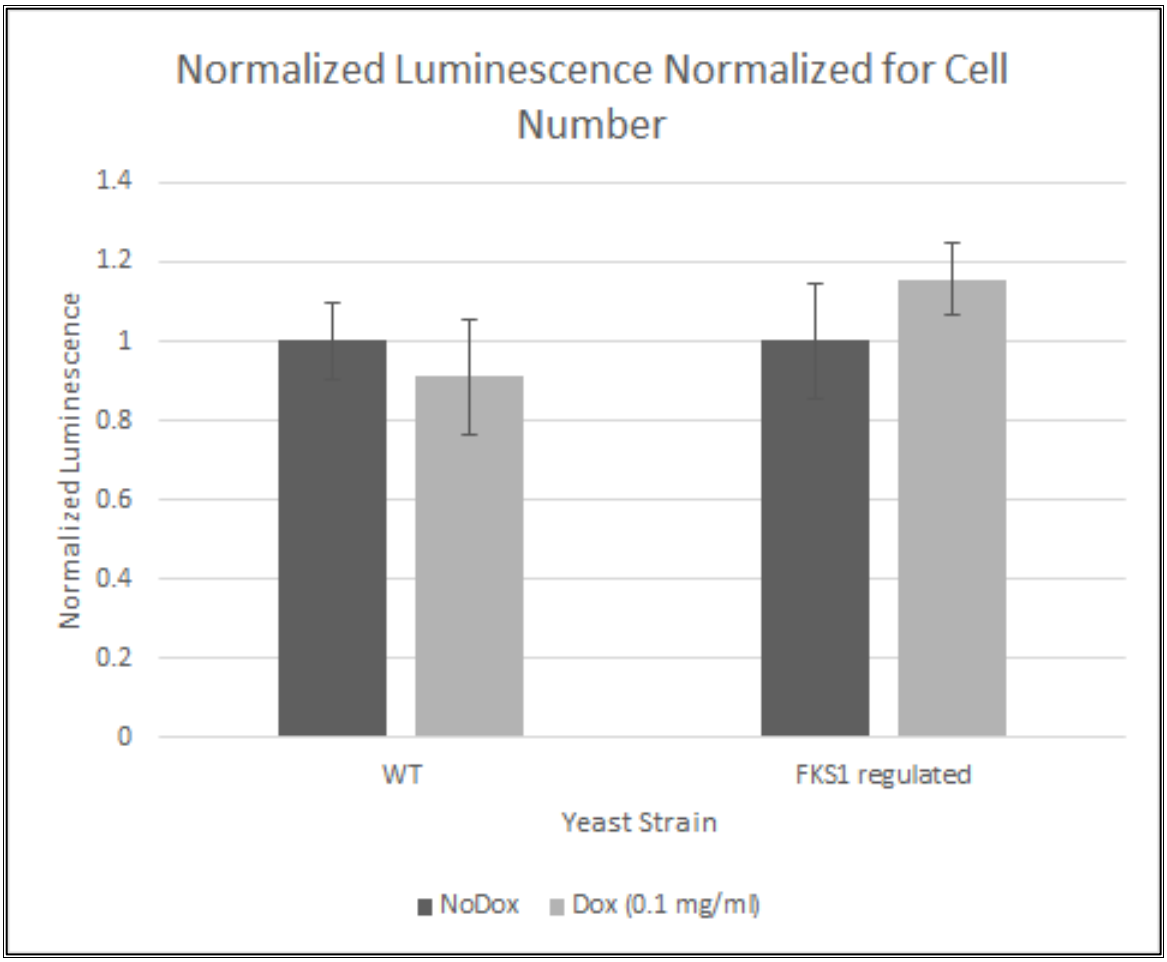

Figure 8: Assay 3 Susceptibility to Lysis. Both wildtype and FKS1 regulated yeast were lysed using bead beating to test their susceptibility to lysis through more rigorous treatment. The doxycycline concentration for both wildtype and FKS1 regulated yeast was 0.1mg/ml. The wildtype yeast measurements show similar bioluminescence in the absence of doxycycline and the presence of doxycycline. The *FKS1* regulated yeast in the absence of doxycycline has lower bioluminescence than the *FKS1* regulated stain in the presence of doxycycline. The p-value was determined to be 0.09584 which is >0.05 leading to not rejecting the null hypothesis that there is no difference in supernatant luminescence between the FKS1 regulated yeast in the presence and absence of doxycycline. These results indicate that the regulated *FKS1* yeast do not exhibit an increased susceptibility to traditional lysing methods in the presence of doxycycline. Error bars show plus and minus one standard deviation, n=3.

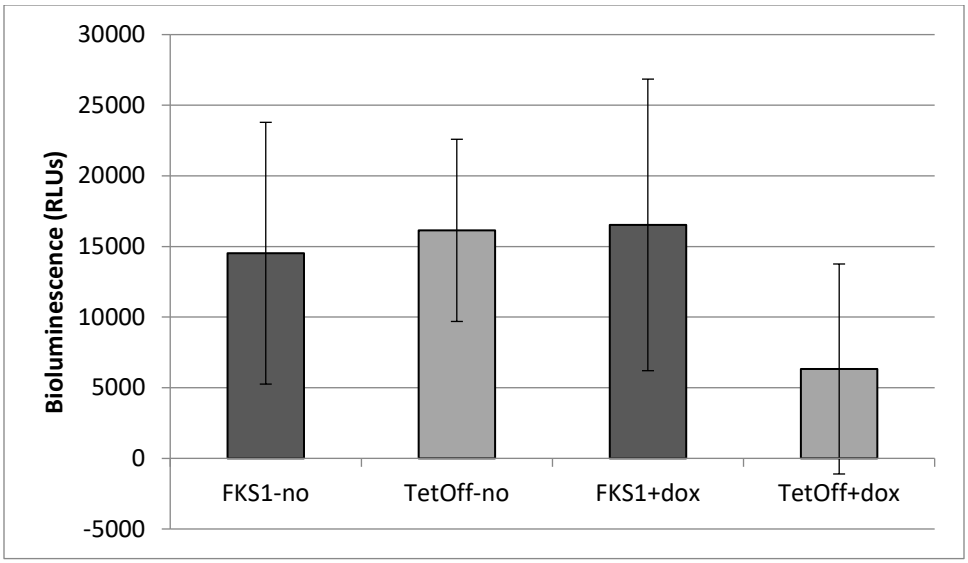

Figure 9: Reporter gene under the control of *FKS1* promoter vs TetO promoter in the absence and presence of doxycycline. Analysis of the two different strains of yeast in figures 15 and 16 illustrates the amount of bioluminescence in each environment (doxycycline vs no doxycycline). FKS1-no and Tetoff-no columns represent the strains of yeast with the FKS1 promoter and the Tetoff promoter in the absence of doxycycline. FKS1+dox and Tetoff+dox represent the strains of yeast with the FKS1 promoter and the Tetoff promoter in the presence of doxycycline. The measurements show that the FKS1-no and Tetoff-no columns have similar levels of bioluminescence (p-value=0.5472). As such, similar native activity levels of the *FKS1* promoter and the tet-off promoter. FKS1+dox column illustrates that even though doxycycline is present, the targeted gene (CBG reporter gene controlled by the *FKS1* promoter rather than tet-off promoter) does not alter the promoter's ability to drive gene expression. The Tetoff-no column illustrates the TetO system will drive expression of the targeted gene (CBG reporter gene) in the absence of doxycycline. This is compared to the Tetoff+dox column which shows a reduced bioluminescence in the tet-off regulated CBG reporter (p-value=8.313x10^-05). The error bars are plus and minus one standard deviation.

