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Investigating the Biorisk of Genetically Engineered *Thermosynechococcus Elongatus* BP1

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INVESTIGATING THE BIORISK OF GENETICALLY ENGINEERED *THERMOSYNECHOCOCCUS ELONGATUS* BP1

by

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ABSTRACT

INVESTIGATING THE BIORISK OF GENETICALLY ENGINEERED *THERMOSYNECHOCOCCUS ELONGATUS* BP1

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Cyanobacteria, also known as blue-green algae, are an ancient group of microorganisms that use simple materials, such as sunlight, carbon dioxide and water, to produce energy while providing oxygen to the atmosphere by performing photosynthesis. Synthetic biology approaches have been employed with cyanobacteria as a platform to produce a range of products, such as biofuels, by inserting a series of genes into the cyanobacterial genome that will allow the conversion of metabolic intermediates to such desired products. Although these methods are promising, it is important to understand any potential bio-risk they pose. This research evaluates the potential bio-risk of genetically engineered thermophilic *Thermosynechococcus elongatus* BP1 (BP1) by investigating key areas of concern regarding genetically engineered microorganisms.

The first aim of this research study assesses the ability for horizontal gene transfer from engineered BP1 to wild-type bacteria, which is a major route of gene movement between microorganisms in nature. This was accomplished by co-culturing the engineered BP1 with *E. coli* DH5α, screening them by plating on solid media with antibiotics and verifying transfer of the transgene cassette by PCR. The second research aim characterizes the growth and survivability of engineered BP1 in varying temperatures and non-continuous lighting by monitoring the optical density over a 4-week period and calculating the colony-forming units (CFUs) during the cold and warm seasons. The final aim investigates the fate and stability of the

transgenes within the genome of BP1. This was achieved by monitoring the presence and expression of the kan resistance gene monthly for 24 months using molecular biology techniques, such as PCR and RT-PCR. Illumina-based whole-genome sequencing and comparative analysis of the one-year sample was also performed to determine if any mutations were acquired within the transgene cassette. Major findings include identifying that cyanobacteria can horizontally transfer transgenes to bacteria, they do not survive well outside laboratory conditions and the transgenes within the genome remain stable for years. This body of work provides valuable insight and practical knowledge for the biotechnology community and policy makers to safely regulate the use of genetically engineered organisms for research and commercial production.

This dissertation is dedicated to my parents, Johnny Lee and Mouri Lisa Barnes for their unwavering love, guidance, support and encouragement throughout life and especially during the doctoral program. Without them – this would not be possible.

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ABBREVIATIONS

3D Three Dimensional

ADH Alcohol Dehydrogenase

BDH Butanol Dehydrogenase

CFU Colony-Forming Units

DNA Deoxyribonucleic Acid

EVMS Eastern Virginia Medical School

GE Genetically Engineered

GMO Genetically Engineered Organisms

HGT Horizontal Gene Transfer

KIVD Alpha-Ketoisovalerate Decarboxylase

mRNA Messenger Ribonucleic Acid

OD_{730} Optical Density at 730 nm

PCR Polymerase Chain Reaction

PDC Pyruvate Decarboxylase

pKA pUC57-KIVD-ADH plasmid

pKB pUC57-KIVD-BDH plasmid

qPCR Quantitative Polymerase Chain Reaction

RT-PCR Reverse-Transcriptase Polymerase Chain Reaction

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CHAPTER I

INTRODUCTION

The study of genes and genomes can hold an enormous amount of a valuable information and has become a corner stone in many aspects of the biological and chemical sciences. According to the World Health Organization, genetics is the study of heredity or the passing of genetic material from parent to offspring, while genomics is the study of genes, their relationship, and their overall influence on organisms. There has been tremendous progression in genomic sequencing technology which has allowed a significant number of organisms from all the kingdoms of life to be sequenced. This information has revealed evolutionary history and is also used in very practical terms to genetically engineer to perform a wide range of jobs, such as insects designed to fight viral epidemics and development of animals for less immunogenic organ transplants [1-5]. The application of genetic engineering, however, has both ethical and bio-risk components. In this dissertation, the application of genetic engineering from a bio-risk perspective is investigated and can be seen to reveal foundational insights in genetics as well as inform the development of policy for its safer use.

CYANOBACTERIA

Cyanobacteria as a Model Organism

Cyanobacteria is a phylum of ancient prokaryotes found in a diverse range of aquatic and non-aquatic ecosystems. These microorganisms are known to perform oxygenic photosynthesis by using abundant, natural resources such as water, carbon dioxide and sunlight as a means of energy production while providing oxygen to the atmosphere. Evidence of cyanobacteria found

in fossils have dated the microorganisms between 2.5 to 3.5 billion years old and has also been suggested they played an integral role in the Great Oxidation Event, essentially oxygenating our world and giving rise to the foundation for other types of organisms to inhabit Earth [6, 7]. Ecologically, cyanobacteria are important as they are primary producers involved in fixing organic carbon. As cyanobacteria perform photosynthesis, atmospheric carbon is converted into organic carbon which is subsequently used as building block for many biomolecules [8, 9]. Nitrogen fixation is another task for many strains of cyanobacteria, where atmospheric nitrogen (N_2) is converted to ammonium ions (NH_4^+) [10, 11]. Cyanobacteria have inhabited a wide range of aquatic environments, including freshwater, marine and brackish and are also able to thrive in environments of extreme conditions, such as hot springs, high salinity, and low temperatures. Their ability to adapt to a variety of different ecosystems over billions of years has resulted in vast genetic diversity among different strains [12, 13]. Due to their important biological functions, adaptability and versatility, cyanobacteria have been used as a model organism in many facets of research including photosynthesis, carbon and nitrogen fixation, circadian clocks, and biotechnology [14-20].

Synthetic Biology and Genetically Engineered Cyanobacteria

Synthetic biology is an interdisciplinary subset of biochemistry that integrates engineering methods and biological systems, allowing the design of novel pathways or production of a desired molecule or material [21, 22]. These organisms are commonly known as genetically engineered or genetically modified organisms (GMOs), as their DNA has been altered within a laboratory setting. There are many ways to manipulate DNA including mutagenesis, gene insertion, gene fusion or gene knockout. Many of these techniques are

possible by recombinant DNA technology [23]. The most common organisms for genetic engineering include bacteria, crop plants, insects, and animals [24-27]. Table 1 outlines examples of GMOs and their uses. Genetically engineered (GE) organisms have been applied to many different sectors in society, ranging from research and agriculture to medicine and biotechnology.

Synthetic biology has been employed within cyanobacteria as an alternative platform for renewable energy source. Fourth-generation biofuels are produced within genetically engineered cyanobacteria that enables the conversion of metabolic intermediates to biofuels and/or bioproducts from sunlight, CO_2 and water [28-30]. Photosynthetic production of biofuels and/or bioproducts in this manner would represent a new type of agriculture for bioenergy, which has shown many advantages such as the decreased competition for arable land, and its eco-friendly nature in that it is net-carbon neutral [31-36].

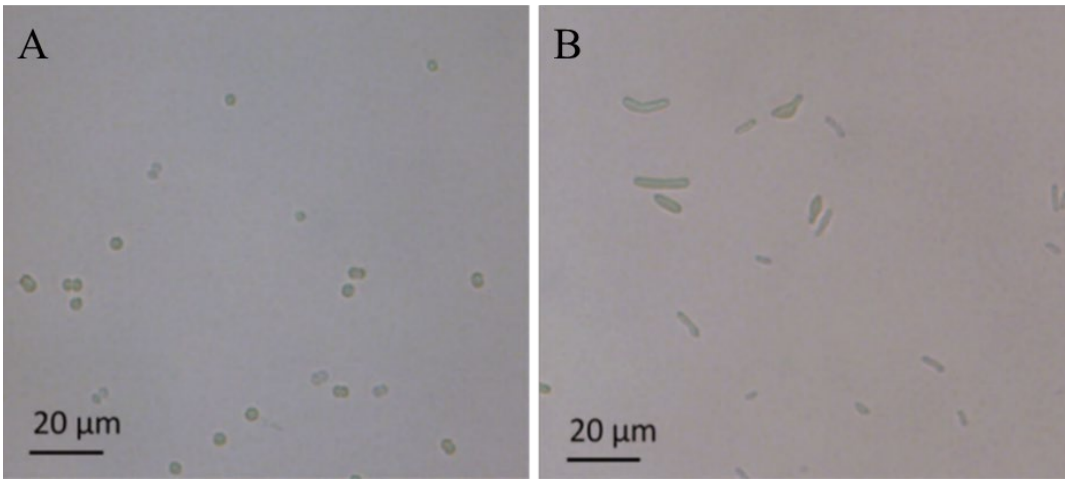

Figure 1. Mesophilic Strains of Cyanobacteria Commonly Used for Biotechnology Applications. Light Microscopy images of (A) *Synechocystis* PCC6803 (green circular cells) and (C) *Synechococcus elongatus* PCC7942 (rod-shaped cells). This figure was adapted from [37].

Type	Name	Genetic Change	Purpose	Reference
Bacteria	*Salmonella typhimurium*	Expression of cytolysin A gene (*clyA*) within an attenuated strain of *S. typhimurium*	Inhibit tumor growth and increase tumor necrosis in targeted cancer therapy	[38]
	Bacillus thuringiensis	Expression of a chimeric *chiA74Δsp*:*cry1Ac* gene	Increase production of δ-endotoxins used as an insecticide	[39]
Plants	*Carica papaya* (Papaya)	Expression of the coat protein (CP) gene of papaya ringspot virus (PRSV) 5-1	Increase tolerance and resistance to PRVS	[40]
	Phaseolus vulgaris L. (Bean)	Expression of the *AC1* gene of bean golden mosaic virus (BGMV)	Increased tolerance and resistance to BGMV	[41]
Insects	*Cochliomyia hominivorax* (New World screwworm)	Expression of a tetracycline repressible female-lethal gene system containing transformer (*tra*) gene	Eradication of the New World screwworms through sterilization and ending its endemic that devasted livestock	[42]
	Bombyx mori (Silkworm)	Expression of a truncated pierisin-1A gene (P1A) gene from *Pieris rapae* (cabbage butterly)	Increased bioproduction of silk proteins that can be utilized as biomaterial for biomedical and industrial applications	[43]
Animals	Pig	Silence allele of the 1,3-galactosyltranferase locus (GGTA1)	Decrease immunogenicity for organ transplant recipients	[44, 45]
	Chicken	Expression of humanized anti-HER2 monoclonal antibody (mAb) gene	Bioproduction of human mAb for biomedical applications	[46]

Table 1. Examples of Genetically Engineered Organisms. This lists an example of type and name of engineered organisms, their genetic manipulation, and their purpose.

In addition, the engineering design of the microalgae can be used to render the process of fuel production more efficiently for a variety of biofuels in different species [47-51]. *Synechocystis* PCC6803 and *Synechococcus elongatus* PCC7942 are two strains of cyanobacteria that have been highly utilized in the production of biofuels and/or products, including alcohols, biosensors, and saccharides (Figure 1) [37, 52-56]. The reason why these strains are commonly used is that their genomes have been sequenced and are adaptable to the insertion and expression of foreign genes as many molecular biology tools developed to assist in the genetical engineering of these strains of cyanobacteria [57-59]. One of many examples of a synthetic pathway designed for biofuel production is ethanol production within *Synechococcus sp*. 7002, where proteins pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) were expressed to encourage the conversion of pyruvate to ethanol and ethanol production was detected for up to 30 days (Figure 2) [47].

