Enabling β-Carotene Production in *Saccharomyces cerevisiae* KTP Through Genetic Engineering

Major Qualifying Project

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Abstract

The human gut microbiome contains thousands of microbiota, including yeasts, that supply the body with nutrients and play a large role in the immune system. With advances in microbiology and genetic engineering, researchers are exploring how to engineer the human gut microbiome for better health outcomes. Many different microbes are being investigated, among them yeasts. In this work, we focused on the yeast *Saccharomyces cerevisiae* strain KTP because it benefits the human gut microbiome and protects against disease. Previous work has successfully inserted plasmids into *S.cerevisiae* KTP for fluorescent protein expression. Therefore, we set a goal of using the other efficient genome engineering strategy, homologous recombination, to engineer a metabolic pathway in the strain. We chose the β-carotene pathway because KTP already produces the precursor necessary to produce β-carotene. We were able to clone the pathway, but we found that pathway integration was not successful in *S.cerevisiae* KTP. Future work could put the pathway on a plasmid or try different approaches to increasing homologous recombination efficiency. Once a successful transformation into *S.c.* KTP is made, the production of β-carotene could be quantified using HPLC. In summary, this work is an initial step towards metabolic pathway engineering in a probiotic yeast strain, ultimately promising new human health treatments.

Acknowledgements

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Background

Probiotics & Their Importance

Microbiome Overview

Established at birth, each human gut has a unique profile of microbiota that develops and changes through all stages of life[1]. The human microbiome is teeming with more than 2000 species of microbiota[2]. Its principal constituents are bacteriome, virome, and mycobiome[3], with bacteriome making up 93.5% [2]. Thanks to the vast majority of the microbiome being bacteria, most of what is known of the microbiome is determined from studies of the bacteriome of adults living a Westernized lifestyle[3]. There is still much to learn about the microbiome, as research has proven that enterotypes, body mass index (BMI), exercise frequency, lifestyle, and cultural and dietary habits all play a role in the varying compositions of the gut flora[3]. Compared to the bacteriome, the mycobiome is believed to be less stable and more susceptible to changes due to environmental factors[4].

Probiotics in the Microbiome and Their Role in Human Health

As knowledge of the human microbiome increases, the prospect of using probiotic microbes to treat various diseases has gained interest. The ability of the gut microbiota to supply the body with essential nutrients, reduce inflammation, promote nerve function and regulate the human immune system could allow probiotic supplementation to serve as a promising strategy to prevent or treat illness[5]. The introduction of a living species rather than a chemical drug has been proven effective in treating diabetes and preventing transmission of HIV in women, among other uses[6,7].

From a host perspective, probiotic effector molecules can have multiple mechanisms of action, most importantly the enhancement of the epithelial barrier function, modulation of the immune system, modulation of systemic immune responses, signaling via the central nervous system (CNS), and modulation of the composition and activity of the microbiota already present in the gut[8].

β-carotene is a molecule that is metabolized to vitamin A in the human body. The introduction of a bacterium that produces β-carotene to the microbiome has been proposed as a possible treatment to children with vitamin A deficiency, as the β-carotene will metabolize to vitamin A[9].

Yeast as a Probiotic and its Implications

In recent years, some species of yeast have been identified as probiotic microbes. One notable species is *Saccharomyces cerevisiae* (*S.cerevisiae*). *S.cerevisiae* commonly enters the body as it is often utilized to ferment bread, beer, and wine[10]. *S. cerevisiae* has a few lesser known strains that have probiotic applications, including *S. boulardii* and *S.cerevisiae* KTP[10]. When introduced correctly, these strains can benefit the human gut biome and even protect

against disease and medical conditions[10]. In clinical trials, *S.boulardii* has shown to improve immunity, support the gut microflora in humans, and show promising improvements in individuals suffering from *C.difficile* infections and Crohn's disease[10]. *S.c.* KTP has shown to inhibit a range of harmful *Candida albicans* strains in the human gut biome[11]. There are some risks associated with introducing yeast to the microbiome that warrant some caution. A fungal infection in the blood has occurred in immunocompromised patients treated with *S.boulardii*. There have also been cases of auto-brewery syndrome reported in patients, but it is generally understood that safe and small doses of *S.boulardii* are in fact safe for humans[10].

Engineering Yeast

Scientists have the capability to genetically engineer different organisms and alter their metabolic pathways, including yeast. Success has been found in *S.cerevisiae* strains when inserting plasmids directly[12]. A study by a previous MQP student found that the KTP strain of *S.cerevisiae* has a higher expression of GFP and RFP than 288C when the same plasmids were inserted[12]. The KTP strain showed stronger promoters and terminators over the 288C strain, with different promoters introduced yielding different effects on the expression ratio[12]. Further, the KTP strain was found to work better than 288C under a variety of pH conditions, including lower pHs in the 3-4 range that would be found in the gastrointestinal tract. The KTP strain also demonstrated a higher sensitivity to higher salt concentrations compared to 288C[12].

In recent years, modular cloning or MoClo has risen to the forefront of experimental systems as a way to assemble complex DNA sequences that code for genetic expression of desired genes and pathways. Modular cloning involves the use of Type IIS restriction enzymes BsaI and BbsI to isolate and assemble basic DNA parts in a linear fashion[13]. Each of the basic parts is stored in a destination vector in *E. coli*, with BsaI sites on either side of the part to allow the parts to be isolated and combined with other parts[13]. Level Zero of the Type IIS reaction involves isolating individual promoters, terminators, and genes from their storage vectors. The Level One reaction that follows combines a promoter, and terminator into a new transcription unit on its own vector[13]. The schematic in **Figure 1** below gives a visual representation of the Level Zero and Level One Type IIS reactions.