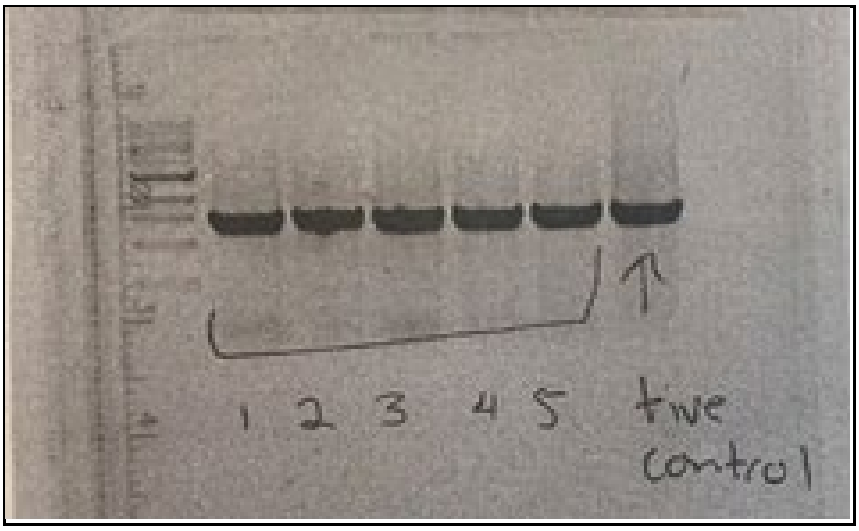

Figure 10: Confirmed Construction of pRS306-CYC-tTA. The tet-activator element from the new construct, pRS306-CYC-tTA, was screened in 5 candidate colonies by PCR using primers Pcyc(Not)5 and Tcyc(Nhe)3 in lanes labeled 1-5. The PCR product was 1.6kb which confirmed correct size as positive control, p416 CYC-tTA, also showed 1.6kb. The far-left lane contained 1kb Ladder (NEB N3232S).

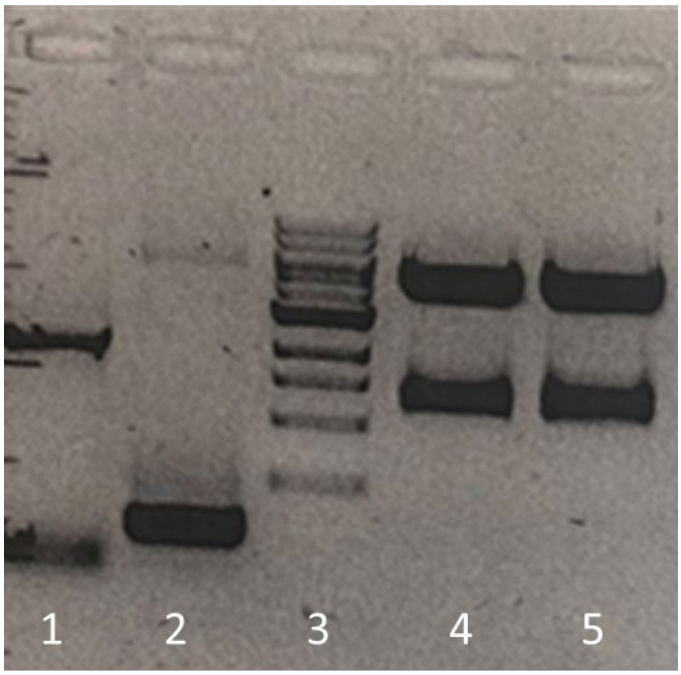

Figure 11: Confirmed Construction of pRS306-CYC-tTA. A restriction digest provided further confirmation of pRS306-CYC-tTA using ECORI restriction enzyme to give band pattern of 4.6 and 1.3 kb**.** Lanes 4 and 5 confirm successful digest pattern. *Lane 1:* PCR amplified KanMX deletion. *Lane 2:* Tet-off promoter amplification using primers ptOFF(xma)5 and ptOFF(Bgl)3. PCR amplify the 200bp tet-off promoter. *Lane 3:* contained 1kb Ladder (NEB N3232S).

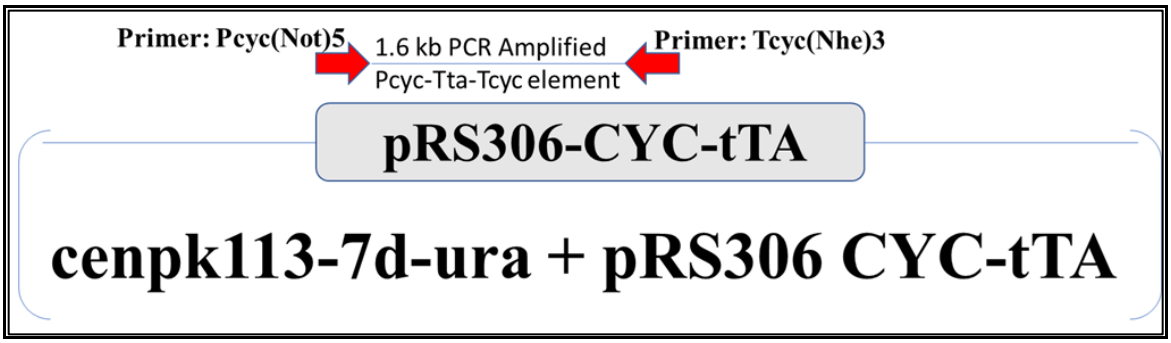

Figure 12: Representation of Confirming Constructs by PCR Screen. New yeast constructs were confirmed by running PCR screens where the primers amplified the insertion in question.