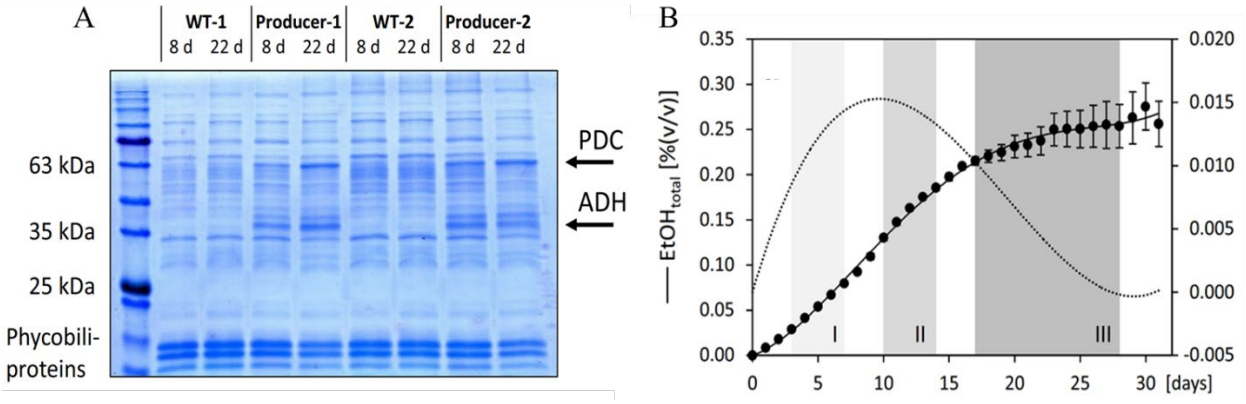

Figure 2. Example of Ethanol Production in GE Cyanobacteria. (A) Presence of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) proteins was detected via Western Blot and (B) ethanol production within GE *Synechococcus sp*. 7002. This figure was adapted from [47].

***Thermosynechococcus elongatus* BP1**

The model organism used in these studies is *Thermosynechococcus elongatus* BP1 (referred to as *T. elongatus* BP1). This strain is a rod-shaped, unicellular strain of cyanobacteria that was isolated from Japan and commonly found to inhabit hot springs. *T. elongatus* BP1 is thermophilic in nature and is shown to have an optimum growth between 55 and 57 °C (Figure 3) [60]. The genome sequence of *T. elongatus* BP1 is also published genome sequence, revealing that its genome is about 2.6 MB long that consists of 2475 protein coding regions [61]. The photosystems within *T. elongatus* BP1 has been extensively investigated to elucidate how photosynthesis occurs at such high temperatures [62-64]. Its thermophilic characteristic is of interest for our studies as it can provide a natural safeguard mechanism if it were to somehow be released from laboratory containment after genetic engineering.

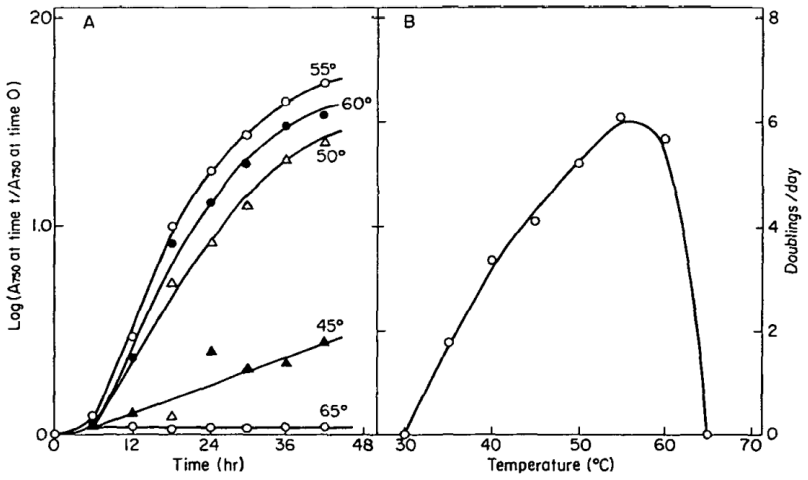

Figure 3. Growth of *T. elongatus* BP1. (A) Growth curves of *T. elongatus* BP1 were determined at 45 °C (closed triangles), 50 °C (open triangles), 55 °C (open circles), 60 °C (closed circles) and 65 °C (open circles) by measuring optical density at 750 nm. (B) Doublings per day (d^{-1}) were measured during exponential phase of *T. elongatus* BP1. This figure was reproduced from [60].

BIO-RISK ASSESSMENT OF GENETICALLY ENGINEERED CYANOBACTERIA

According to the World Health Organization, bio-risk assessment is the process of identifying acceptable and unacceptable risks and their potential consequences. Although synthetic biology and GE tools have been instrumental in solving many significant problems as explained previously, there has been a rising concern surrounding organisms whose DNA has been modified. From a scientific and general public perspective, some of these apprehensions involve toxicity, rise in antibiotic resistance, ecological and environmental effects as well as health risks for humans [65]. From a federal, state and institutional regulatory standpoint, there is a concern that the increased activity in the area of genetic engineering requires greater oversight as the use and availability of synthetic biology and GE technology has dramatically increased over time. The government and regulatory bodies must also be vigilant and update regulatory policies to prevent the potential for misuse [66]. These issues highlight the importance of understanding the potential bio-risks if GMOs are to be used in a large-scale capacity in a manner that is safe.

GE cyanobacteria are no exception and may also pose certain potential risks if they were to break physical containment and escape to the environment. Some of the risks associated with GE cyanobacteria include their competition and persistence in nature, the ability to transfer their modified genes to other bacteria and ultimately negative impacts on aquatic populations or human and animal health [67-71]. Figure 4 is a schematic that depicts the flow of assessing bio-risks pertaining to GE cyanobacteria. First, it is important to understand if GE cyanobacteria escape to the environment, will they dominate, persist or die? If they can survive outside of laboratory conditions, do they have the potential for horizontal gene transfer (HGT) to other organisms that give the recipient a selective advantage? If they show the possibility of either of

these, how can it impact the ecosystem? Many of these concerns remain unresolved to this day and it is vital to address them as the use of these organisms continues to increasel. Additionally, the use of GE organisms by the industry can occur on large scales, thus potentially having greater impact on the enviornment if it is not properly regulated.

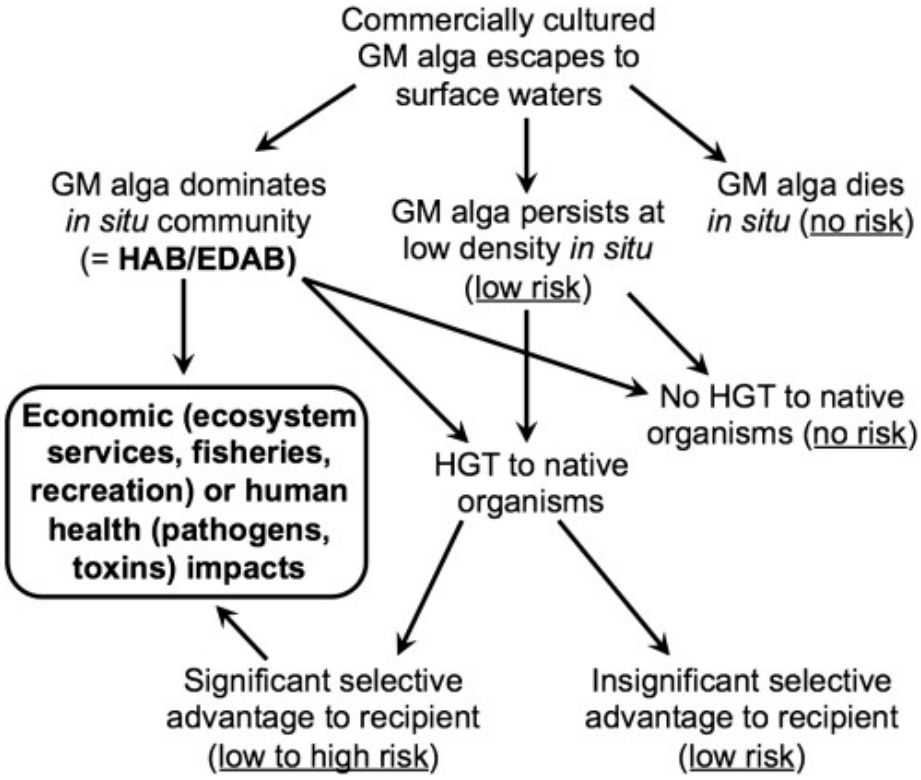

Figure 4. Schematic of Bio-Risk Assessment of GE Cyanobacteria. HAB = harmful algal blooms, EDAB = ecosystem disruptive algal bloom. This figure was adapted from [69].

RESEARCH AIMS

The experimental and computational studies presented in this dissertation aims to address three areas of critical concern involving cyanobacterial genetics and engineering. The first addresses

the question of HGT from cyanobacteria to other bacteria. It is through this mechanism that genes are naturally shared and unwanted or unintentional transfer of foreign and designer genes has the possibility of occurring. The ramifications of which can have unintended consequences such as transfer and acquisition of antibiotic resistance to medically relevant bacteria. The second aim investigates the ability of GE cyanobacteria to survive outside of laboratory conditions in an effort to understand the ramifications should they escape containment and develop new strategies of containment. The third aim seeks to understand the persistence and fate of transgenes within the chromosome of GE cyanobacteria. In this avenue of investigation, a window to uniquely monitor and understand the impact of transgenes is opened.

EXPERIMENTAL APPROACHES

Transformation by Electroporation

Transformation is the process of introducing foreign DNA to a microorganism and it is a vital step in the generation of GE organisms. There are many transformation methods including natural transformation, chemical transformation and physical transformation [72]. Natural transformation involves active uptake of exogenous DNA by microbial cells under physiological conditions [73]. Chemical transformation involves the use of chemicals, such as $CaCl_2$ and polyethylene glycol, to drive the transfer of negatively charged DNA in host cells [74]. Electroporation is type of physical a technique that involves short but high-voltage electrical pulses that increases the permeability of the plasma membrane, allowing exogenous DNA to enter the cell [75, 76]. This electroporation method has been used for many strains of cyanobacteria, which allows genetic manipulation to be achievable [77-81]. Electroporation was used as our preferred method of transformation to produce the GE *T. elongatus* BP1 used in the following studies of this dissertation.

Molecular Biology Techniques for Genetic Analysis

The Polymerase Chain Reaction (PCR) is a common molecular biology technique that allows the targeted amplification of small segments of DNA as a key preparatory technique for DNA and RNA analysis [82-84]. The essential components for PCR includes DNA source as a template for amplification, forward and reverse primers that span the area of interest, nucleotides, and thermal stable DNA polymerase enzyme to synthesize the new strands of DNA [85]. These components are placed into a PCR tube and the reaction is placed into a thermocycler to perform the enzymatic assay [86]. There are 3 main steps in a PCR cycle: denaturation, annealing and elongation (Figure 5).

I. In the denaturation step, temperatures are elevated to above 90 °C, which disrupts the hydrogen bonds within double-stranded DNA and allows them to separate to individual strands for primer annealing.
II. The annealing step involves the lowering of temperatures to facilitate binding of primers to region of DNA that is to be amplified.
III. During the extension step, temperatures are adjusted to a temperature ideal for DNA polymerase to add nucleotides in a 5’ to 3’ to synthesizing a new stand of DNA complementary to template DNA.

During this process, the PCR product is doubled with each cycle and resulting in significant amplification of the targeted region of DNA for analysis. Since the PCR principle was developed in 1990, it has been utilized for numerous applications including medical diagnostics, detection of infectious diseases and forensic analysis [87-91]. There have also been many variations of PCR developed that allow for site-specific mutagenesis, gene expression and quantification [92-94].