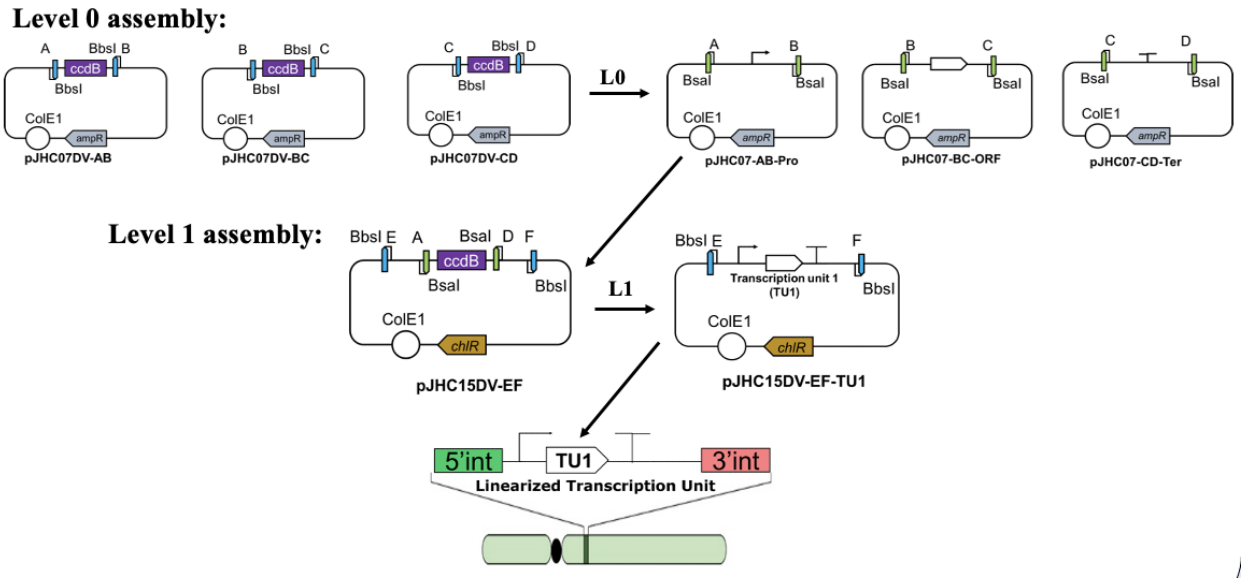


Figure 1: *A diagram of Level 0 and Level 1 Type IIS reactions.*

One method of modular cloning called CIDAR MoClo, developed by the CIDAR Lab at Boston University, involves using a library of basic DNA parts that can be assembled to make transcription units in thousands of different combinations. CIDAR MoClo provides equal cloning efficiency to traditional modular cloning with a 90 minute reaction time compared to 5 hours. This combinatorial library allows for the tuning of gene expression to the desired level[14]. Glycerol stocks of modularly cloned transcription units can be made to create a secondary library for further experimentation and multi-gene assemblies[14].

Plasmid Integration

Plasmid integration is a successful method to introduce genes into *Saccharomyces cerevisiae* and *E.coli*. Plasmids are defined as circular, double stranded DNA molecules that naturally exist in bacterial cells and in some eukaryotes[15]. Plasmids have a variety of lengths of thousands of base pairs, which can be used to identify the plasmid. When bacteria divides, all of the plasmids within the cell wall are copied[15]. DNA fragments or genes can be inserted into a plasmid vector which can then be inserted into bacteria using transformation. Each engineered plasmid contains a selection marker, promotor, and terminator[16]. Antibiotics are commonly used as a selective agent to help reduce genetic instability[17].

Homologous Recombination

Homologous recombination is a DNA repair mechanism used in eukaryotes to repair broken DNA. It can fix DNA damage that includes DNA gaps, double-stranded breaks, and interstrand crosslinks[18]. The mechanism to fix broken DNA involves ligating the strands of DNA at overlapping sequences[19]. Although all eukaryotes use homologous recombination to

fix DNA, *S.cerevisiae* strains have the most efficient mechanism for homologous recombination. Because of this, homologous recombination can be used for DNA transformation in synthetic biology[19]. In *S.cerevisiae*, if there are 20 to 40 base pairs of identical sequence the enzymes used in homologous recombination will ligate the sequences together and embed them within the host genome[19].

Metabolic Pathways

The possibilities within metabolic engineering are endless. Theoretically, any metabolite has a metabolic pathway that can be introduced to a host organism, which makes determining a metabolite to produce a difficult choice. Ideally, the metabolite would benefit the health of humans, as well as, have a well established metabolic pathway. One class of potential metabolites are carotenoids. The main carotenoids that benefit health are alpha carotene, β-carotene, lycopene, lutein, β-cryptoxanthin, and zeaxanthin[20]. Carotenoids are essential parts of the human diet, which means that humans can not synthesize them from the initial reactants. One of the health benefits of carotenoids is that they are antioxidants[20]. Antioxidants are molecules that can inhibit oxidation and free radicals, which left unchecked would cause serious health issues and could lead to diseases such as cancer[20].

Vitamin A, also known as retinol, is essential to eyesight in humans. However, humans cannot synthesize it from scratch, only from carotenoids, mainly β-carotene. Humans get 60% of their vitamin A from carotenoids provided by vegetables in their diets[20]. Vitamin A is half a β-carotene molecule with a water molecule attached, and humans produce the enzymes that catalyze this reaction[20].

Another benefit of β-carotene is that there are almost no serious consequences of having too much of it. Carotenodermia is when a person's skin has an orange tint in it due to consuming too much β-carotene, but this is reversible and has no serious effects on health [20]. There is also some evidence that suggests that consuming too much β-carotene is linked to an increased risk of lung cancer in people who smoke[20]. However, β-carotene is an essential part of the human diet and the health benefits greatly outweigh the risks.

S.cerevisiae naturally contain the mevalonate pathway, which is the metabolic pathway the synthesizes isoprenoids in eukaryotes, archaea, and some bacteria. The isoprenoid pathway is responsible for synthesizing cholesterol, among other products important for life[21]. Another product of the mevalonate pathway is gerylgeryl diphosphate (GGPP), which is a precursor for carotenoid synthesis, specifically β-carotene[21,22]. There have been multiple studies done in *S.cerevisiae* focuses on increasing β-carotene production by varying different aspects of the mevalonate pathway. For example, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is one of the rate limiting enzymes in the mevalonate pathway[22]. It was found that isoprenoid synthesis increases when the catalytic domain of HMGR is overexpressed in *S.cerevisiae*[22]. It was also determined that the enzymes glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase influence

β-carotene production in *S.cerevisiae*[21]. Both of these enzymes participate in glycolysis, which is upstream from the isoprenoid pathway. Additionally, both of these enzymes produce acetyl-CoA, which is a precursor in isoprenoid synthesis. There were other enzymes that were also found to increase the production of β-carotene that were not directly related to the mevalonate pathway[21].

The synthesis pathway of β-carotene can be seen in **Figure 2**.