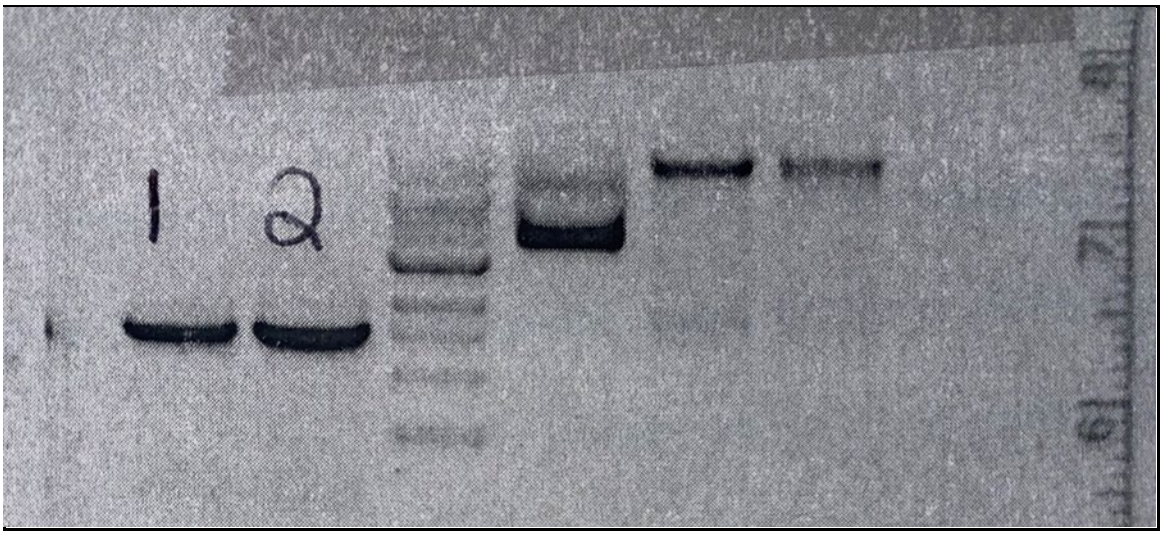

Figure 13: Confirmation of pRS306-CYC-tTA transformed into Yeast.
A PCR screen provided confirmation that the tet-activator element was successfully transformed into the yeast by using primers, Pcyc(Not)5 and Tcyc(Nhe)3, that would produce a 1.6kb product if the insertion was successful. The PCR screen confirmed successful insertion of the tet-activator element into yeast with 1.6kb PCR products in each of two candidate colonies (lanes 1 and 2). Lane 3 contained 1kb Ladder (NEB N3232S).

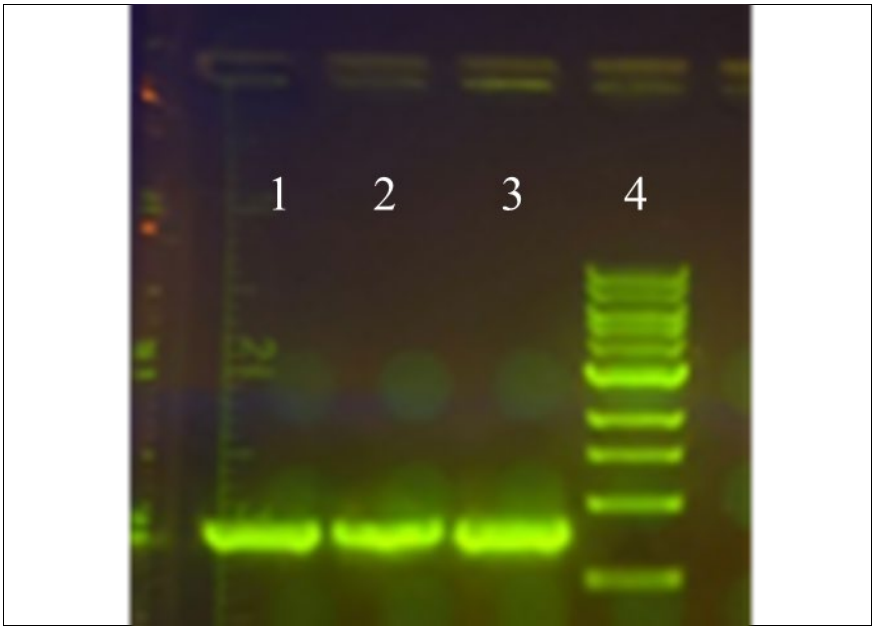

Figure 14: Confirmation of KanMX deletion element transformed into Yeast. A PCR screen provided confirmation that the KanMX deletion element was successfully transformed into the yeast by using primers, KanC and GSC2DS3, that would produce a 1.0kb product if the insertion was successful. The PCR screen confirmed the transformation was successful with 1.0kb PCR products in 3 candidate yeast colonies (lanes 1-3). Lane 4 contained 1kb Ladder (NEB N3232S).

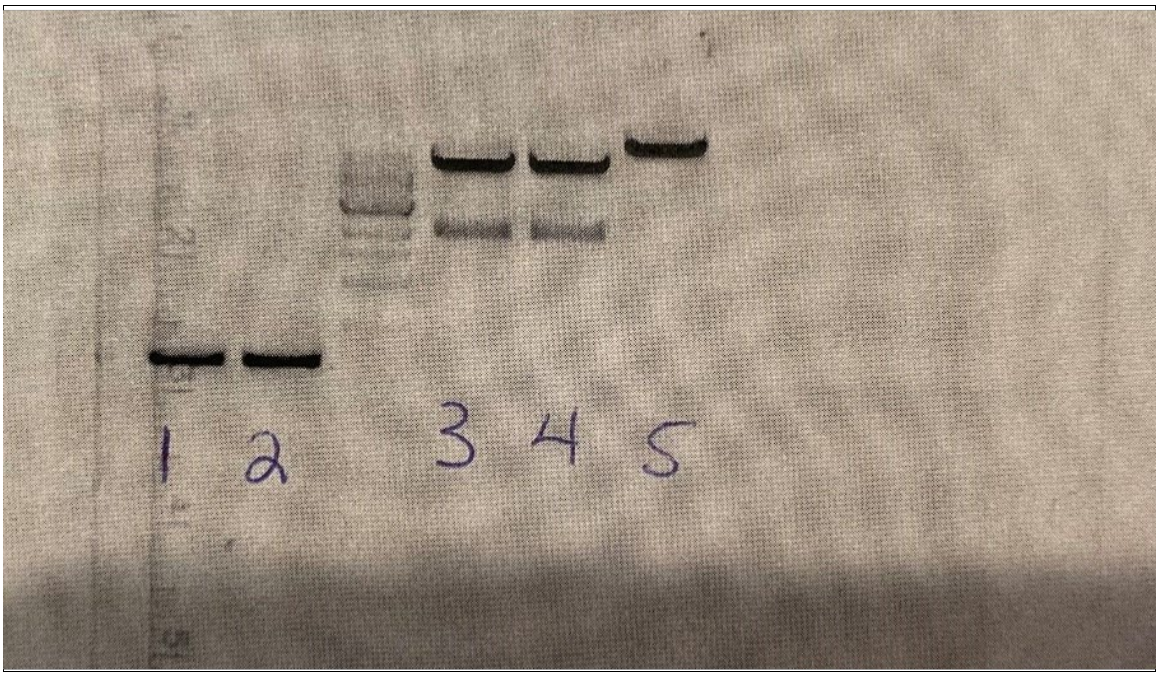

Figure 15: Confirmed Construction of PtOFF-CBG99-hph305. To confirm the plasmid was correct, the DNA was PCR amplified using primers, ptOFF(Xma)5 and ptOFF(Bgl)3, that would produce a 0.2kb product if the insertion was successful. The 0.2kb PCR product (tet-off promoter) in lanes labeled 1 and 2 from two candidate colonies confirm a successful construction of PtOFF-CBG99-hph305. To further confirm, the candidate plasmid was also digested using the restriction enzyme SalI, (lanes labeled 3 and 4). A negative control, pRS305-hph-Ptef-CBG99, was placed in lane labeled 5. The negative control, pRS305-hph-Ptef-CBG99, showed the digest band pattern of 9kb. The band patterns in lanes 3 and 4 (7kb and 2 kb) confirmed a successful ligation.