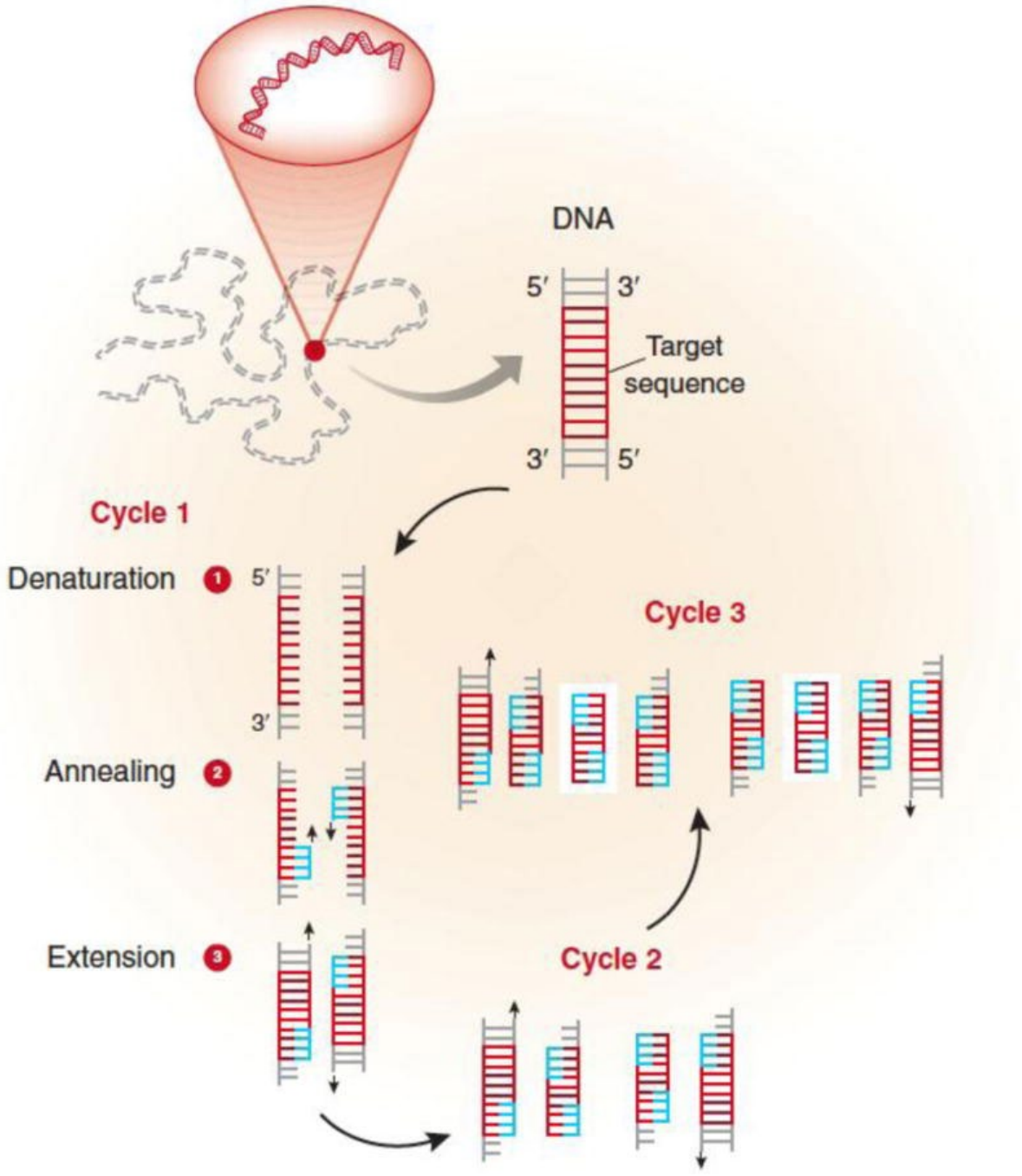

Figure 5. Steps of Polymerase-Chain Reaction. The three main steps of a PCR cycle are; (1) denaturation to separated double-stranded DNA, (2) annealing of primers to DNA flanking the area to be amplified and (3) extension of the complementary strand by DNA polymerase. These steps are repeated, and the PCR product is doubled each cycle. This figure was reproduced from [83].

Reverse Transcriptase-PCR (RT-PCR) is an adaption of PCR that is used to determine the presence or absence of mRNA transcripts and investigate expression of specific genes [95]. The RT-PCR protocol can be broken down into three steps and are depicted in Figure 6. First, total RNA is isolated from cell or tissues and subsequently treated with DNase to eliminate all residual DNA in preparation for RT-PCR [96]. The first step involves the synthesis of complementary DNA (cDNA) from the RNA template by use of an RNA-dependent DNA polymerase, also known as a reverse transcriptase [97]. The second step uses the newly synthesized cDNA as a template for amplification via PCR, which when visualized on an agarose gel can uncover whether a gene is being expressed [95]. RT-PCR is also used as a method of quantifying gene expression by using probes or fluorescent dyes during the amplification process [98].

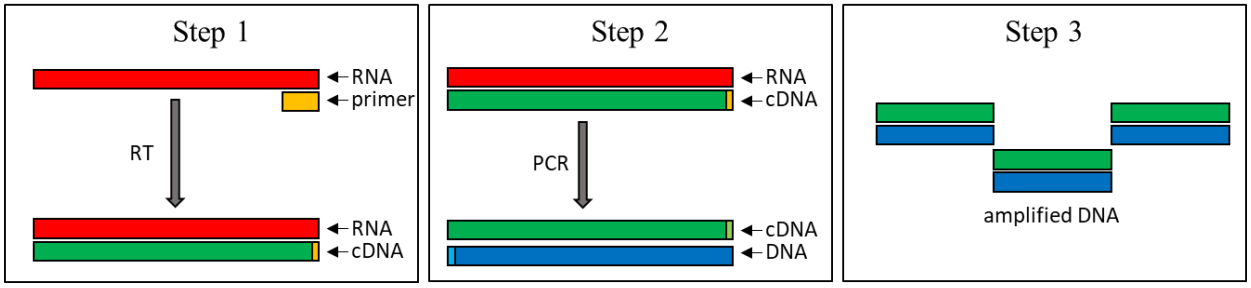

Figure 6. Reverse-Transcriptase PCR. RNA (red) is converted to cDNA (dark green) by use of a reverse primer (yellow). The cDNA is used as a template to synthesize double-stranded DNA (dark blue) in the first PCR cycle followed by amplification of the DNA using specific primer (light blue and light green)

Genome Sequencing Technology

Sequencing is a molecular biology tool that allows the determination of the nucleotide sequence of DNA from smaller fragments (ie. genes) up to small and large genomes. Sequencing has become an innovative tool and a cornerstone to gain insight on rare diseases, cancer and evolution of different organisms. The dawn of sequencing technology began with the development of Sanger sequencing in 1977 [99]. Sanger sequencing employs labeled dideoxy nucleotides (ddNTPs) that lack the 3' hydroxyl group. Their addition to the growing DNA strand during PCR amplification results in the termination of the growing DNA strand due to the inability to form phosphodiester bond with the incoming nucleotide. To determine the order of nucleotides, the fluorescently labeled ddNTPs can be identified through automated sequencing machines. Sanger sequencing technology was utilized to complete the draft of the Human Genome Project in 2001, which was an enormous accomplishment in the field. Since then, the National Human Genome Research Institute developed an initiative for the development of cheaper and faster sequencing technology leading to a boom in what is called next-generation sequencing (NGS) [100-102].

The Illumina sequencing platform is considered second-generation sequencing technology and currently dominates in the industry as it has significantly lower output times and reduction in cost compared to Sanger sequencing [103]. Illumina operates by using a sequencing-by-synthesis (SBS) method where the first step involves preparing a library of adapter-ligated DNA oligomers which are subsequently introduced to a glass slide where bound DNA fragments begin a process called is bridge-amplification [104]. During bridge-amplification, clusters of the same DNA fragment is amplified significantly generating millions of forwards and reverse reads. As the fluorescently labeled nucleotides are added, automated machines record and reads the

fluorescent signals to determine the nucleotide at that position [105]. Although Illumina sequencing produces shorter sequence reads (100-300 bp) when compared to Sanger Sequencing, the idea is that by amplifying many copies of small fragments will ensure the correct base is being called, thus increasing the sequence depth and accuracy of the base call. The processes for Sanger Sequencing and Illumina sequencing platforms are depicted in Figure 7.

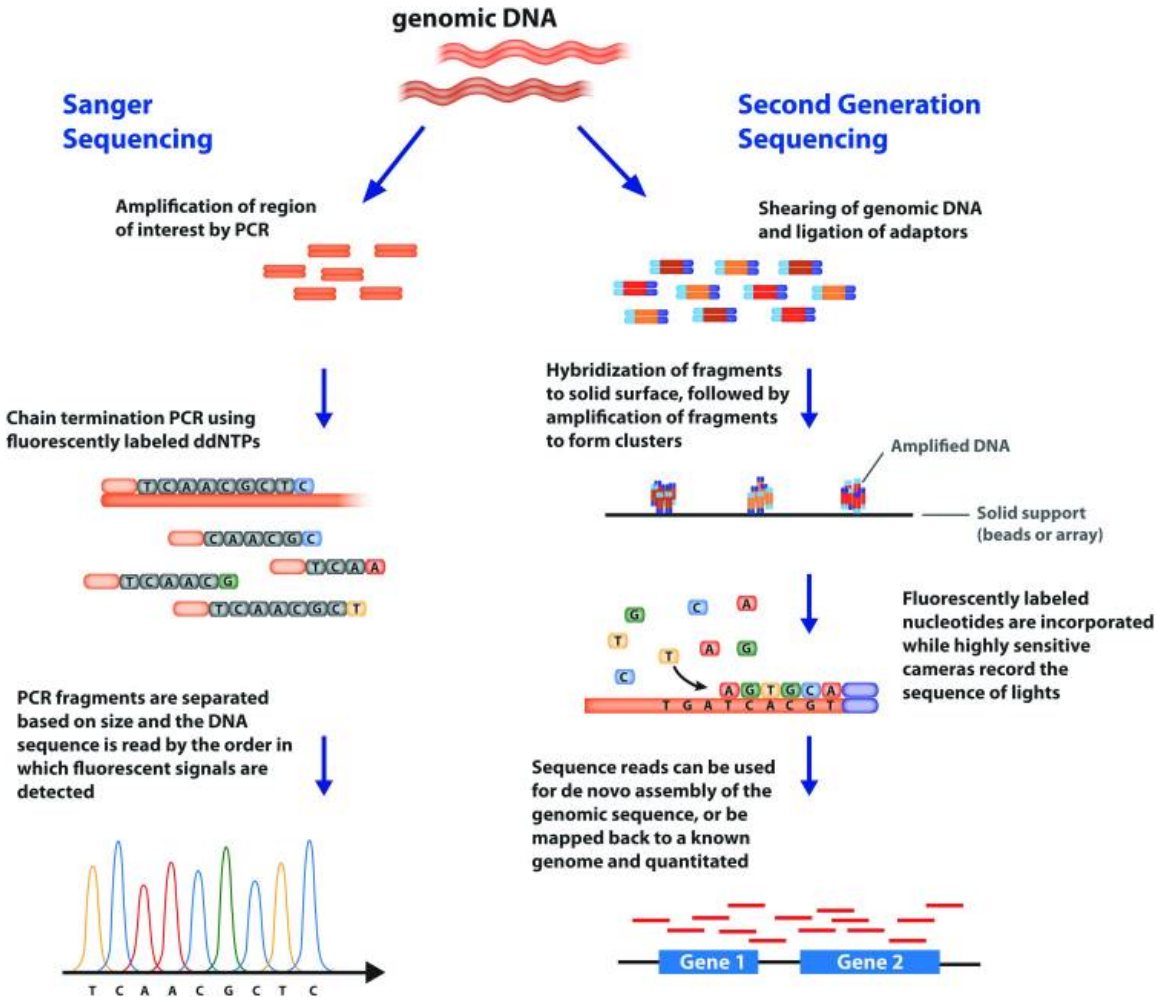

Figure 7. First and Second Generation Next-Generation Sequencing Platforms. This schematic depicts steps involved in Sanger Sequencing and Illumina Sequencing. This figure was adapted from [106].

Genome Assembly

Genome assembly is the process of arranging the short genetic segments called "reads" generated from sequencing in the correct order and merging them to generate an organism's genome. This method may be best described as a process akin to putting the pieces of a jigsaw puzzle together to reveal an entire picture. Genome assembly can be achieved through either comparative assembly or *de novo* assembly (Figure 8). Comparative assembly, also known as reference-guided assembly, allows short and/or long reads to be arranged using an already assembled and published genome [107]. There has been a rapid increase in the number of completed and published genomes available, allowing comparative assembly to become more highly utilized. Comparative assembly is also more commonly used to identify mutations within the nucleotide sequence of an organism with a known genome sequence or to assist in the genome reconstruction of an organism that is a close relative to one that has a published genome.