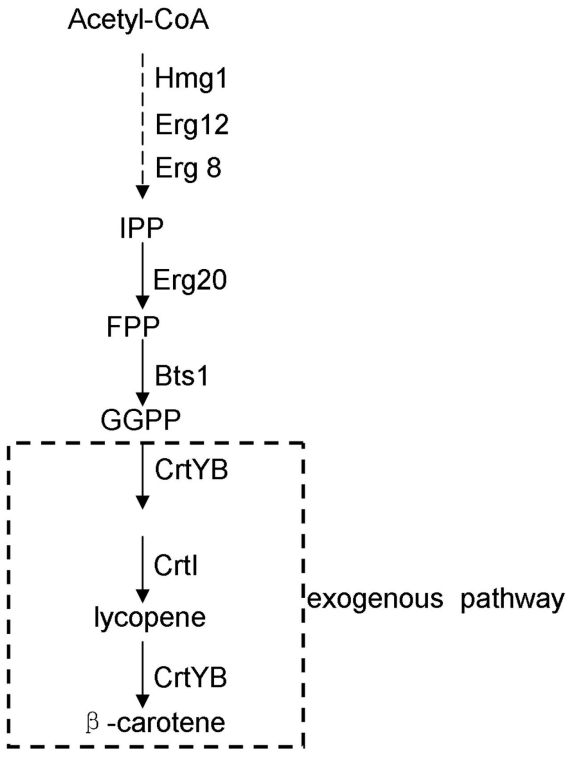


Figure 2: *Synthesis Pathway of β-carotene*[20].

S.cerevisiae naturally make GGPP as part of their mevalonate pathway[22]. To produce β-carotene from this point, three enzymes have to be introduced into the *S.cerevisiae*. Phytoene synthase and phytoene desaturase are the enzymes that turn GGPP into lycopene, which is a carotenoid[22]. Lycopene β-cyclase is the enzyme that converts lycopene into β-carotene. Some species have preferences in codons used during translation, based on what most of their genetic sequence uses. Using this information, studies have been completed to increase β-carotene yield by mutating genes to contain different codons to optimize these preferences[22].

Experimental Goals

The first goal of the research was to successfully create transcription units with the genes to create the β-carotene pathway. Each transcription unit involved a promoter, terminator, and a gene from the β-carotene pathway, either CertI, CertE, or CertYB. High, weak, and inducible promoter and terminator combinations were tested.

Next, the goal was to isolate the transcription units to prepare to be inserted into the *S.cerevisiae* KTP. The individual parts were isolated using the QIAGEN MINIPREP Kit from *E. coli*, and then assembled into transcription units using destination vectors HR1, HR2, HR3. and HR4. These parts were transformed into *E. coli*. PCR was utilized to verify the part lengths, and then parts were enzyme-digested in preparation for transformation into yeast.

The final goal was to successfully transform the pathway into the *S.c.* KTP so that it could produce yeast. After PCR, the parts were digested by an enzyme to increase the likelihood of a successful transformation. The high combinations were also transformed in *S.c.* 288.

Methodology

Experimental Preparation

To prepare for experimentation, growth media was prepared as in the Media Prep section below. YB broth was prepared to be used for *S.c. KTP* growth, and LB broth with the antibiotic chloramphenicol was prepared for E. coli growth. *S.c. KTP* was cultured on YB broth agar plates to be used later on.

Experimental Approach

The experimental procedure used modular cloning of transcription units followed by a homologous recombination transformation method to insert the genes for the β-carotene pathway into *S.c.* KTP. As previously mentioned in the background, homologous recombination can be used as a synthetic biology tool to insert multiple genes into yeast without assembling the whole pathway on a plasmid vector for insertion. As homologous recombination is a natural process for yeast cells, when individual transcription units are introduced on specific homologous recombination vectors with appropriate homologous arms, the yeast should work to combine the genes into the full pathway.

Each transcription unit required a promoter, terminator, and a gene from the β-carotene pathway (crtE, crtI, or crtYB). Each gene was expressed in multiple transcription units with varying promoter and terminator combinations, which can be seen in **Table 1**. Each gene had a strong and weak transcription unit, as well as a promotable transcription unit. The Pgal10 promoter was used with each gene to create an inducible transcription unit, which will result in an inducible pathway, theoretically resulting in high gene expression when galactose is introduced to the transformed cells.

Table 1: *Transcription units created throughout the project.*

Gene	Strong		Weak		Inducible	
	Promoter	**Terminator**	**Promoter**	**Terminator**	**Promoter**	**Terminator**
CrtE	Ptdh3	Trp115a	Ppxr1	Tyox1	Pgal10	Trpl15a
CrtYB	Pfba1	Trp141b	Pykt6	Ttip1	Pgal10	Trp141b
CrtI	Psptdh3	Tefm1	Pmyo4	Teno1	Pgal10	Tvma2

To create the transcription units the individual parts were isolated using the QIAGEN MINIPREP Kit from *E. coli*, and the resulting DNA concentrations were quantified using a

Nanodrop 1. The parts were then assembled into transcription units using the destination vectors HR1, HR2, HR3, and HR4. The HR2 destination vector for each combination contained the antifungal nourseothricin (Nat), and the HR1, HR3, and HR4 vectors contained crtE, crtYB, and crtI, respectively. The transcription units were assembled using a Type IIS reaction.

The resulting transcription units were then transformed into E. coli. The transformed E. coli was cultured overnight then MiniPrepped. A glycerol stock was made of each of the transcription units in their respective destination vectors for repetition of experiments and future work. These stocks are stored in the "Prob1" box in the -80°C freezer.

PCR or BbsI enzymatic digestion was completed for each of the samples, and the PCR or digested results were analyzed on a 0.8% Agarose gel plate. The samples were then purified to further eliminate background.