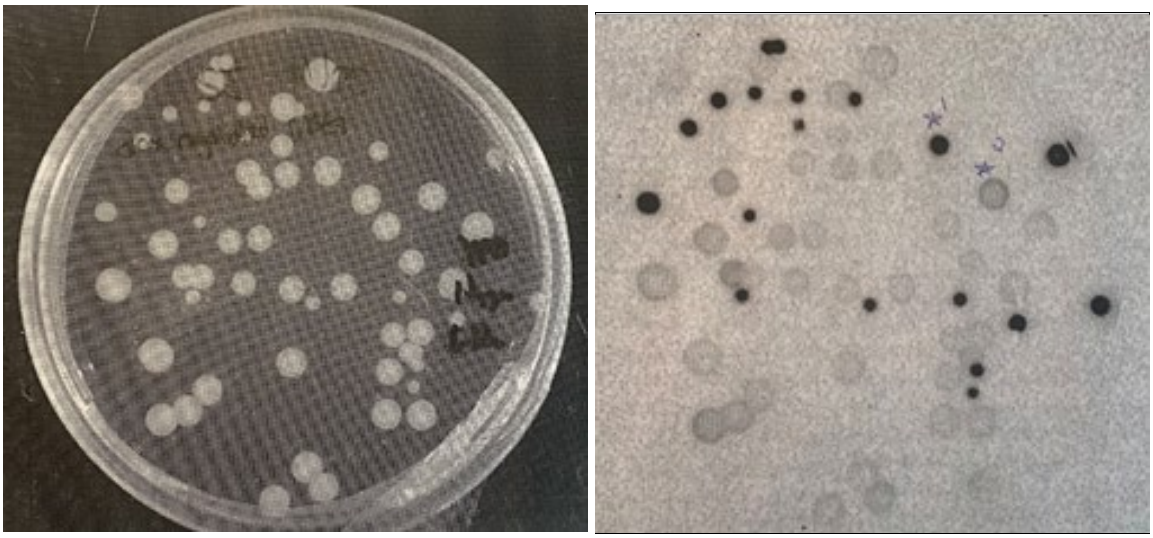

Figure 16: Doxycycline Regulated CBG Yeast Growth plates. *Left:* Brightfield of cenpk113-7d-ura+ pRS306 CYC-tTA+ KanMX deletion+ PtOFF-CBG-Hph305 yeast construct. The yeast was selected on YPD Hygromycin +LH2 plates. *Right:* Bioluminescence of yeast construct cenpk113-7d-ura+ pRS306 CYC-tTA+ KanMX deletion+ PtOFF-CBG-Hph305. The brightfield image was taken to see the amount of yeast growth in the bioluminescence image.

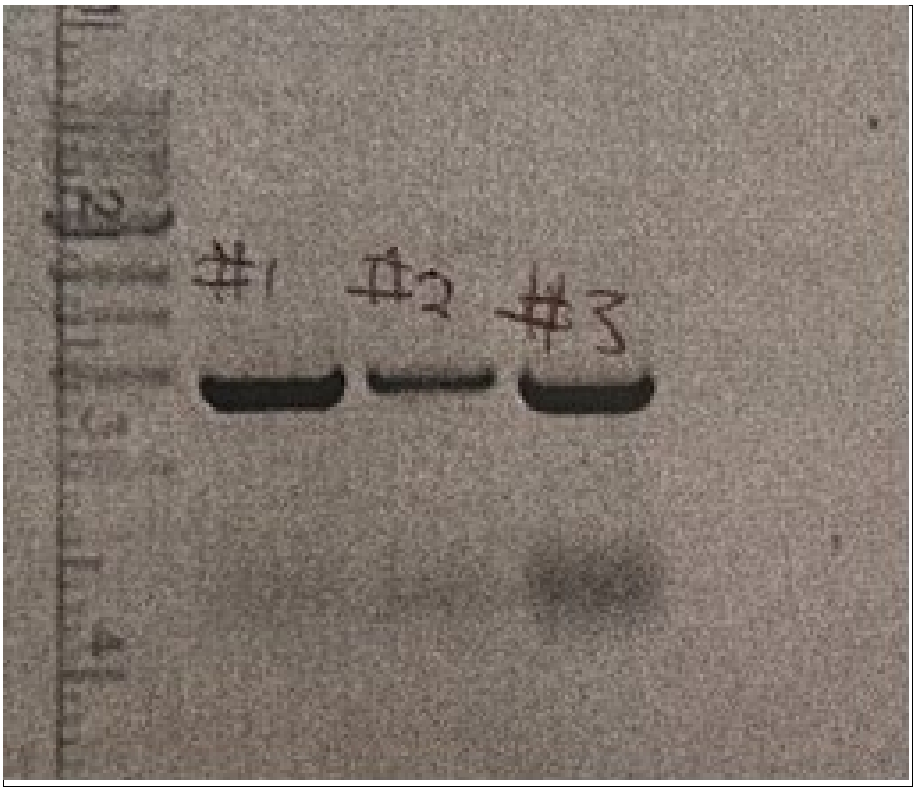

Figure 17: PCR amplified *FKS1* promoter. The FKS1 promoter (1.0kb) was amplified using primers pFKS(Xma)5 and pFKS(Bgl)3. The three lanes (2-4) were testing different genomic preparations for the template source. All of the PCR products were 1.0kb which confirmed the *FKS1* promoter was amplified. The far-left lane contained 1kb Ladder (NEB N3232S).