De novo assembly is the process of reconstructing a genome from the reads directly and does not involve the use of a reference. There are several approaches to *de novo* assembly including Greedy Assembler, Overlap-Layout-Consensus (OLC) and De-Bruijn (Figure 9) [108-111]. The Greedy assembly is one of the earliest and simplest genome assembly methods. The Greedy algorithm begins by joining short sequence reads that have the highest number of matching base pair in their overlap to produce longer contig sequences [108, 109]. Subsequently, the read with next highest scoring overlap is merged into the contig and the process is repeated until there are no more reads/contigs can be joined, producing the genome sequence [109, 110]. Genome assemblers that use the Greedy algorithm includes TIGER [112] and VCAKE [113]. The OLC approach is widely applied to sequence reads generated from Sanger Sequencing and is comprised of three main steps: (1) The overlap step involves the process of determining overlaps

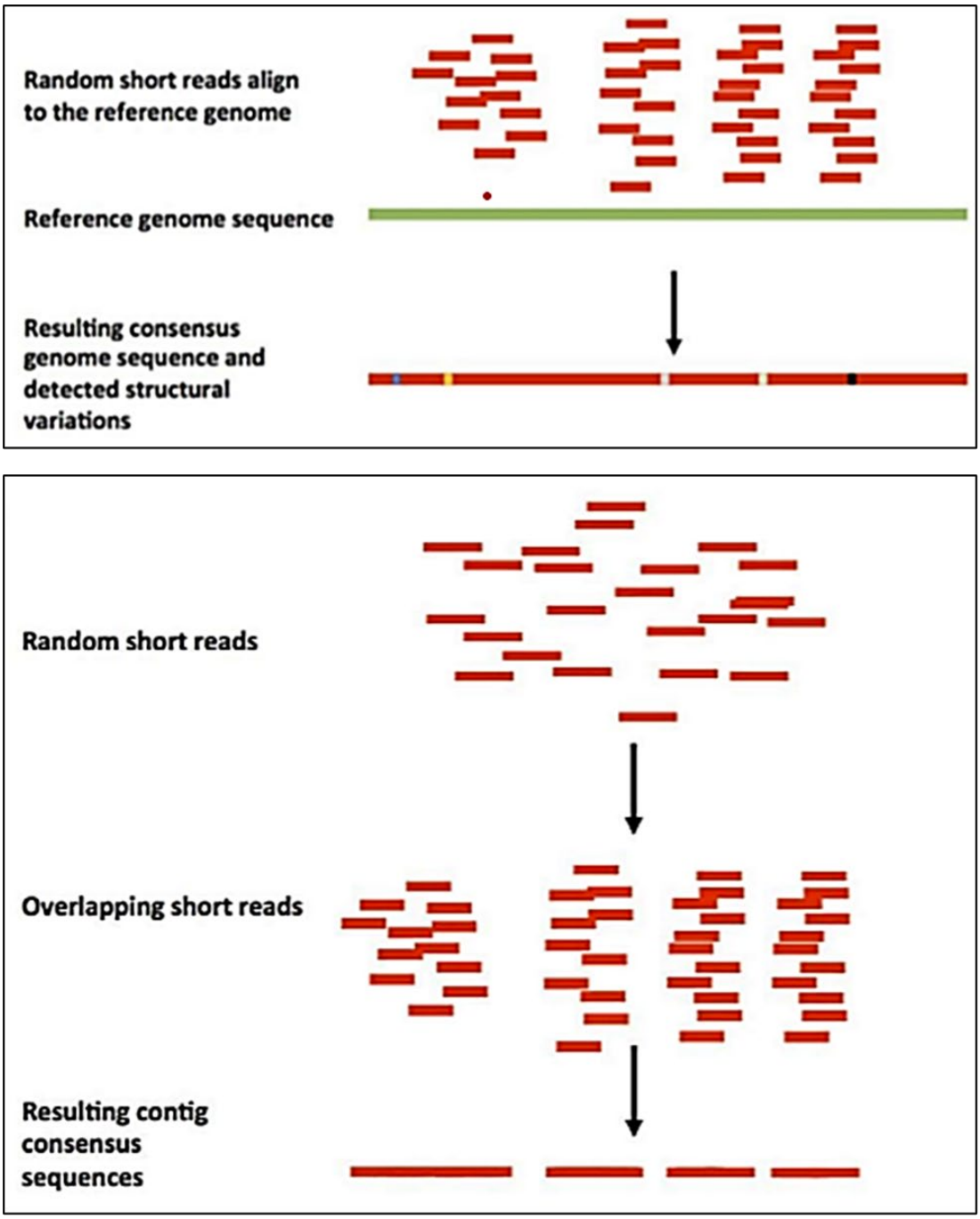

Figure 8. Approaches to Genome Assembly. The schematic depicts the process of comparative genome assembly (top panel) and *de novo* genomes assembly (bottom panel). This figure was adapted from [107].

among all sequencing reads by using software that performs pairwise read comparison. (2) In the layout step, an approximate layout graph of the reads is produced using overlap information results of the first step. (3) Finally, the consensus step employs multiple sequence alignments of reads to produce a precise layout and extrapolate a consensus sequence that is likely the sequence of the genome [109-111]. An example of an OLC genome assembler is Celera Assembler, which was used to assemble the human genome [102, 114]. The De-Bruijn graph assembler is widely applied to genome assembly using short sequences generated from second generation sequencing technology, such as the Illumina platform, and takes advantage of a k-mer approach. Short sequence reads are chopped into shorter overlapping segment and then organized into a de Bruijm graph based on the relationship between neighboring k-mers [110, 111]. Afterwards, erroneous sequences are removed and a global overlap graph, called a Eulerian path, is generated to which the contigs are determined [108, 115]. Examples of assemblers that utilize the de-Bruijn paradigm are ABySS [116], ALLPATHS [117] and SPADES [118].

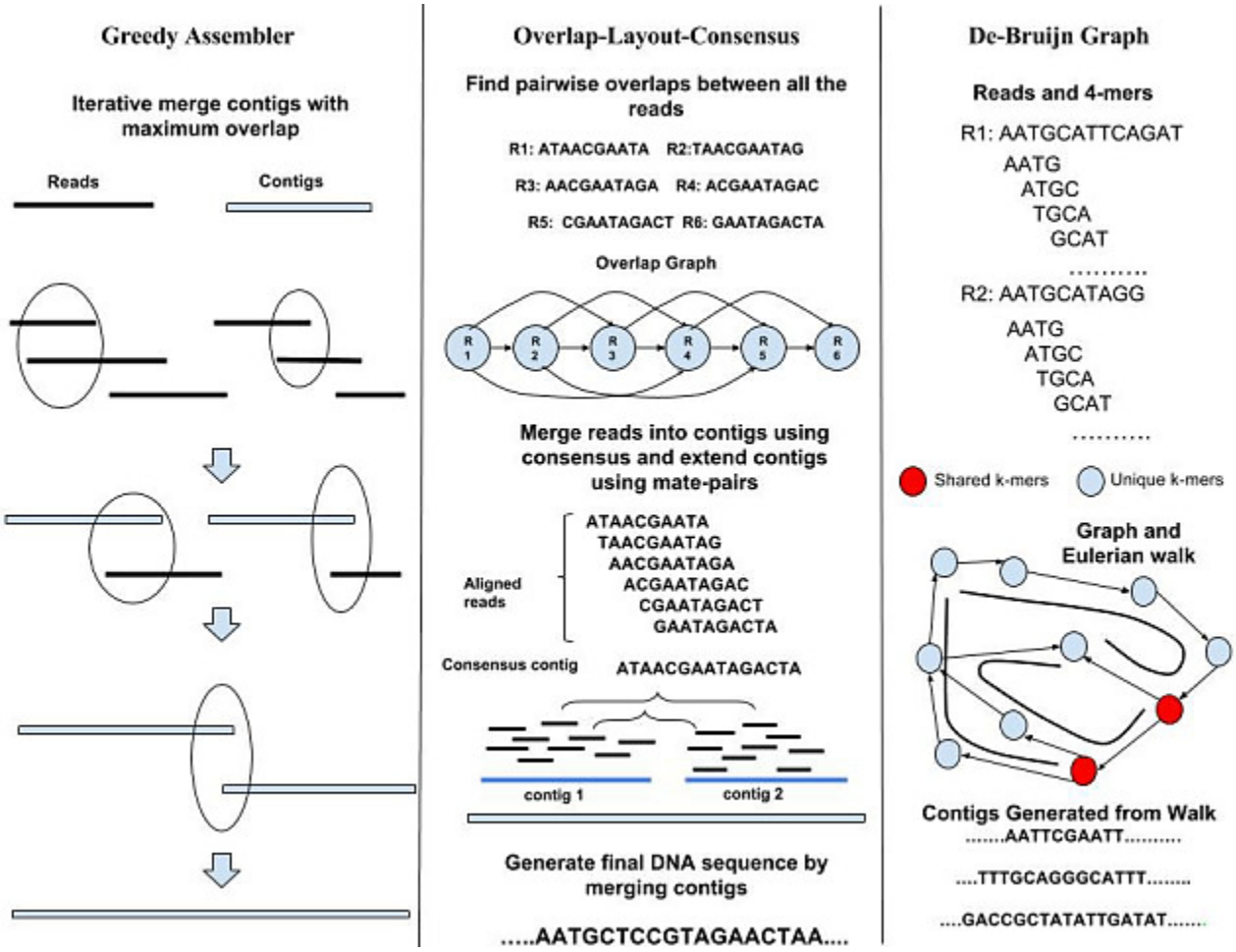

Figure 9. Approaches for *De Novo* Sequencing. These three schematics depict the process and comparison of greedy assembly (left panel), overlap-layout-consensus (middle panel) and de-Bruijn graph (right panel). This figure was reproduced from [110].

CHAPTER II

HORIZONTAL GENE TRANSFER OF PKA PLASMID FROM GENETICALLY ENGINEERED *THERMOSYNECHOCOCCUS ELONGATUS* BP1 TO WILD-TYPE *E. COLI* DH5α

PREFACE

The content in this chapter is reprinted with permissions from "Nguyen TH, Barnes CL, Agola JP, Sherazi S, Greene LH, and Lee JW. Demonstration of Horizontal Gene Transfer from Genetically Engineered *Thermosynechococcus elongatus* BP1 to Wild-Type *E. coli* DH5α. Gene. 2019; 704:49-58". Reported is a modified version of the published manuscript. Permission is provided in Appendix H.

OVERVIEW

In recent years, genetically engineered (GE) cyanobacteria have become a promising and attractive platform for renewable energy as they have to potential to produce valuable products. Biofuel production by photosynthetic organisms has many advantages as they use raw, abundant materials (ie. sunlight, water and CO_2), reduces the competition of land/crops for fuel production or food supply, and has the potential to help alleviate the increase of CO_2 in the atmosphere by being considered net neutral as they use dissolved inorganic carbon to drive their energy production [37, 119, 120]. Photosynthetic production of various biofuels within GE cyanobacteria can be accomplished by inserting a series of genes that will enable the direct conversion of photosynthetic metabolic intermediates to products such as ethanol and butanol [121-123]. The synthetic biology approach with cyanobacteria will help address the major

concerns of fossil fuels, including their inevitable depletion and contribution to the increasing levels of greenhouse gases (ie. CO_2) in the Earth's atmosphere [55, 124-130].

While the photosynthetic biofuel production approach is encouraging, one main concern is the ability of GE cyanobacteria to transfer its modified genes to other bacteria through horizontal gene transfer (HGT). HGT is a natural process where genetic information is transferred between organisms that is not its offspring of the same or different species and is used as a mechanism of adaptation and evolution [131, 132]. If GE organisms were to escape laboratory containment and reach the external environment, there could be a possibility wild-type bacteria acquire transgenes through HGT, such as those designed for biofuel production or even antibiotic resistance, which is widely used for selective transformation for bacteria and could be cause health concerns [67, 68, 133]. In this study, we investigate the potential for horizontal gene transfer between GE *T. elongatus* BP1 and wild-type *E. coli* DH5α. This is accomplished by co-incubating BP1 and *E. coli* and monitoring the HGT of the antibiotic resistance of the pUC57-pKA plasmid, which also contains genes associated with biofuel production (Figure 10).