With the nine transcriptions that were prepared, there are 27 possible combinations that can be made that include each of the unique genes. Nine of the 27 possible combinations were chosen as combinations that would be transformed into *S.c.* KTP to allow for varying levels of gene expression. The nine combinations of transcription units for the transformations were prepared as described in the table below:

Table 2: *Combinations of transcription units studied.*

HR1	HR3	HR4	Strength Combination
Ptdh3-crtE-Trpl15a	Pfba1-crtYB-Trpl41b	Psptdh3-crtI-Tefm1	High-High-High
Ppxr1-crtE-Tyox1	Pykt6-crtYB-Ttip1	Pmyo4-crtI-Teno1	Low- Low- Low
Pgal10-crtE-Trpl15a	Pgal10-crtYB-Trpl41b	Pgal10-crtI-Tvma2	Galactose-Galactose-Galactose
Pgal10-crtE-Trpl15a	Pfba1-crtYB-Trpl41b	Psptdh3-crtI-Tefm1	Galactose-High-Medium
Ptdh3-crtE-Trpl15a	Pgal10-crtYB-Trpl41b	Psptdh3-crtI-Tefm1	High-Galactose-High
Ptdh3-crtE-Trpl15a	Pfba1-crtYB-Trpl41b	Pgal10-crtI-Tvma2	High-High-Galactose
Ppxr1-crtE-Tyox1	Pfba1-crtYB-Trpl41b	Psptdh3-crtI-Tefm1	Low-High-High
Ptdh3-crtE-Trpl15a	Pykt6-crtYB-Ttip1	Psptdh3-crtI-Tefm1	High-Low-High
Ptdh3-crtE-Trpl15a	Pfba1-crtYB-Trpl41b	Pmyo4-crtI-Teno1	High-High-Low

The transformation into *S.c.* KTP was completed according to the YoungLab protocol below, and were plated on previously prepared YPD agar plates made with nourseothricin for antibiotic selection. The plates were incubated at 30°C for at least 4 days before examination for colonies. A visual overview of the methodology is provided in the graphic below.

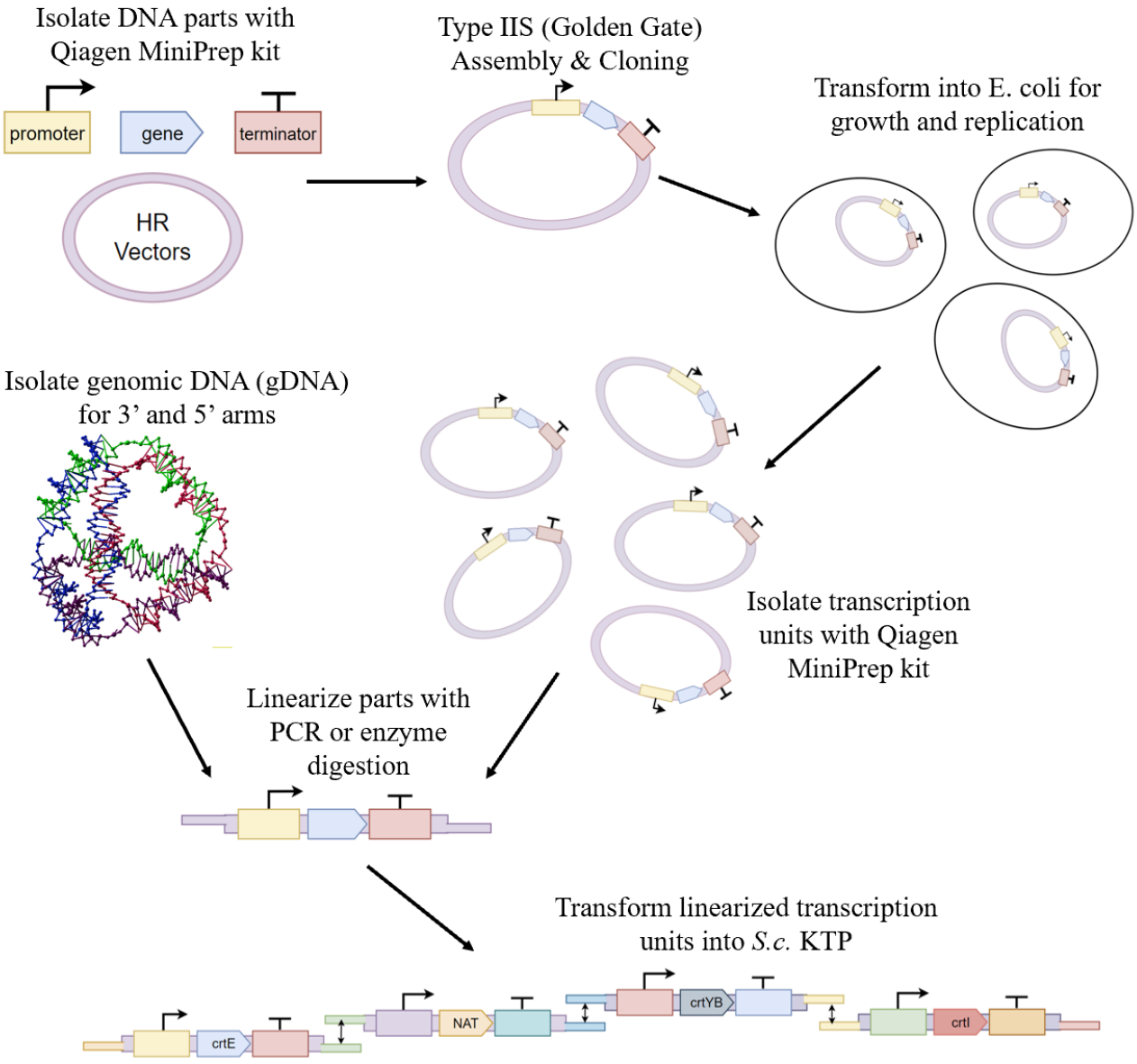


Figure 3: *The schematic above shows the general process for the methodology.*

Step-by-Step Protocols

YPD Liquid Growth Media & Plates Preparation *(yeast extract peptone + dextrose (D-glucose))*

Materials

- MilliQ diH_2O
- Yeast Extract Peptone (YEP)
- Agar
- Petri Dishes
- Autoclave
- Glucose
- Nourseothricin (Nat)

YPD Liquid Growth Media Prep

1. Using a graduated cylinder add 450 mL diH_2O (MilliQ) to a clean 1 L bottle.
2. Weigh 15 g of YEP (yeast extract peptone) and add to the bottle.
3. Screw cap onto bottle then unscrew ½ turn. Tape cap with autoclave tape. Autoclave using liquid cycle 2.
4. Remove bottle chrome autoclave after slow exhaust is complete. Tighten bottle cap. Let the bottle cool until it is warm but not hot to the touch.
5. Add 50 mL of 10x glucose (200 g/L in diH_2O, sterile filtered), tighten cap. Mark that glucose has been added on the label.