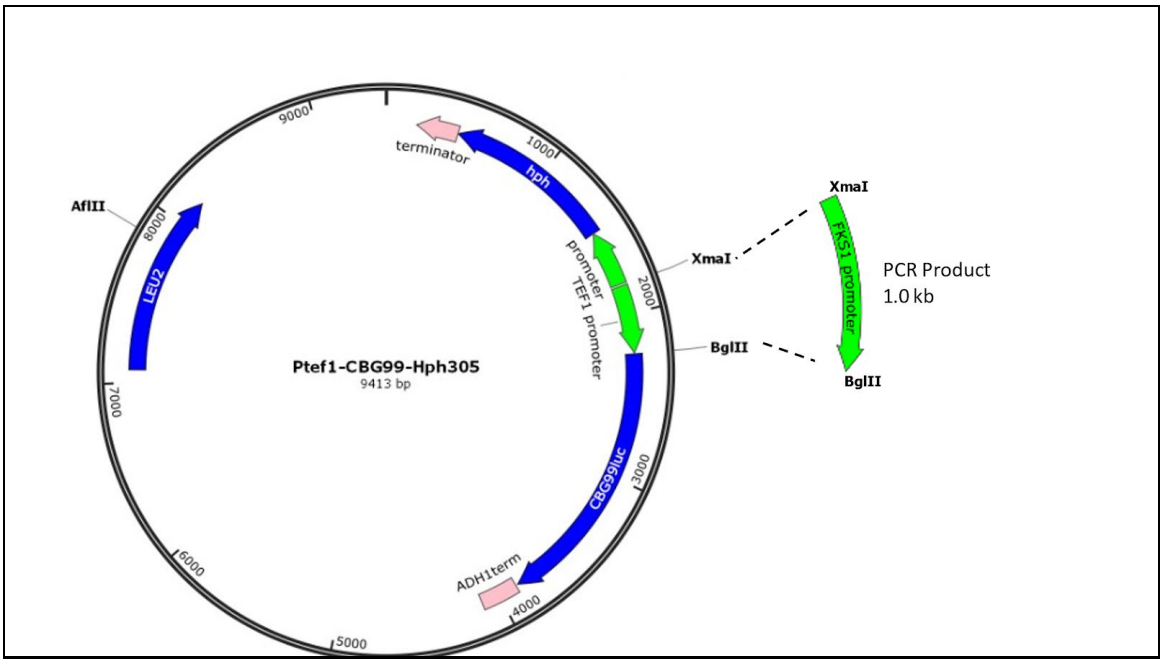

Figure 18: Construction of pFKS1-CBG99-Hph305, a bioluminescent reporter to reveal natural levels of FKS1 expression. The plasmid pFKS1-CBG99-Hph305 was built by adding a 1.0 kb PCR product containing the FKS1 promoter to the plasmid pRS305-hph-Ptef-CBG99 using the restriction sites XmaI at the 5' end and BglII at the 3' end. This PCR product replaced the TEF1 promoter that was previously in the plasmid.

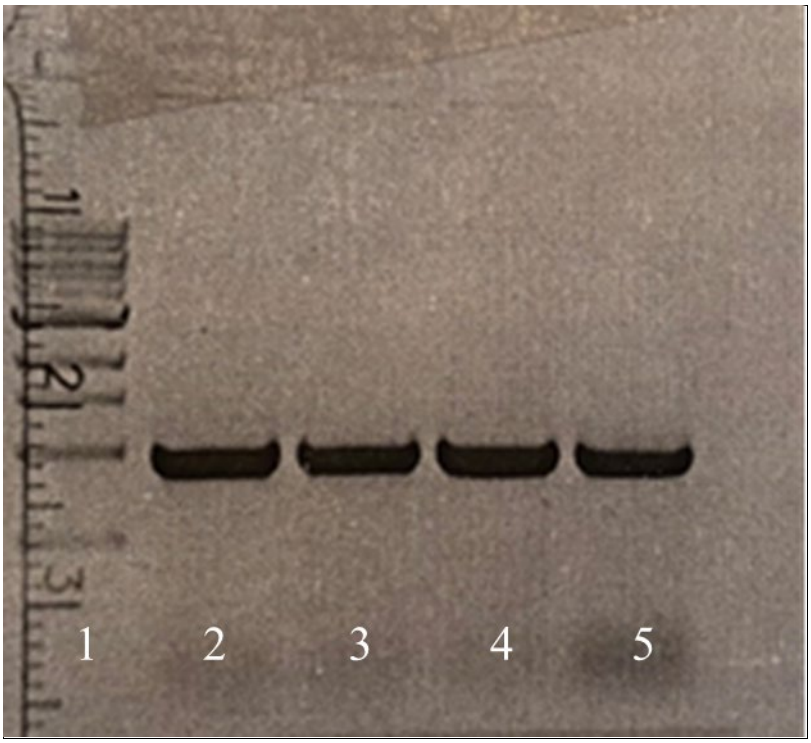

Figure 19: Confirmation of Construct pFKS1-CBG-Hph305. Successful construction of pFKS1-CBG-Hph305 was confirmed by PCR amplifying the FKS1 promoter using primers pFKS(Xma)5 and pFKS(Bgl)3 from four candidate colonies. The 1.0kb band confirms the construction successfully ligated the FKS1 promoter with the plasmid pRS305-hph-Ptef-CBG99. The far-left lane contained 1kb Ladder (NEB N3232S).

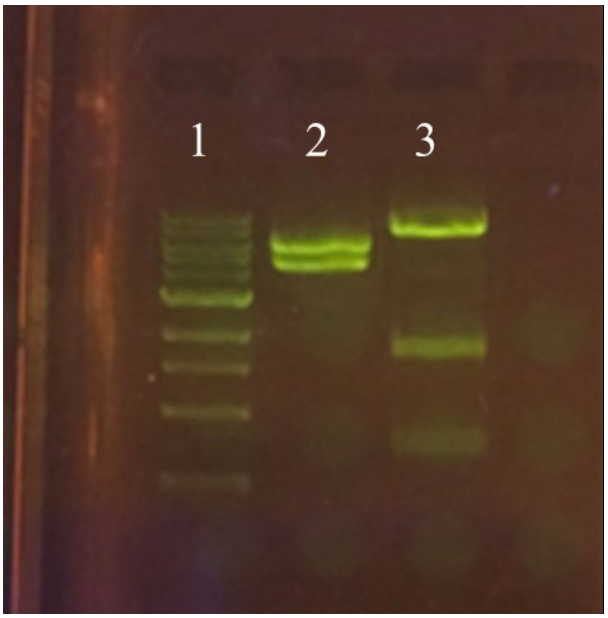

Figure 20: Further confirmation of pFKS1-CBG-Hph305 using Restriction Digest. The digest of pFKS1-CBG-Hph205 with restriction enzymes ApaI. The predicted digest pattern of 7.5kb, 1.8kb, and 0.7 kb was seen in lane 3, confirming a successful construction. Lane 2 was a candidate plasmid that did not turn out to be successful. The far-left lane contained 1kb Ladder (NEB N3232S).

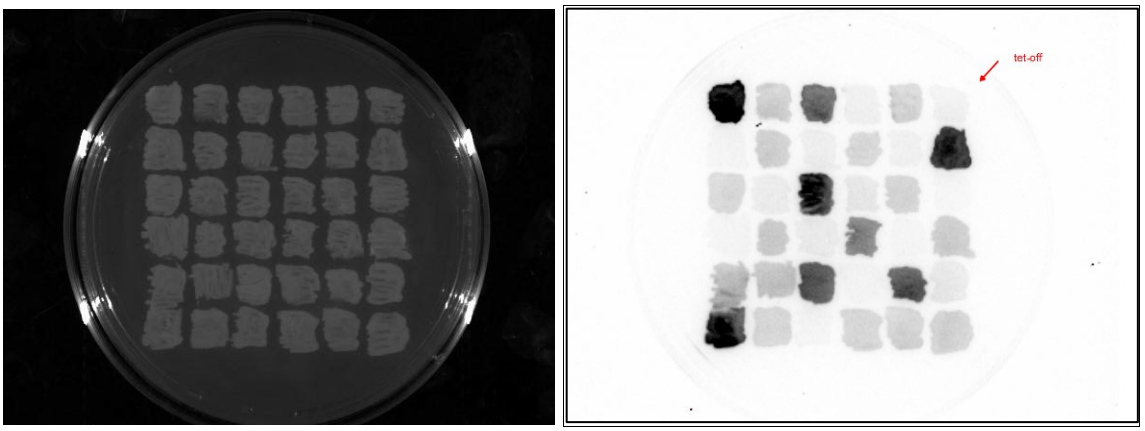

Figure 21: Yeast's Response to Doxycycline Using Bioluminescent Reporter with the FKS1 promoter compared to the tet-off promoter (Doxycycline Present) *Left:* Brightfield image taken to indicate the amount of growth of the two strains (FKS1 promoter vs Tet-off promoter) of yeast alternately patched in a grid format onto an agar medium that contained doxycycline. The brightfield image supports the bioluminescence image by showing the amount of yeast growth when the bioluminescence was analyzed. *Right:* The plate was imaged by a ChemiDoc camera that takes sequential pictures of plate bioluminescence. The picture of yeast bioluminescence was taken when the plate had been in dark for 30 sec. The red arrow indicates the start of the alternately patched pattern with tet-off promoter yeast.