MATERIALS AND METHODS

Constructing pUC57- pKA Plasmid

The original pUC57-pKB (pKB) plasmid was designed by Dr. James W. Lee and synthesized in collaboration with GeneScript for an earlier Lee lab project. The pKB plasmid included a modified alpha-ketoisovalerate decarboxylase (KIVD) gene (NCBI_AAS49166.1) from *Lactococus lactis* and a butanol dehydrogenase (BDH) gene (NCBI_BA000016.3) from *Clostrium perfringens* followed by a rubisco rbcS 3' UTR terminator from *T. elongatus* BP1.

The pKB plasmid also included a heat-tolerant kanamycin (kan) resistance gene as a selection marker under the control of the the *cpc* gene continuous promoter of *T. elongatus* BP1 and the *slpA* gene promoter of *Thermus thermophilus*. The pUC57-pKA plasmid is similar to the pUC57-pKB except it contains a new synthetic gene encoding for the NADH-dependent alcohol dehydrogenase (ADH) from butanol dehydrogenase instead of the butanol dehydrogenase gene. To achieve the pUC57-pKA plasmid, the pKB-vector DNA fragment and ADH-insert was amplified by PCR resulting in 7.8 kb and 1.1 kb bands respectively, including the ClaI and SbfI restriction sites. The pKA-vector and ADH-insert were subsequently isolated from the gel using Invitrogen PureLink® Gel Extraction kit, digested with ClaI and SbfI restriction enzymes and ligated together to form the pUC57-pKA plasmid (Figure 10). The new plasmid was then cloned into *E. coli* cells for amplification and extracted using the Promega PureYield Plasmid Miniprep kit. PCR was then used to verify correct ligation of the DNA fragments. The work in this dissertation falls under the IBC# 14-002 and #19-002.

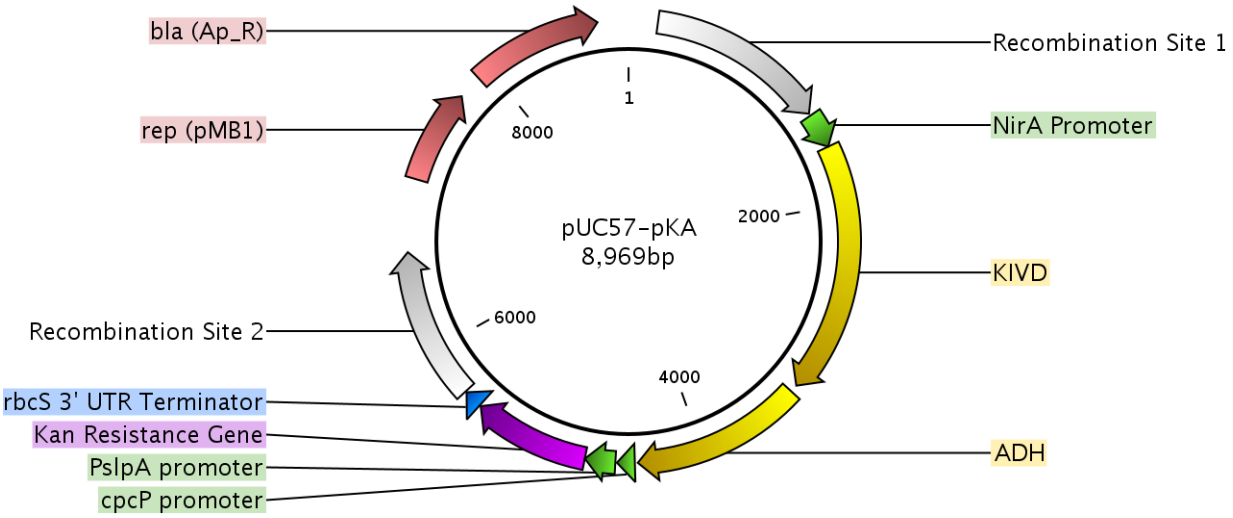

Figure 10. Plasmid Map of the pUC57-pKA Plasmid. Depicted in CLC Genomics Workbench software including the upstream and downstream recombination sites (white), inducible and continuous promotors (green), biofuel producing genes (yellow), antibiotic selection marker (purple) and terminator (blue). This figure was adapted from [134].

Transformation of *Thermosynechococcus elongatus* BP1

Transformation of the pUC57-pKA plasmid into *T. elongatus* BP1 was achieved by electroporation. Wild-type *T. elongatus* BP1 cell were collected by centrifugation and resuspended in water to reach an optical density (OD_{730}) of 20. A 400 µL sample of wild-type *T. elongatus* BP1 and 1 µg of pUC57-pKA plasmid were mixed in an electroporation cuvette. Electroporation conditions using BioRad PowerPad Basic were at 5kV/cm, 200 Ω and 25 µF. After pulsing, cells were transferred to 5 mL of pre-warmed BG-11_0SA media and incubated at 45 °C for 24 hours. Transformed cells are then spread on BG-11_0SA agar media containing kanamycin (40 µg/mL). After 10-14 days of photoautotrophic incubation, colonies containing the kan resistance gene formed on the plates as green colonies. Individual colonies were streaked on

fresh BG-11_0SA plates as well as grown in BG-11_0SA liquid with kanamycin and verified via PCR. The recombination sites within the transgene cassette are to assist in homologous recombination that will integrate the genetic material (regulatory sequences and genes) between these sites into the chromosome of *T. elongatus* BP1, extending this region from 2.4 kb to 6.7 kb (Figure 11).

Horizontal Gene Transfer Study

The stock culture of *T. elongatus* BP1-pKA transformant were photoautotrophically grown at 45 °C in the Percival environmental chamber in BG-11_0SA media containing kanamycin (40 µg/mL) and wild-type *E. coli* DH5α were grown at 37 °C in a shaking incubator in LB broth. *T. elongatus* BP1-pKA cells were pelleted by centrifugation, the supernatant was discarded, and cell were resuspended in fresh BG-11_0SA media without antibiotics. Both *T. elongatus* BP1-pKA and wild-type *E. coli* cells were resuspended in their respective media to a final concentration to 10^7 cells/mL. To prepare the coculture, 15 mL of *T. elongatus* BP1-pKA cell suspension in BG-11_0SA was mixed with 15 mL of wild-type *E. coli* DH5α in LB for a total volume of 30 mL. The co-cultures were incubated in a shaker at 37 °C under continuous light source.

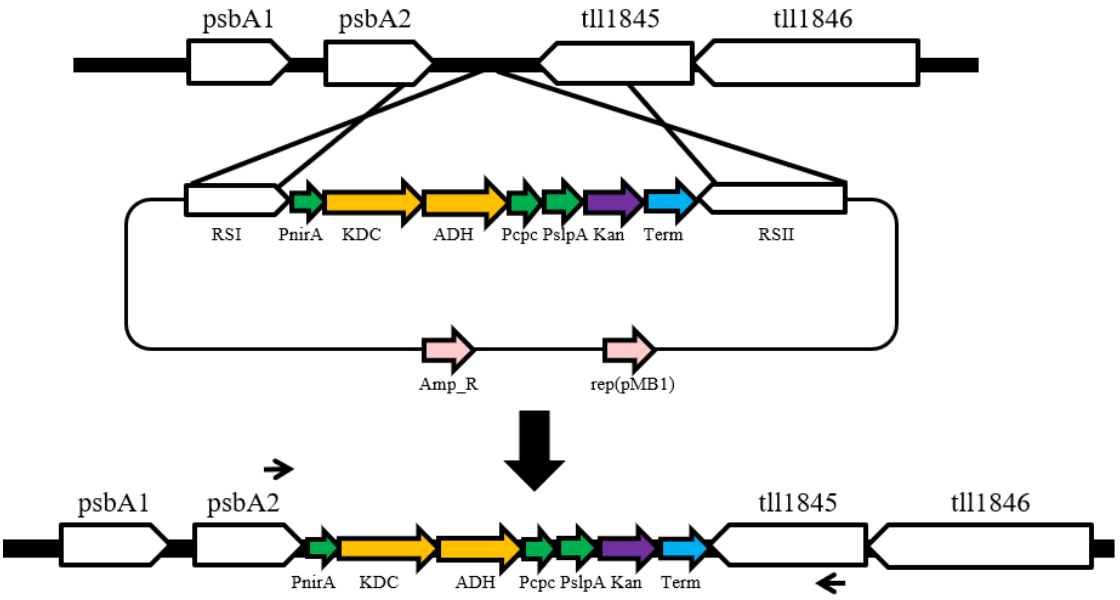

Figure 11. Integration of pUC57-pKA Transgenes into Genome of *T. elongatus* BP1. Schematic of homologous recombination between the pUC57-pKA plasmid and the *T. elongatus* BP1 genome. Primers (black arrows) to amplify from the beginning of the upstream recombination site to the end of the downstream recombination site reveal two distinct sizes: the natural wild-type genome of *T. elongatus* BP1 results in a 2.4 kb size and extends the region to 6.7 kb if the gene cassette is integrated. This figure was adapted from [134].

Two control cultures were also prepared for this study. Control 1 or the "*E. coli* Only" control consisted of 15 mL of wild-type *E. coli* DH5α and 15 mL of BG-11$_0$SA media without *T. elongatus* BP1-pKA cells. Control 2 or the "BP1 Only" control consisted of 15 mL of *T. elongatus* BP1-pKA and 15 mL of LB broth without wild-type *E. coli* DH5α cells. The controls were also incubated in a shaker at 37 °C simultaneously with the co-cultures. After 48 hours of incubation, a 100 µL sample from each liquid co-culture is collected, spread on LB agar plates containing kanamycin and placed in the incubator at 37 °C and observed for colony formation.

The resulting colony formation on LB agar plates containing kanamycin suggests that the kan resistance gene was horizontally transferred from GE *T. elongatus* BP1-pKA. Images of the flask preparation are shown in Figure 12.

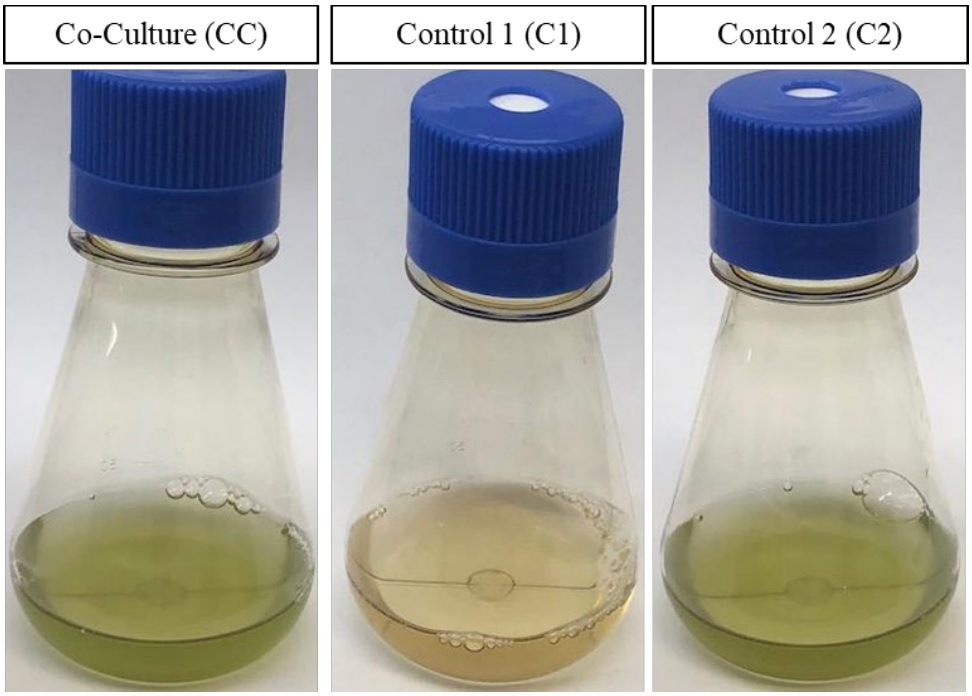

Figure 12. Flask Preparation for Horizontal Gene Transfer Study. The flasks were prepared as followed: The co-culture (CC) contains both *E. coli* and GE *T. elongatus* BP1-pK cells, the control 1 (C1) is *E. coli* DH5α only and the control 2 (C2) is GE *T. elongatus* BP1-pKA cell only. All cultures were prepared in a 1:1 ratio of BG-11_0SA and LB media in a 30 mL volume.