YPD Agar Plates Preparation

1. Using a graduated cylinder add 450 mL diH_2O (MilliQ) to a clean 1 L bottle.
2. Weigh 15 g of YEP (yeast extract peptone) and 10 g agar and add both to the bottle.
3. Screw cap onto bottle then unscrew ½ turn. Tape cap with autoclave tape. Autoclave using liquid cycle 2.
4. Turn on the water bath and set it to 60°C.
5. Remove bottle chrome autoclave after slow exhaust is complete. Tighten bottle cap.
6. Place bottles in the water bath for 15 min.
7. Add 50 mL of 10x glucose (200 g/L in diH_2O, sterile filtered), tighten cap. Mark that glucose has been added on the label.
8. Add antibiotic (depending on yeast species, for the experimentation nourseothricin was used).
9. Pour warm media into 10 cm plates until the media covers the plate bottom.
10. Once the media has solidified, flip plates. Let sit overnight.
11. Label with final concentration of antibiotic then store in 4 °C fridge.

Inoculation

Materials

- Growth Media
- Frozen Stock Solution or Colony
- Falcon Tubes
- Serological Pipette

Inoculation from a Frozen Stock of E. coli

1. On a lab bench sterilized by a flame, label falcon tubes for each sample. Use a serological pipette to aliquot 5 mL of LB growth media to each tube.
2. Use a 10 µL pipette tip to scrape off a small chunk of frozen stock.
3. Drop the pipette tip with the frozen cells on it into the falcon tube and incubate overnight in a gently spinning rack at 37ºC .

Inoculation from a Colony

1. Mark a prominent colony with a marker on the bottom of the dish.
2. Use a 10 µL pipette tip to lightly scrape and pick up the marked colony without puncturing the agar.
3. Drop the pipette tip into the falcon tube and incubate overnight in a gently spinning rack at the proper growth temperature for the organism (37ºC for *E. coli*, 30ºC for *S. c. KTP*).

Mini Prep

Materials

- Qiagen Zyppy MiniPrep Kit
- QIAprep 2.0 Spin Column
- Benchtop Centrifuge

Protocol

1. Pellet 1-5 mL bacterial overnight culture by centrifugation at 3200 xg for 5 min at room temperature (15-25ºC).
2. Resuspend the pelleted bacterial cells in 250 µL Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250 µL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Let stand for 2 min. Do not allow the lysis reaction to proceed for more than five minutes.
4. Add 350 µL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
5. Centrifuge for 10 min at 13,000 rpm in a table top microcentrifuge.
6. Apply 800 µL supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 30-60s and discard the flowthrough.
7. Wash the QIAprep 2.0 spin column by adding 0.5 mL of Buffer PB. Centrifuge for 30-60s and discard the flowthrough.

8. Wash the QIAprep 2.0 spin column by adding 0.75 mL of Buffer PE. Centrifuge for 30-60s and discard the flowthrough.
9. Centrifuge for 1 min to remove the residual wash buffer.
10. Place the QIAprep 2.0 column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µL Buffer EB to the center of the QIAprep 2.0 spin column, let stand for 1 min and centrifuge for 1 min.

Yeast Genomic DNA Isolation

Materials

- Ice
- Zymolyase
- Wizard Genomic DNA Purification Kit
- Centrifuge
- 70% Ethanol
- 100% Isopropanol
- 50mM EDTA
- Falcon Tubes

Procedure

1. Inoculate cells in 5 mL YPD overnight in a spin drum at 30ºC.
2. Add 5 mL of culture into a 15 mL Falcon tube.
3. Centrifuge at 500 xg for five minutes. Remove supernatant with pipette.
4. Resuspend the cell pellet using 1.5 mL of 50 mM EDTA.
5. Add 37.5 µL of 5U/µL zymolyase & gently piet four times to mix.
6. Incubate the sample at 37ºC for 60 minutes to digest the cell wall. Allow them to cool to room temperature.
7. Transfer to 1.5 mL and centrifuge for 1 min at 1000xg.
8. Add 1.5 mL of Nuclei Lysis Solution to the cell pellet and flick to mix.
9. Incubate at room temperature for 30 min.
10. Add 7.5 µL of RNase A solution and flick to mix. Incubate at 37ºC for 15 minutes.
11. Add 500 µL of Protein Precipitation Solution and invert gently to mix.
12. Let the sample sit on ice for 5 minutes.

Type IIs Assembly

Materials and solutions:

- DNAse free water
- Ligase buffer
- BsaI enzyme
- MiniPrepped DNA Parts
- T4 DNA Ligase

Procedure:

1. Make stock dilutions of parts at 20 fmol/µL.
    a. Use NEBiocalculator with bp lengths from the Master Strain Database and Benchling.

2. Complete Type IIs assembly using this volume table:

Table 3: *Volume Table for Type IIs Assembly***.**

Final Units	Component	Stock Units	Vol to add per rxn (µL)
	DNase free dH2O		7.9-N
1 X	Ligase Buffer	10X	1
20 fmol	DNA parts (N)	20 fmol/µL	1*N
1U/µL	Bps1 or Bsa1	10 U/µL	1
8U/µL	T4 DNA ligase HC	20 U/µL	0.4
		Total	**10.3**

3. Once all parts have been added to respective PCR tubes, set a thermocycler to the settings below. Once the reaction has finished, store at -20ºC.

Table 4: *Thermocouple conditions for Type IIs Assembly.*

Temperature Setting	Time
37ºC	90s
16ºC	3min
50ºC	5min
80ºC	10min

Transformation into E. coli

Preparation

1. Collect ice
2. Label PCR Tubes (one for each Type IIS reaction completed previously)

Procedure

1. Get cells from -80ºC freezer and thaw on ice for 5 minutes

2. Add 10 µL of cells to the new labeled PCR tubes
3. Add 4 µL of the Type IIs reaction solution to the respective PCR tube
4. Let sit on ice for 30 min
5. Heat shock at 42 ºC for 30 seconds in the thermocycler
6. Let sit for 5 minutes then add 100 µL of SOC medium
7. Put in 37 ºC for one hour
8. Label petri dishes (with flame running)
9. Pipette all onto plate, add 5-6 glass beads and shake until plates are dry
10. Put in 37 ºC overnight

Glycerol Stock Preparation

Materials

125 mL deionized water

125 mL glycerol

500 mL cells suspended in growth media

Procedure

1. Pour both into a 250 mL beaker
2. Stir with medium sized stir bar until homogenous without mixing lines
3. Pour into vacuum filter head and vacuum filter
4. Put the cap on the sterile glycerol in the presence of a flame
5. To an empty 1 mL glycerol stock vial, add 500 µL of cells and 500 µL of 50% glycerol.
6. Store in -80ºC freezer until use.