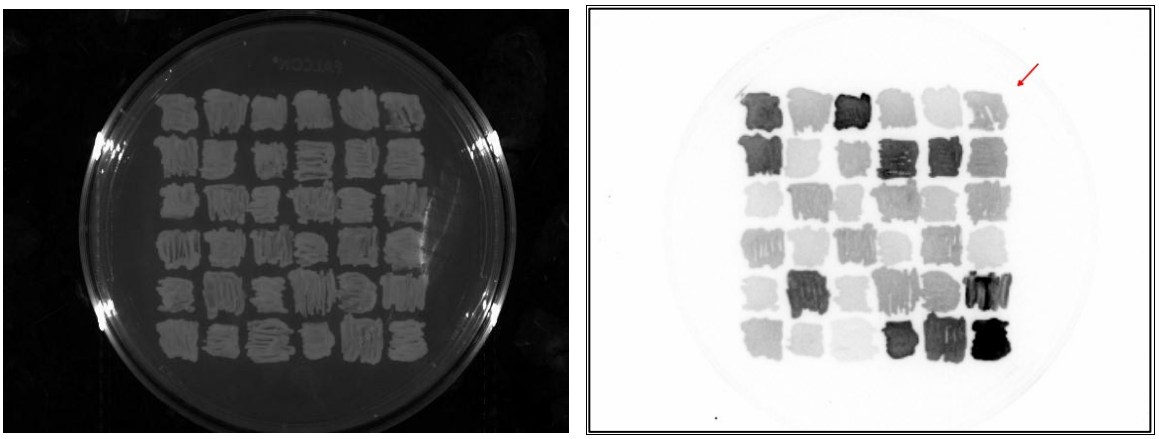

Figure 22: Yeast's Response to Doxycycline Using Bioluminescent Reporter with the FKS1 promoter compared to the tet-off promoter (Doxycycline Absent). *Left:* Brightfield of the two strains (FKS1 promoter vs Tet-off promoter) of yeast alternately patched in a grid format onto an agar medium that did not contain doxycycline. The brightfield image supports the bioluminescence image by showing the amount of yeast growth when the bioluminescence was analyzed**.** *Right:* The plate was imaged by a ChemiDoc camera that takes sequential pictures of plate bioluminescence. The picture of yeast bioluminescence was taken when the plate had been in dark for 20 sec. The red arrow indicates the start of the alternately patched pattern with tet-off promoter yeast.

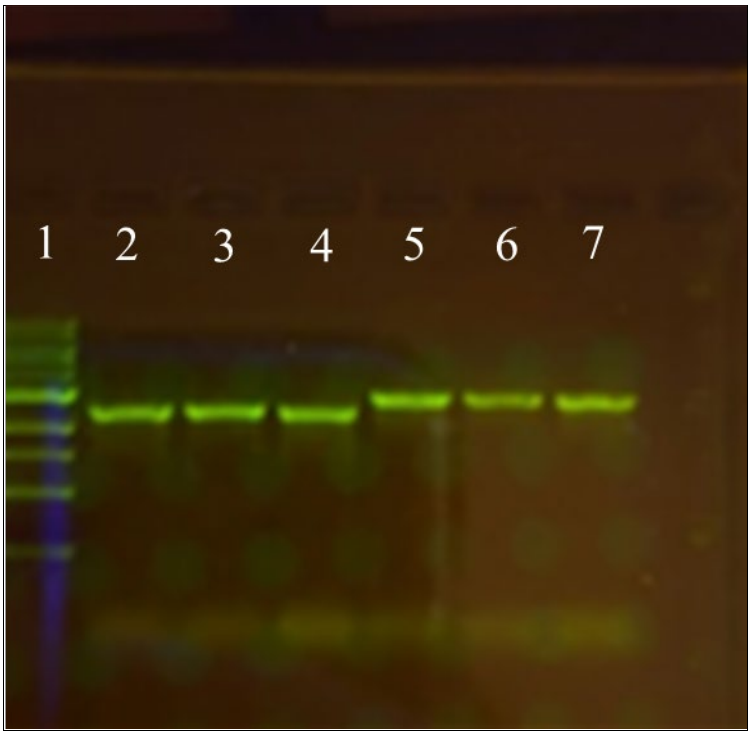

Figure 23: PCR amplified Left and Right halves of *FKS1* gene. *Lanes 2-4* Bands of 2.6kb show the left half of PCR amplified *FKS1* gene using primers, FKS1(Bam)5 and FKS1mid(Afl)3. *Lanes 5-7* Bands of 3.0kb shows right half of PCR amplified *FKS1* gene using primers, FKS1mid(Afl)5 and FKS1(Nhe)3. The far-left lane contained 1kb Ladder (NEB N3232S).

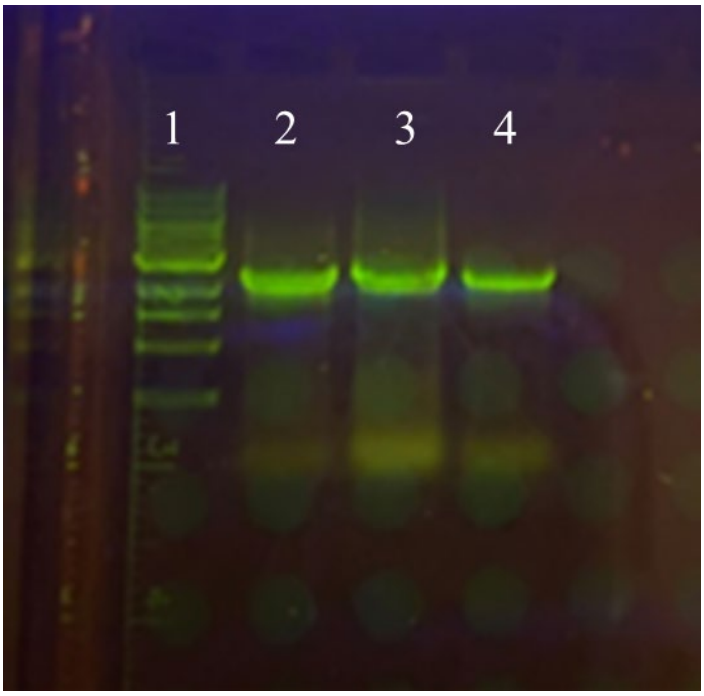

Figure 24: Confirmation of pAllet+left half of *FKS1* gene Construct. 2.6kb bands confirmed ligation using primers, FKS1(Bam)5 and FKS1mid(Afl)3, that amplified the left half of the *FKS1* gene. Lane 4 shows positive control, left half of *FKS1* gene PCR product (2.6 kb). The PCR products (2.6kb) in lanes 2 and 3 provide confirmation that the left half of the FKS1 gene was successfully inserted into pAllet for the two candidate colonies screened. The far-left lane contained 1kb Ladder (NEB N3232S).