To further verify the presence of the of the pUC-57 transgenes within *E. coli*, PCR was performed using total DNA and plasmid DNA isolated from *E. coli* colonies on the LB agar plates containing kanamycin. Primers were used to amplify a 1.1 kb band that includes the *splA*

and *cpc* continuous promoters with the kan resistance gene and a 4.0 kb band that includes kan resistance gene to the ampicillin resistance gene on the pUC-57 vector (Figure 13). The PCR amplicons were separated on a 1% agarose gel by electrophoresis and visualized.

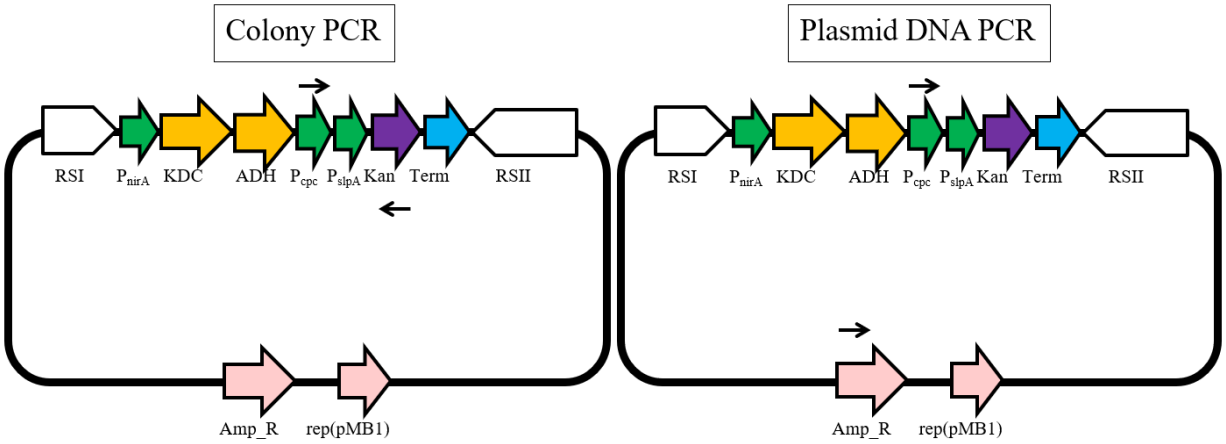

Figure 13. Schematic of PCR to Verify Horizontal Gene Transfer. Total DNA was used to amplify the kan resistance gene and plasmid DNA was used to amplify from the designer cassette to part of the pUC57-vector. Primers are indicated by the black arrow. This figure was adapted from [134].

E. coli DH5α and pUC57-pKA Control Study

To ensure that the transfer of the plasmid was from GE *T. elongatus* BP1 and was not a result of *E. coli* DH5α naturally taking the plasmid from the liquid media, a control experiment was carried out as followed. 15 mL of wild-type *E. coli* DH5α cells in LB at the concentration of 10^7cells/mL was added into 15 mL of BG-11_0SA medium without GE *T. elongatus* BP1, followed by the addition of a known amount of pKA plasmid DNA (10 ng, 100 ng, 1000 ng).

The cultures were incubated at 37 °C under continuous photosynthetic light intensity of about 8 μE m^{-2} s^{-1} for 4 days. The cells were then plated on to LB agar control plates and LB agar plates with kanamycin. These plates were then incubated at 37 °C. This experiment was plated in triplicates.

RESULTS AND DISCUSSION

The pUC57-pKA Transgene Cassette Successfully Integrated *T. elongatus* BP1 Genome

The first aim of this project was to generate GE T. elongatus BP1 that has successfully integrated the transgene cassette within their chromosome. After transforming the pUC57-pKA into *T. elongatus* BP1, green colonies began to grow on the plate (Figure 14a). These colonies were re-streaked onto fresh BG-11_0SA agar plates and grown in liquid BG-11_0SA media. To verify integration of the transgene cassette into *T. elongatus* BP1 chromosome, genomic DNA was extracted and PCR primers for within the upstream recombination site to a position on the chromosome outside the downstream recombination site were used to amplify the insertion site. The GE *T. elongatus* BP1-pKA showed two bands: a smaller size at 2.4 kb band and a large size 6.7 kb band (Figure 14b). The latter indicates that the transgene cassette indicates integration of the transgene cassette. Because *T. elongatus* BP1 is polyploid, the two bands indicated that there are copies chromosomes that have integrated the transgene cassette while others have not. Plasmid DNA was also extracted from the transformant cells to verify presence of the plasmid. PCR primers from within the kan resistance gene of the transgene cassette to the ampicillin resistance gene of the pUC-57 vector were used and resulted in the amplification of a 4.0 kb band. This confirms that the pUC57-pKA plasmid is present. In summary, the GE *T. elongatus* BP1-pKA was verified and could be used in subsequent studies.

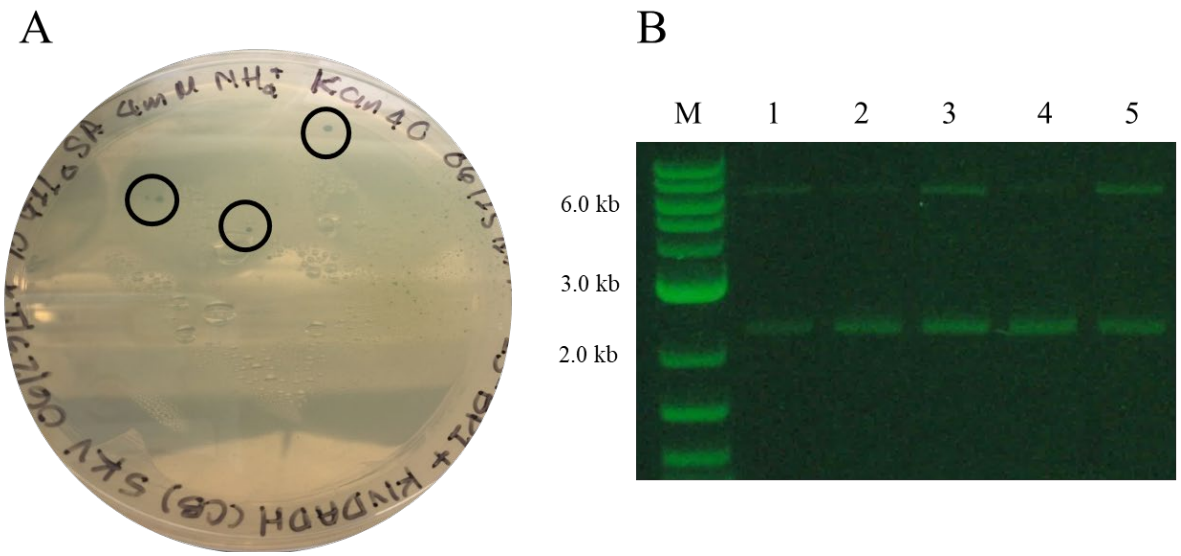

Figure 14. Transformation of *T. elongatus* BP1. (A) Photo of transformant green colonies (circled) on BG-11_0SA solid media with kanamycin after electroporation with the pUC7-pKA plasmid. (B) Colony PCR was performed to verify integration of pKA transgenes in *T. elongatus* BP1. Primers to amplify the insertion site were used resulting in a 6.7 kb or 2.4 kb DNA band indicating chromosomes with integration and no integration, respectively. The lanes are marked as follows: M = molecular weight marker, lanes 1-5 are transformant *T. elongatus* BP1 colonies from BG-11_0SA agar containing kanamycin. This figure was adapted from [134].

Demonstration of Horizontal Gene Transfer to Wild-Type *E. coli* DH5α

The GE *T. elongatus* BP1 formed was used to investigate if and how readily transgenes from cyanobacteria can be horizontally transferred to bacteria. In this aim, the *E. coli* DH5α strain was selected due to its common use in research and *E. coli* has a ubiquitous presence in nature. The experiment involved the co-incubation of wild-type *E. coli* DH5α and GE *T. elongatus* BP1-pKA in a 1:1 ratio in their respective media for a period of time and subsequently plated on LB agar plates with and without kanamycin. The plating from the co-culture flasks showed confluent

growth of LB agar plates without kanamycin and milky colonies appeared on the LB agar plates containing kanamycin (Figure 15b). The presence of these colonies suggests they have received the transgene cassette with the kan resistance gene from the GE *T. elongatus* BP1 cells. These colonies were re-streaked and grown for total and plasmid DNA isolation to verify transfer for the pUC57-pKA plasmid via PCR. The expected band for the continuous promoters and kan resistance gene within the transgene cassette was seen for several colonies while it was not present for wild-type control *E. coli* DH5α (Figure 16a). Another primer pair was designed to amplify a portion of the designer transgene cassette and pUC57 vector and resulted in the expected band for one colony from all those tested (Figure 16b). These results validate the horizontal transfer of the antibiotic resistance gene; however, it may not be present in the pUC57-plasmid form as there was only one positive result for the plasmid DNA PCR.

There were two controls designed to understand how each cell type behaves in the mixed media. Control 1 only contained *E. coli* DH5a and showed growth on LB agar without kanamycin and did not grow on BG-11_0SA control and LB plates with kanamycin 40 µg/mL (Figure 15a). These results imply that the *E. coli* DH5α are still viable after 48 hours of incubation in mixed media containing LB and BG-11_0SA, cannot grow on BG-11_0SA media alone, and the wild-type *E. coli* have not developed a spontaneous mutation allowing cells to grow on LB agar plates containing kanamycin. Control 2 only contained GE *T. elongatus* BP1 and when plated, there was no growth on LB agar plates with and without kanamycin (Figure 15a). These results indicated that the GE *T. elongatus* BP1-pKA stock culture was clean of any contaminant bacteria and further that *T. elongatus* BP1 is not able to form colonies on LB agar media, suggesting that any colonies we observe on the LB agar plates with kanamycin are not those of cyanobacteria. Overall, the results of the control suggest that any colonies seen on LB

agar plates containing kanamycin are the results of horizontal transfer from GE *T. elongatus* BP1-pKA.