PCR Protocol

Materials

- Q5 2X Mastermix
- Forward Primer
- Reverse Primer
- E-Gel EX Agarose gel
- DNA Free Water
- PCR Tubes
- QIAquick PCR Purification Kit

PCR Procedure

1. Dilute the primers to a 10mM concentration, template must be diluted to 1ng/µL and genomic DNA must be diluted to 200g/µL.
2. In each PCR tube add:
    a. 25µL of Q5 2X Mastermix (add last)
    b. 2.5µL of Forward Primer
    c. 2.5µL of Reverse Primer
    d. 1µL of Template

e. 19µL of DNA free water

3. PCR tubes were grouped by temperature and time, as shown in **Appendix Number**. For varying times, the maximum extension time in the temperature group was used.

PCR Purification Procedure

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, ph 5.0, and mix. The color will turn yellow.
2. Place a QIAquick column in a 2 mL collection tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 3-60s.
4. To wash, add 750 µL Buffer PE to the Q1Aquick column and centrifuge for 30-60s.
5. Centrifuge the Q1Aquick column once more in the 2 mL collection tube for 1 min to remove the residual wash buffer.
6. Place each Q1Aquick column in a clean 1.5mL microcentrifuge tube.
7. To elute DNA, add 50 µL Buffer EB or water to the center of the Q1Aquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µL elution buffer to the center of the Q1Aquick membrane, let the column stand for 1 min and then centrifuge.
8. If the purified DNA is to be analyzed on a gel, add 1 volume of loading dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Gel Electrophoresis of PCR results

1. Load 20 µL DNA free water to each gel slot.
2. Load 10 µL of the marker was added with 10 µL of water to the Marker slot.
3. Load 2 µL of sample into each plate.

Enzyme Digestion

Materials

- Plasmid
- 10X Cutsmart Buffer
- BbsI Enzyme
- Nuclease Free Water

Procedure

1. To prepare the reaction add the following reagents:

Table 5: *Components for Enzyme Digestion.*

Component	Vol (μL)/reaction
Plasmid	40
10X Cutsmart Buffer	5
Bbsl Enzyme	2
Nuclease Free Water	3

2. Vortex briefly to make sure the solutions are adequately mixed
3. Incubate the reaction mixture at 37ºC for 10h and 65ºC for 10 min using the thermocycler.

SC Transformation

Materials

- Nuclease free water
- 100 mM Lithium Acetate (Filter Sterilized)
- 1.0 M LiAc (Filter Sterilized)
- Salmon Sperm DNA (10 ug/uL) *Prepare by boiling for 5 minutes. Can keep as aliquots and re-thaw 3-4 times.
- PEG 3350 (50% w/v) (filter sterilized)
- YPD liquid media and YPD w/ antibiotic or CSM knockout selection plates
- Clean, sterile 100mL shake flasks
- Rattler plating beads

1. Inoculate 10 mL of YPD with SC cells from glycerol stock. Grow until saturation at 30°C in 100mL shake flask overnight.
2. Inoculate 5 mL of YPD to a cell density of OD~0.25. Scale in appropriate shake flask for number of transformations.
3. Incubate tubes on a rotating drum until OD~1.0 (approximately 4 hours).
4. Prepare DNA solution for each transformation in a PCR tube.
    a. Linearize DNA if needed by restriction enzyme digestion or PCR
    b. 0.1-10 μg of each DNA part (never go over 50 total μL)
    c. Add nuclease free water up to 50 μL
5. Pellet cells in centrifuge at 500 x g for 5 minutes.
6. Pour off supernatant, and resuspend in 2.5 mL of sterile water and centrifuge again.
7. Pour off the water and resuspend the cells in 100 μL of 100 mM LiAc and transfer to a 15 mL centrifuge tube.
8. Pellet cells in microcentrifuge at 500 x g for 30 seconds. Pipette off supernatant.

9. Resuspend cells to 50 μL in about 40 μL of 100 mM LiAc and flick to mix.
10. Add in the following order:
    a. 240 μL PEG 3350, then flick briefly.
    b. 36 μL of 1.0 M LiAc, then mix by flicking.
    c. 5 μL of Salmon Sperm DNA (10 μg/uL).
    d. The DNA mixture from step 4. Flick until each pellet is mixed completely.
11. Incubate at 30 C for 30 minutes. Add 35 μL of DMSO.
12. Heat shock in a water bath at 42 C for 15 minutes.
13. For antibiotic selection:
    a. Centrifuge at 500 x g for 30 seconds and remove transformation mix with a pipette.
    b. Pipette 1.0 mL of YPD into each tube and resuspend the pellet by mixing gently.
    c. Transfer mix to 4 mL of YPD in a Falcon tube. Incubate at 30 C overnight.
    d. Plate 200 μL cells onto YPD w/ NAT plates. Spread with 5-7 Rattler plating beads.
    e. Incubate at 30ºC for 2-4 days to recover transformants.

Results and Analysis

Nine unique transcription units were created from the promoters, genes, and terminators designated in **Table 1**. These transcription units were initially verified with E-Gel EX agarose gel electrophoresis to be around the expected base pair lengths. Gels were run after both PCR and enzyme digestion. Since a cleanup was done on the PCR samples, the bands on the gel in **Figure 4** appear much cleaner, and there is only one band per sample. Contrarily, the enzyme digested samples in **Figure 5** display multiple bands, as there are still other DNA components that create a significant background when read by a gel.