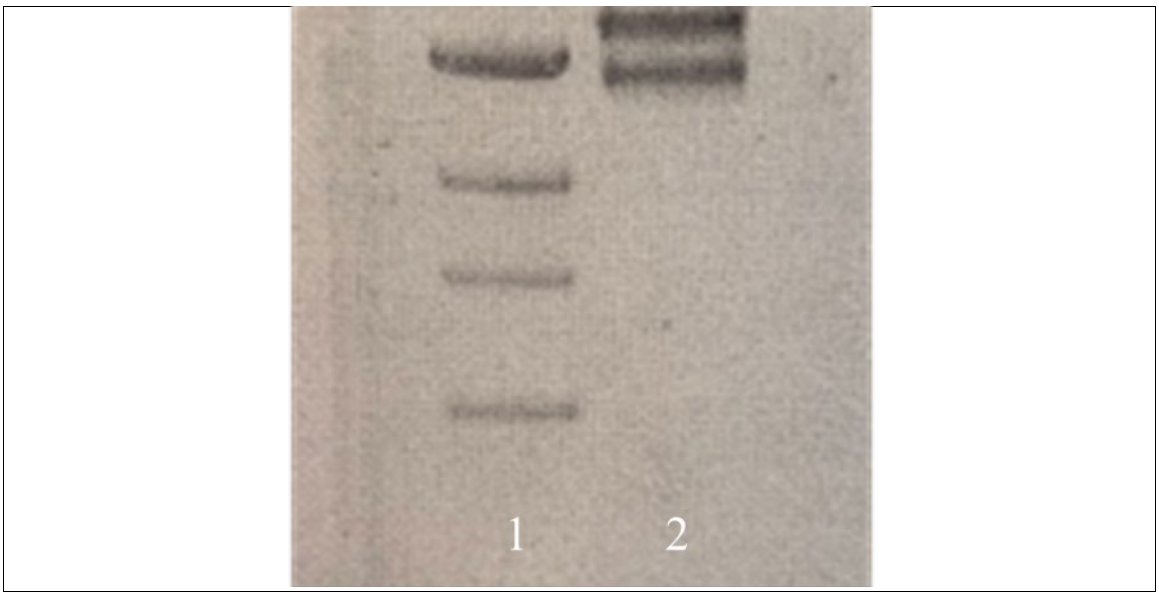

Figure 25: Confirmation of construct pToff-*FKS1*-Hph305 using Restriction Digest. Plasmid ptOFF-*FKS1*-Hph305 was digested using restriction enzymes AflII and BamHI. The correct band pattern confirmed successful ligation of ptOFF-CBG-Hph305 and the whole *FKS1* gene.

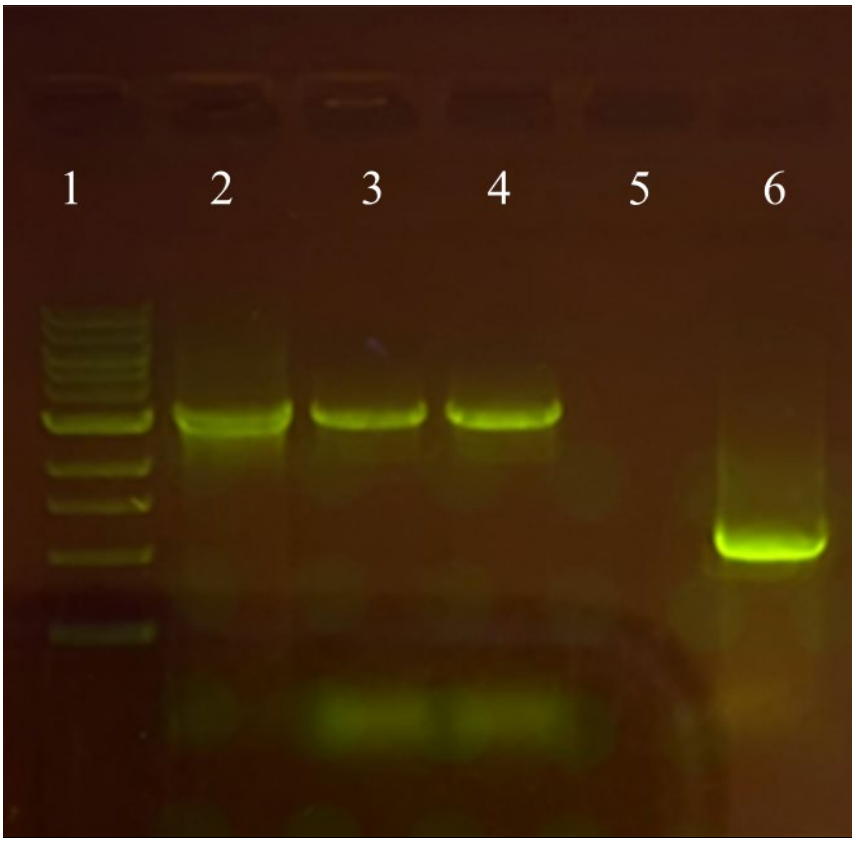

Figure 26: Confirmation of pToff-*FKS1*-Hph305 transformed into Yeast. A PCR screen provided confirmation that plasmid pToff-*FKS1*-Hph305 was successfully transformed into the yeast by using primers, ADHIterm(Not)3 and FKS1mid(Afl)5, that would produce a 3.3kb product if the insertion was successful. The PCR screen (lanes 2 and 3) of two candidate colonies confirmed the transformation was successful with 3.3 kb PCR products compared to the 3.3 kb positive control ptOFF-*FKS1*-Hph305 in lane 4. Lane 6 shows the PCR amplified NAT gene (1.1kb) from pYM-N9 using primers, Nat(FKS1us)5 and Nat(FKS1da)3. The far-left lane contained 1kb Ladder (NEB N3232S).

Figure 27: Confirmed native *FKS1* knockout. To confirm the native *FKS1* knockout was successful, the yeast construct was PCR amplified using primers, FKS1koscrn5 and FKS1koscrn3. The band pattern of ~1.7kb confirmed a successful knockout of the native *FKS1* gene for 3 of the 4 candidate cultures tested. The far-left lane contained 1kb Ladder (NEB N3232S).