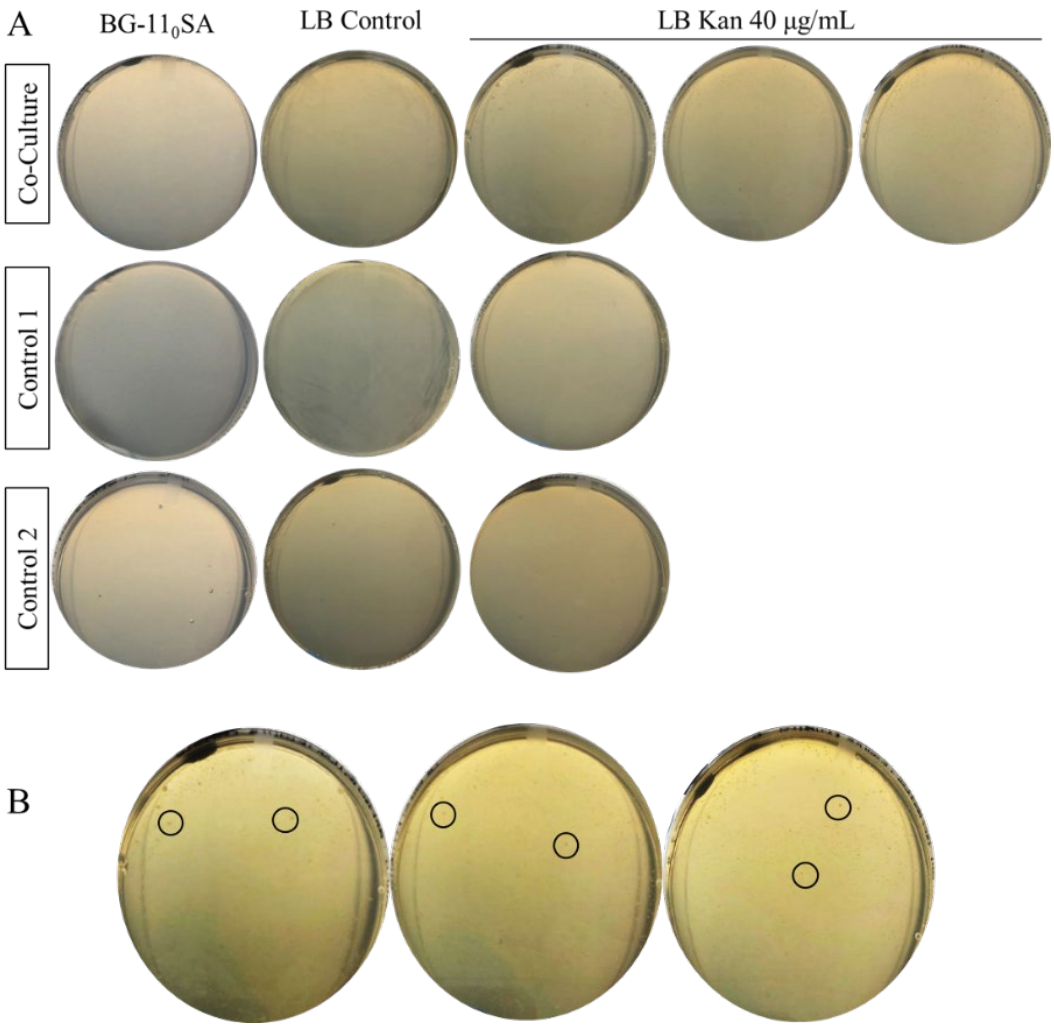

Figure 15. Horizontal Gene Transfer Study Plating. (A) After 2 days of incubation at 37 ℃, a sample of the co-culture, control 1 and control 2 are plated on BG-11$_0$SA control, LB control and LB kanamycin 40 µg/mL solid media. (B) Closer images of *E. coli* DH5α on antibiotic plates. If transfer of the pUC57-pKA to *E. coli* occurs, colonies on the LB kanamycin 40 µg/mL plates (indicated by black circle). This figure was adapted from [134].

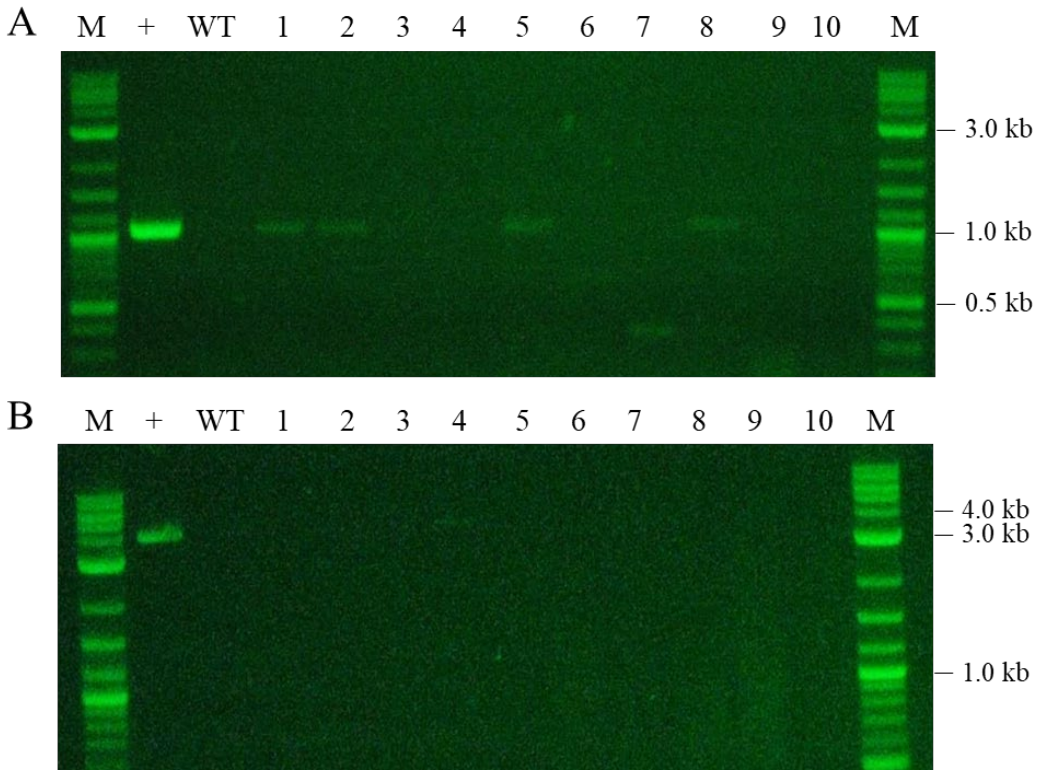

Figure 16. Verification of pUC57-pKA Plasmid Transfer to Wild-Type *E. coli* DH5α. (A) Colony PCR was conducted where total DNA was used to amplify a 1.1 region that includes the kan resistance gene. (B) Plasmid DNA PCR was used to amplify a 4.0 kb region from the *cpc* promoter to the ampicillin resistance gene. The lanes are marked as follows: M = molecular weight marker, + = pKA plasmid, WT = wild-type *E. coli* total or plasmid DNA, lanes 1-10 are selected *E. coli* colonies from LB agar plates containing kanamycin. This figure was adapted from [134].

E. coli DH5α Does Not Uptake pUC57-pKA Plasmid Naturally

While it is known that wild-type *E. coli* DH5α is not naturally competent, in order to ensure that the cassette was indeed horizontally transferred into *E. coli* from *T. elongatus* BP1 transformants, we also carried out a key control experiment to test whether wild-type *E. coli*

DH5α can naturally take up free-floating DNA from the liquid medium. *E. coli* DH5α was incubated with a known amount of pUC57-pKA plasmid using the same media condition and LB:BG-11_0SA medium ratio of the HGT study (Figure 17). The maximum amount used in the controls were 1000 ng of plasmid DNA per 30 mL incubation treatment because this was the amount of plasmid DNA used in electroporating *T. elongatus* BP1. Therefore, in the event of plasmid being carried over, 1000 ng would be the maxium possible quantity of plasmid in the supernatant of co-cultures. The results from plating these *E. coli* cells on LB agar plates with and without kanamycin showed that these *E. coli* cells were only able to grow on LB plates without kanamycin but there were no colonies observed on LB plates containing kanamycin (Figure 18). This indicates that wild-type *E. coli* DH5α cells under our experimental conditions do not have the ability to take in DNA from the media but has to be introduced into *E. coli* via other processes, such as conjugation with the GE *T. elonguatus* BP1 cells.

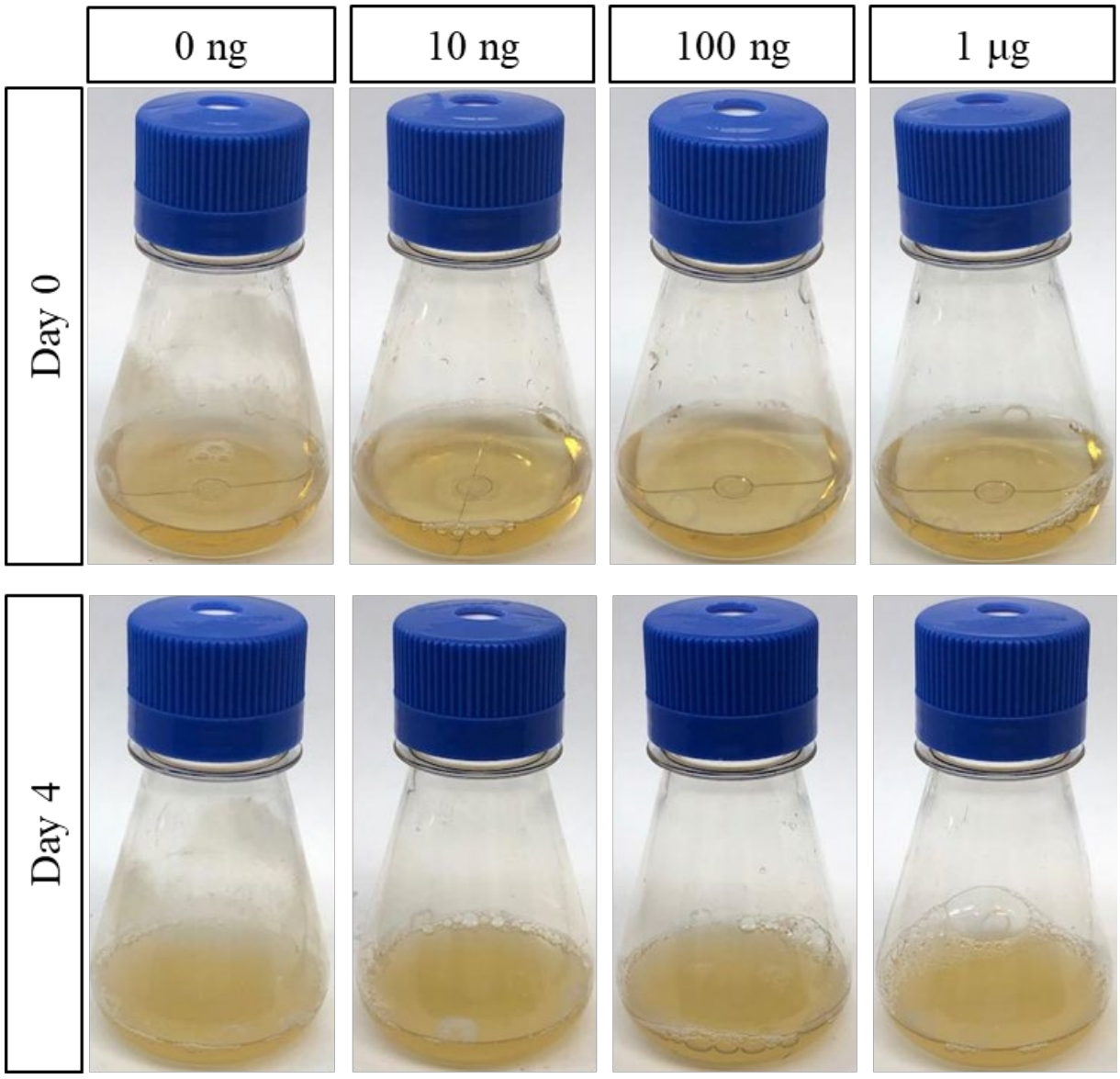

Figure 17. Flask Preparation for Plasmid DNA Control Experiment. *E. coli* DH5α cells were incubated with pUC57-pKA plasmid (0 ng to 1 µg) in 1:1 ratio of BG-11_0SA and LB for 4 days at 37 °C. Shown are the results from Day 0 and Day 4 using increasing plasmid concentrations.