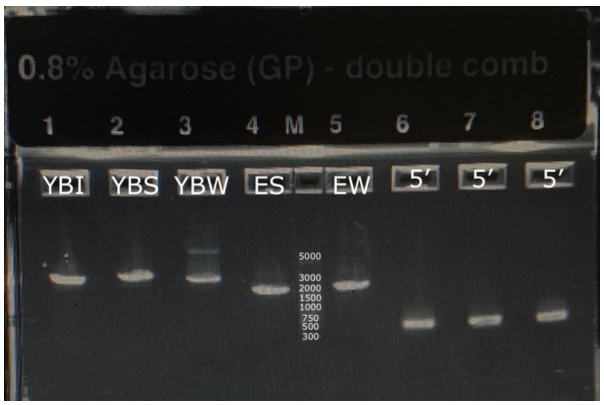


Figure 4: *PCR results on a 0.8% E-Gel EX agarose gel.*

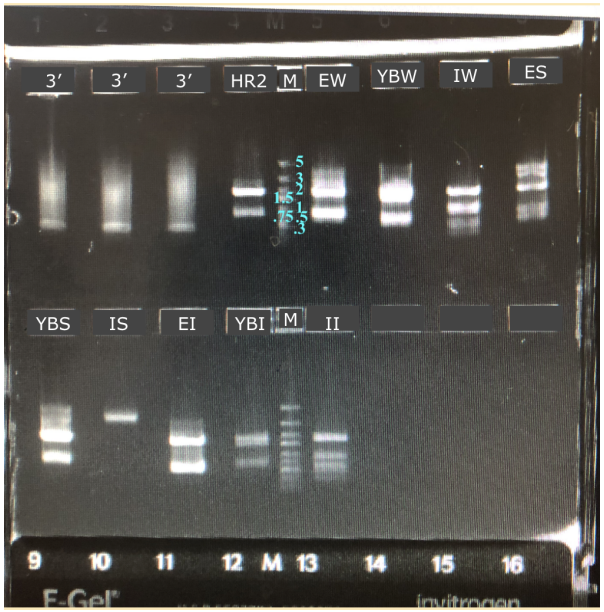


Figure 5: *Enzyme digestion results on a 0.8% E-Gel EX agarose gel. The sample in the well labeled IS was not successfully digested, as the only present bar did not move from 5 kb.*

On both figures, the well labeled “M” contains the nucleotide ladder to which the samples can be compared. As labeled, the ladder can measure DNA lengths with a maximum size of 5000 base pairs (5 kb) and a minimum of 300 base pairs (0.3 kb). **Table 6** below gives the relative lengths that each of the transcription units should have lined up to. These estimated lengths were compared to the gels to determine if assembly was successful. The figures displayed above are not representative of all the trials that were completed, as further gels were ran for the samples that did not appear as expected, and samples were remade until all nine transcription units, the antibiotic resistance gene (HR2), and both homologous arms (3’ and 5’) appeared at the correct lengths.

Table 6: *Transcription units and the predicted base pair length.*

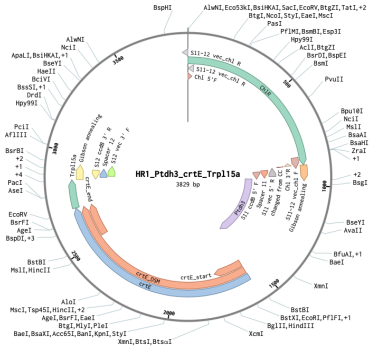
Transcription Unit	Estimated Length (bp)
ES	1979
YBS	3490
IS	2596
EW	2182
YBW	3020
IW	2686
EI	1705
YBI	3265
II	2371
HR2	1220
5’ homologous arm	550
3’ homologous arm	550

After ensuring that all the necessary parts were the correct, transformations into *S.c.* KTP were attempted multiple times with no success. Changes in the transformation protocol were made after each trial to rule out the possibility of technical error causing the transformation failure. Fresh lithium acetate and PEG was used for each trial, and for one trial a 42°C heat shock of 1 hour was used instead of the usual 15 minutes to ensure that the cell walls were penetrable. The first trial was completed with DNA that had been linearized

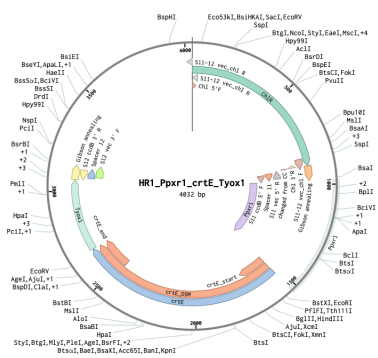
with PCR, and the following trials were completed with DNA that has been prepared with BbsI enzyme digestion.

To understand whether the issue in transformation lay in the transcription units or the nature of KTP itself, the same transcription units were transformed into *S.c*. S288C, with no avail. The lack of successful transformation into S288C revealed that the issue was likely an error in the transcription units themselves that prevented the integration of the genes, therefore hindering expression.

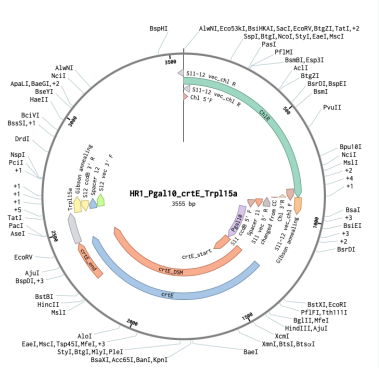
All of the transcription units were sent to Quintara Biosciences for DNA sequencing to try to figure out the problem. While they were being sequenced with Sanger sequencing, plasmid maps were assembled using Benchling for each plasmid. These plasmid maps can be seen in **Figure 6** and were used to compare the sequencing results.