Appendix A-2: Tables

Table 1: List of Primers

Name	Code	Annealing Temperature
Aconf5	AGTGTGTGACACGAAATTTCAAGATA	65
Dconf3	CGTGTCTTTCTATGTGTATCACCTG	65
GSC2us5	CGGTATGATGCAAATGAGGTG	65
KanB	CTGCAGCGAGGAGCCGTAAT	69
KanC	TGATTTTGATGACGAGCGTAAT	64
GSC2ds3	GGAGAGAAGCGTTACTATTTGATC	64
Pcyc(Not)5	actactGCGGCCGCtAAAGCTGGAGCTCATTTGG	61
Tcyc(Nhe)3	actactGCTAGCAAATTAAAGCCTTCGAG	60
PtOFF(Xma)5	actactCCCGGGTCCCTATCAGTGATAGAGAG	52
PtOFF(Bgl)3	actactAGATCTATTGATCCGGTAATTTAGT	53
Pfks(Xma)5	actactCCCGGGCCTCTGTGCATTGGTTTGTG	63
Pfks(Bgl)3	actactAGATCTGGTCTGACCGTTGTATGAAAG	61
FKS1(Bam)5	actactGGATCCATGAACACTGATCAACAACCT	57
FKS1(Spe)3	actactACTAGTTATTTTATAGTTGACCAGGTC	56
Nat(FKS1us)5	TAGCCTTCATTTACCAAACAGGAACTAGCGTATA TCATTAGACATGGAGGCCCAGAATAC	63
Nat(FKS1ds)3	TGCTTTTGGATAGAATATCAGTAAAATCAAGCGT TCAAGCTGCCGGTAGAGGTGTGGTC	64
FKS1us(ext)5	AGCTGTTTTAACCGACTACGAAGTTCTCCATTCT CGAACACTAGCCTTCATTTACCAAAC	54
FKS1ds(ext)3	CGAACGGTATTTGCAACATCTTGAGAGTTTCTGG TCTACTTGCTTTTGGATAGAATATCA	55
FKS1koScrn5	GCGTTTGATGAAGCACAGG	65
FKS1koScrn3	CAGAATTACTGACACCGAAAGC	63
FKS1mid(Afl)3	aatttctCTTAAGGACAGCAGAATTCTTTCCG	59
FKS1mid(Afl)5	tgctgtcCTTAAGAGAAATTATTCGTGAAG	57
FKS1(Nhe)3	actactGCTAGCTTATTTTATAGTTGACCAGGTC	56

Table 2: List of Plasmids

Plasmid Name	Relevant Notes	Source
p416CYC-tTA	Episomal plasmid containing Tet Activator gene	Cuperus et al., 2015
pRS306-CYC-tTA	Tet Activator gene added to yeast integrating plasmid pRS306	This work
pCM181-LUC1	Single Tet-off operator/*CYC1* promoter (PtOFF) driving Luciferase reporter	Stan Fields, Univ. Washington
pRS305-hph-Ptef-CBG99	Integrating plasmid pRS305 containing Hph hygromycin selectable marker and *TEF1* promoter driving *CBG99* CDS	Robertson lab
PtOFF-CBG99-Hph305	pRS305-hph-Ptef-CBG99 with PtOFF promoter replacing *TEF1* promoter	This work
pFKS1-CBG99-Hph305	PtOFF-CBG99-Hph305 with FKS1 promoter replacing PtOFF promoter	This work
pAllet	Cloning vector with extensive multiple cloning site	Reichard Thesis, 2017, Robertson Lab
pAllet+left half *FKS1*	pAllet containing first half of *FKS1* CDS	This work
pAllet+*FKS1*	pAllet containing complete *FKS1* CDS	This work
ptOFF-*FKS1*-Hph305	PtOFF-CBG99-Hph305 with complete *FKS1* CDS replacing *CBG99* CDS	This work
pYM-N9	Source for *NAT*, nourseothricin selectable marker	Janke et al., 2004
pRS304-ShBle-Ptef-CBG99	Integrating plasmid pRS304 containing ShBle zeocin selectable marker and *TEF1* promoter driving *CBG99* CDS	Robertson Lab

Table 3: Bioluminescence of Yeast with Doxycycline Regulated Reporter Gene. Measurements show stunted bioluminescence in the colonies with doxycycline compared to the colonies without doxycycline.

TUBE	Bioluminescence measured in relative light units (RLU's)							
	10:30 AM	**11:30 AM**	**12:30 PM**	**2:30 PM**	**5:30PM**	**6:30PM**	**8:00 AM**	**9:00 AM**
1 Colony 1 +Dox	1.3K	7.17K	6.12K	5.54K	16.48K	35.1K	2,819K	1,500K
2 Colony 1 + No Dox	1.4K	8.65K	8.96K	14.09K	72.6K	168.1K	17,081K	17,811K
3 Colony 2 +Dox	6.9K	19.57K	23.20K	28.70K	27.58K	49.8K	2,301K	2,561K
4 Colony 2 + No Dox	6.8K	22.44K	36.41K	281.95K	186.07K	301K	19,662K	17,746K

Table 4: Assay 1 - Cell Concentrations

	OD600 (theoretical undiluted)			
	Wild Type strain		**FKS1 regulated strain**	
	No Dox	**Dox**	**No Dox**	**Dox**
	3.096	3.016	4.2	3.008
	3.096	3.128	4.232	3.032
	3.272	3.176	4.272	3.208
Mean:	3.154667	3.106667	4.234667	3.082667
Standard Deviation:	0.101614	0.082106	0.036074	0.109203

Table 5: Assay 1 – Cell Concentrations (Normalized Measurements)

	OD600 (normalized)			
	WT strain		FKS1 reg strain	
	No Dox	Dox	No Dox	Dox
	0.981403	0.956044	0.991814	0.710327
	0.981403	0.991547	0.99937	0.715995
	1.037194	1.006762	1.008816	0.757557
Mean:	1	0.984784	1	0.72796
Steandard Deviation:	0.032211	0.026027	0.008519	0.025788

Table 6: Assay 2 - Presence of Luciferase Enzyme in Supernatant measurements show amount of luciferase in supernatant

No Dox	Dox
0.607 (thousand RLUs)	0.587 (thousand RLUs)
0.458 (thousand RLUs)	0.355 (thousand RLUs)
0.488 (thousand RLUs)	0.837 (thousand RLUs)

Table 7: Assay 3 - Susceptibility to Lysis

	Raw Luminescence (thousand RLUs)			
	WT strain		FKS1 reg strain	
	No Dox	Dox	No Dox	Dox
	1156	991	7137	6209
	975	1049	8661	6771
	998	765	6525	5811
Mean:	1043	935	7441	6263.667
Standard Deviation:	98.53426	150.0533	1099.971	482.3291

Table 8: Assay 3 - Susceptibility to Lysis (Normalized Measurements)

	Normalized Luminescence Normalized for Cell Number			
	WT strain		FKS1 reg strain	
	No Dox	Dox	No Dox	Dox
	1.108341	0.964824	0.959145	1.14626
	0.934803	1.021292	1.163956	1.250012
	0.956855	0.744794	0.876898	1.072784
Mean:	1	0.910303	1	1.156352
Standard Deviation:	0.094472	0.14609	0.147826	0.089044