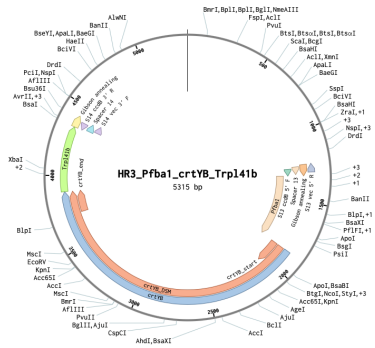
a. ES



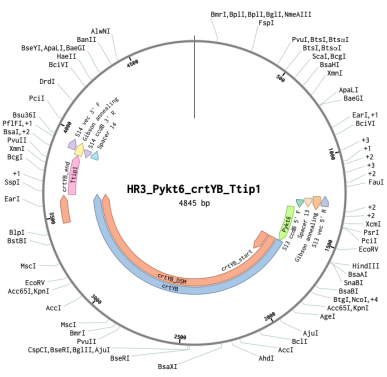
b. EW



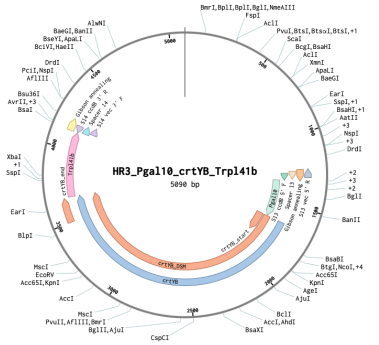
c. EI



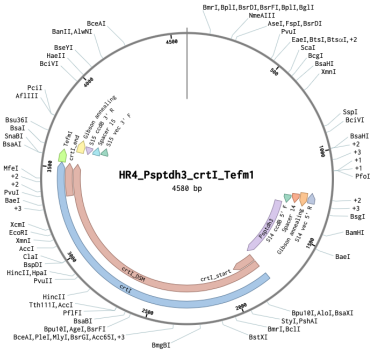
d. YBS



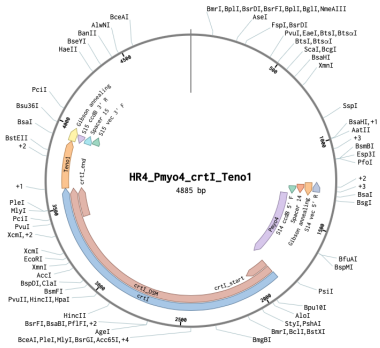
e. YBW



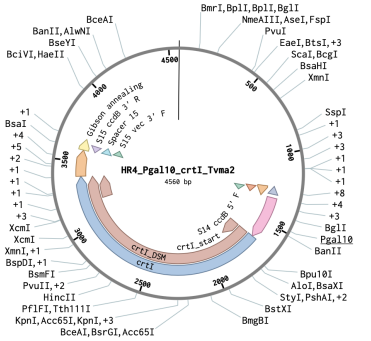
f. YBI



g. IS



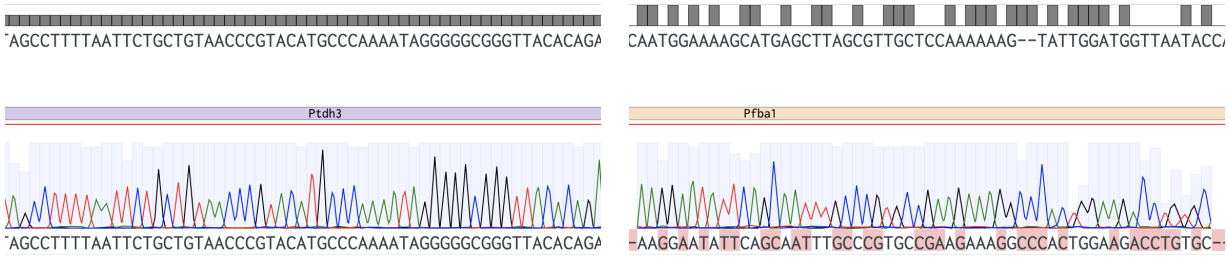
h. IW



i. II

Figure 6: *Plasmid maps created for each transcription unit. (a)-(i) .*

Benchling was used to align the plasmid maps and the sequencing results for each transcription unit. The MAFFT multiple sequencing alignment program was used. An example of a good alignment and a not so good alignment can be seen in **Figure 7**.



(a) (b)

Figure 7: *Alignment of sequenced transcription units compared to their respective plasmid maps.* ***(a)*** *ES forward primer sequence compared to the ES plasmid map.* ***(b)*** *YBS forward primer sequence compared to the YBS plasmid map.*

As seen in **Figure 7**, the nucleotides highlighted in red do not match the template strand, which is from the plasmid map. Looking at the differences between (a) and (b), some of the sequences aligned better than others. **Table 7** has the percent identity of the sequenced alignment to the plasmid maps for all the transcription units created.

Table 7: *Percent identity of plasmid maps and sequenced alignment*

Gene	**Strong**		**Weak**		**Inducible**	
	Forward	Reverse	Forward	Reverse	Forward	Reverse
CrtE	98.41%	97.91%	98.61%	97.82%	97.33%	97.02%
CrtYB	20.98%	29.25%	94.9%	98.11%	92.9%	98.21%
CrtI	59.62%	93.2%	68.21%	96.72%	96.34%	97.02%

Looking at the values within **Table 7**, the transcription units for CrtYB strong, CrtI strong, and CrtI weak did not align properly. This suggests that there may have been errors in creating these transcription units and may explain why all of the transformation attempts were not successful.

Conclusions and Recommendations

Although there was not a successful transform into the KTP, transcription units were created that can be used for future work. Although a few transcription units may need to be remade, from the sequencing it was possible to determine that six of the transcription units created were correctly aligned with the predicted sequences from Benchling. Once all nine of the transcription units are confirmed to have the correct sequences, future groups can attempt transformation via homologous recombination. If this does not work, instead of homologous recombination, a future group can use plasmid integration after doing a Level 2 Type IIS reaction to put the whole gene pathway into one vector. Additionally, further investigation can be made into possible reasons why homologous recombination is not a successful method of transformation into S.c. KTP for the β-carotene pathway when the established lab protocol is used.

Once transformation into *S.c.* KTP is successful, future work could involve quantifying the amount of β-carotene synthesized using HPLC. The HPLC data will allow for quantitative comparison of expression levels between transcription units. Then, studies could be performed to determine the efficiency of transformation into *S.c.* KTP and experimentation could be completed to optimize the process. From here, the high, low, and inducible combinations could be studied to determine which is the most useful for a probiotic.

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Appendices

Table 8: *Settings and lengths for PCR Reaction.*

Promoter	Gene	Terminator	Length (bp) including overhangs	Temp. (°C)	Time per cycle (min)
Ppxr1	CertE	Tyox1	2182	64	1.5
Pykt6	CertYB	Ttipl	3020	63	1.5
Pmyo4	CertI	Tenol	2686	61	1.5
Ptdh3	CertE	Trpl15a	1929	64	1
Pfba1	CertYB	Trpl141b	3490	61	1.5
Psptdh3	CertI	Tefm1	2596	61	1.5
Pgal 10	CertE	Trpl15a	1705	64	1
Pgal 10	CertYB	Trpl41b	3265	63	2
Pgal 10	CertI	Tvma2	2371	61	1.5
5’ HR			550		
HR-2			1220		
3’ HR			550		

Table 9: *Nomenclature*

Abbreviation	Definition
crtE	geranylgeranyl diphosphate synthase
crtI	phytoene desaturase
crtYB	bifunctional enzyme phytoene synthase and lycopene cyclase
S.c.	*Saccharomyces cerevisiae*
E.c.	*Escherichia coli*
PEG	Polyethylene glycol
HPLC	High pressure liquid chromatography