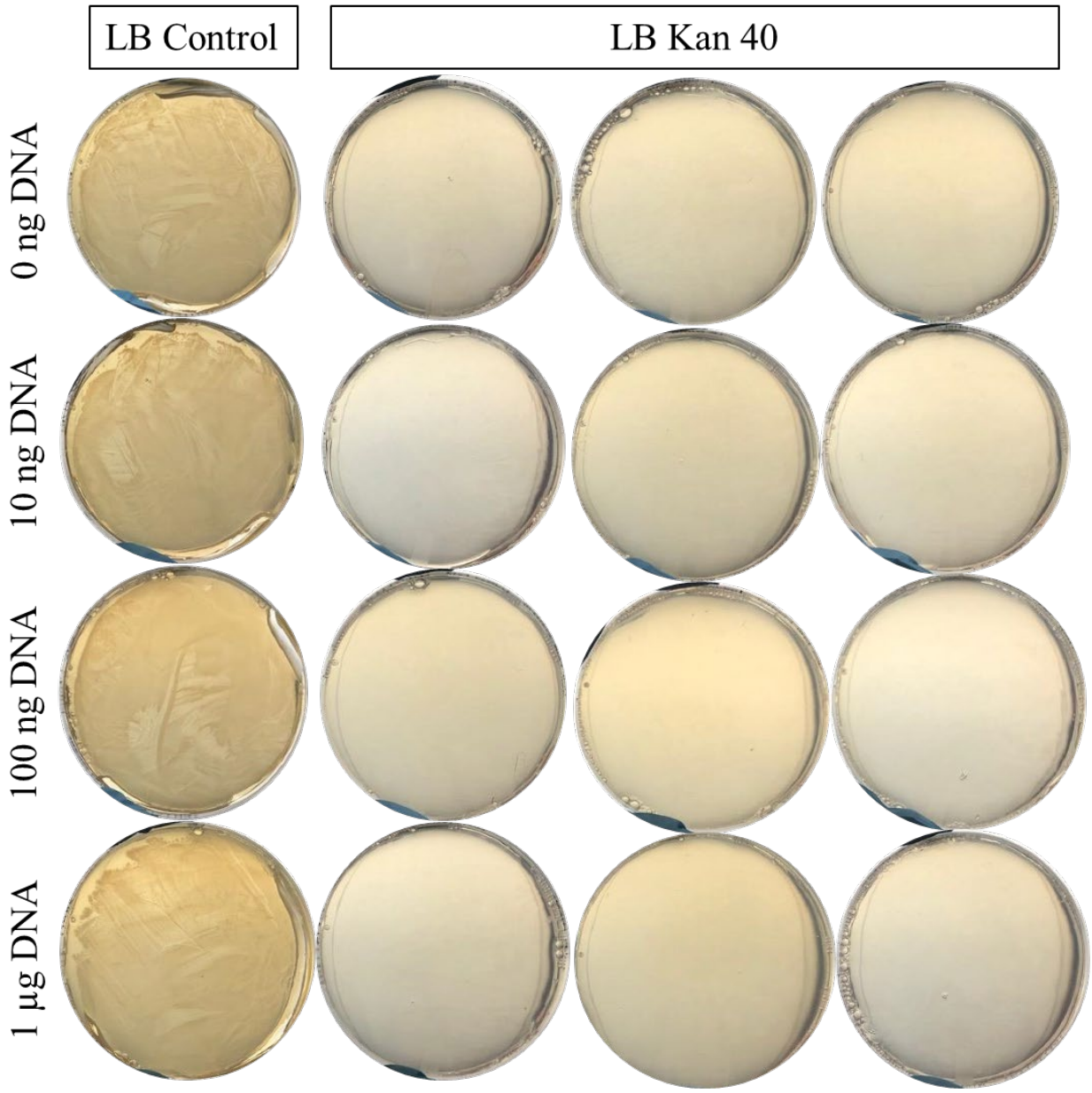

Figure 18. Plasmid Control Experiment Plating. After 4 days of liquid incubation with *E. coli* DH5a and varied amount of plasmid DNA, a sample of the culture was plated on a series of LB control and LB kanamycin 40 µg/mL plates to screen for colonies that have acquired the kan resistance gene.

Conclusion

Horizontal gene transfer of antibiotic resistance genes to bacteria is a major concern regarding human health, as it can lead to the development of multidrug resistant bacteria [135, 136]. As of 2019 *Clostridium difficile* and methicillin-resistant *Staphylococcus aureus* (MRSA) are the top threats according to the Centers of Disease Control and Prevention [137]. *C. difficile* is a gram-positive, spore-forming bacteria known to colonize the large intestines and produce exotoxins proteins, *TcdA* and *TcdB,* that causes severe inflammation and diarrhea in infected individuals [138]. *C. difficile* causes hospital-acquired infection and there were around 15,500 cases of *C. difficile* infections leading to 29,300 deaths in 2011 within the United States [139, 140]. This is a huge health and economic burden, costing patients and hospitals billions of dollars [139, 140]. Recently, several strains of *C. difficile* are beginning to acquire resistance to many antibiotics and has been shown to transfer its antibiotic resistance to other opportunistic pathogens within the intestine through conjugation [141, 142]. *Staphylococcus aureus* is another gram-positive bacterium that is known to be a human health threat and huge economic burden, especially since its developed resistance to methicillin, which also involves horizontal gene transfer [143-145]. These are just a couple of examples contributing to the rise of antibiotic resistance and highlights the importance in combating this issue. In the development of GE organisms, other selection methods besides antibiotic resistance genes should be investigated and utilized, as the use of antibiotic resistance genes has shown to have the potential to horizontally transfer between bacteria and contribute the ongoing antibiotic resistance crisis. Thus, assessing the bio-risk of genetically engineered microorganisms, such as cyanobacteria, is essential for reducing the spread of antibiotic resistance.

At the conclusion of the bio-risk aim in this chapter, which focused on assessing horizontal gene transfer, we were able to genetically transform wild-type *T. elongatus* BP1 with the pUC57-pKA plasmid and verified its presence within *T. elongatus* BP1-pKA in both plasmid form and integrated into its genome. After co-incubation with wild-type *E. coli* DH5α, LB agar plates containing kanamycin resulted in *E. coli* DH5α colonies, suggesting that *E. coli* was able to acquire the pUC57-pKA plasmid via HGT, and was further verified though PCR. Horizontal gene transfer has been shown to occur between *E. coli* and cyanobacteria and was previously reported in literature; however, the direction that the transfer occurs is from *E. coli* to cyanobacteria [146, 147]. To date, there are no other published works that show the horizonal transfer of antibiotic resistance genes from GE cyanobacteria to wild-type *E. coli*. The plasmid DNA control reveals that it cannot uptake plasmid from the media, indicating that HGT is more than likely carried out through conjugation, which involved direct contact between cells [148-150]. Results from total DNA PCR studies showed that many *E. coli* colonies possessed the kan resistance gene, but plasmid DNA PCR only revealed that one colony has the full pUC57-pKA plasmid. Although it is evident that the resistance to kanamycin antibiotic feature of transgene plasmid from GE *T. elongatus* BP1-pKA was horizontally transferred to wild-type *E. coli* DH5α – the fate of these transgenes once transferred to *E. coli* DH5α is not entirely clear as to if it integrates into the genome or remains as a plasmid. Further study is needed to assess potential integration sites within the *E. coli* genome or does it preferentially remain at a plasmid.

This research was part of a larger project involving two plasmids (pUC57-pKA and pUC57-pKB) and published in [134]. Cherrelle Barnes performed all experiments and controls for the pUC57-pKA (pKA) plasmid, which is presented in this dissertation. Thu Nguyen-Jones (graduate student, Dr. James Lee's group at Old Dominion University) performed all the

experiments and controls for the pUC57-pKA (pKB) plasmid, which is presented in her dissertation [151]. As described in the materials and methods, the main difference between these plasmids used is that the pKB plasmid contains a butanol dehydrogenase (BDH) gene from *Clostrium perfringens* with the aim to produce butanol while the pUC57-pKA (pKA) contains an alcohol dehydrogenase from *Thermoplasma volcanium* GSS1. These complimentary studies did not demonstrate any stark differences experimentally and both plasmids resulted in transfer of the antibiotic resistance gene wild-type *E. coli* DH5α from GE *T. elongatus* BP1.

CHAPTER III

SURVIVABILITY OF GENETICALLY ENGINEERED *THERMOSYNECHOCOCCUS ELONGATUS* BP1 IN VARIED TEMPERATURE AND LIGHT CONDITIONS

PREFACE

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OVERVIEW

Biocontainment to limit the potential spread of GE organisms is an important aspect of bio-risk assessment and should be considered if they are to be used for commercial purposes. One method for biocontainment of GE organisms is utilizing synthetic biology approaches, which have been investigated for containment of genetically engineered cyanobacteria have been investigated [152, 153]. Some examples include additional genetic manipulation to nucleases, CO_2-concentrating genes and toxin-antitoxin systems to produce "kill-switches" [154-157]. We propose that biocontainment may be within the natural characteristic the organism itself, by using its natural properties as a growth barrier such as producing alcohol in thermophilic cyanobacterium. This approach would reduce the need for further genetic manipulation, which

could reduce the time, cost and labor of the GE organism. It is also important to study the fitness of the GE cyanobacteria in natural environments in order to tell how such species would behave if exposed to the environment.

T. elongatus BP1 is considered to be a thermophilic strain of cyanobacteria and has exhibited an inability to survive in temperatures below 30 °C [60], and its thermophilic nature may serve as natural biosafety mechanism that could limit the spread of GE cyanobacteria exposed to the environment without additional genetic modifications. Currently, available literature is lacking in knowledge regarding the use of thermophilic cyanobacterium as a natural biocontainment approach. Upon further study to assess the fitness of wild-type as well as GE forms of *T. elongatus* BP1 at select lower temperatures, we aim to understand the behavior of the *T. elongatus* BP1 transformants containing a cassette of foreign genes outside of laboratory containment, as there may be a difference between them. In this study, we evaluate the growth and survivability of a wild-type and GE *T. elongatus* BP1 monitored in the greenhouse of Old Dominion University in Norfolk Virginia during two different periods of the year with different temperature ranges; cool temperatures (15.44 - 25.30 °C) from March 4th, 2019 to April 1st, 2019 and warm temperatures (31.42 - 36.27 ºC) from July 1st, 2019 to July 29th, 2019.

MATERIALS AND METHODS

Growth Study of Wild-Type and GE *T. elongatus* BP1

A set of wild-type *T. elongatus* BP1, wild-type *Synechocystis* PCC 6803 and GE *T. elongatus* BP1-pKA cultures were freshly prepared in triplicates with a cell concentration of 10^{6-7} cells/mL. To prepare the cultures for the cool and warm growth studies, the initial concentration of the cells was determined by cell counting using a microscope and hemocytometer. Upon

determination of the initial concentration in the stock, calculations using $M_1V_1=M_2V_2$ was used to determine the volume of liquid cultures from the stock needed in order to achieve cell concentrations of 5 x 10^6 and 1 x 10^7 cells/mL in a total volume of 75 mL for each culture. One set of cultures is in laboratory conditions, where the light source is constant, and temperature is controlled. For this environment, cultures were placed in the Percival environmental chamber (for the wild-type and GE *T. elongatus* BP1) at ~42.2 °C and in the shaking incubator (for the *Synechocystis* PCC 6803) at 25ºC. Greenhouse conditions are more similar to a natural environment, in that there is a dark/light cycle and varied temperatures. For this environment, cultures were placed in a greenhouse within the Arthur & Phyllis Kaplan Orchid Conservatory at Old Dominion University. The study was in the greenhouse at cool temperatures (March 4th to April 1st, 2019) and warm temperatures (July 1st to July 29th, 2019). The temperature of the greenhouse was monitored using the digital temperature sensor connected to the LinkConn software and the day lengths during the experiments were found at Time and Date website (Figure 19; Table 2). The liquid cultures were incubated in greenhouse or laboratory conditions for a 28-day period. To determine the increase or decrease in cell density, the optical density at 730 nm of the liquid cultures was measured on day 0 and weekly for the duration of the study using the microplate reader.

Survivability Study of Wild-Type and GE *T. elongatus* BP1

Survivability is the ability for cells to recover from the exposure of greenhouse conditions when placed back in laboratory conditions. To prepare the survivability assay, a 0.5 mL sample of wild-type and GE cyanobacteria cultures in greenhouse or laboratory conditions was added to 2.5 mL of fresh BG-11 media in each well of a 24-well Costar plate in duplicates

and placed in the Percival environmental incubator (~42.2 °C). After inoculation in the Percival environmental chamber, the multi-well plates were observed and photographed weekly for a 3-week period to monitor for evidence of growth. The optical density at 730 nm was also measured weekly during incubation in the Percival environmental chamber.

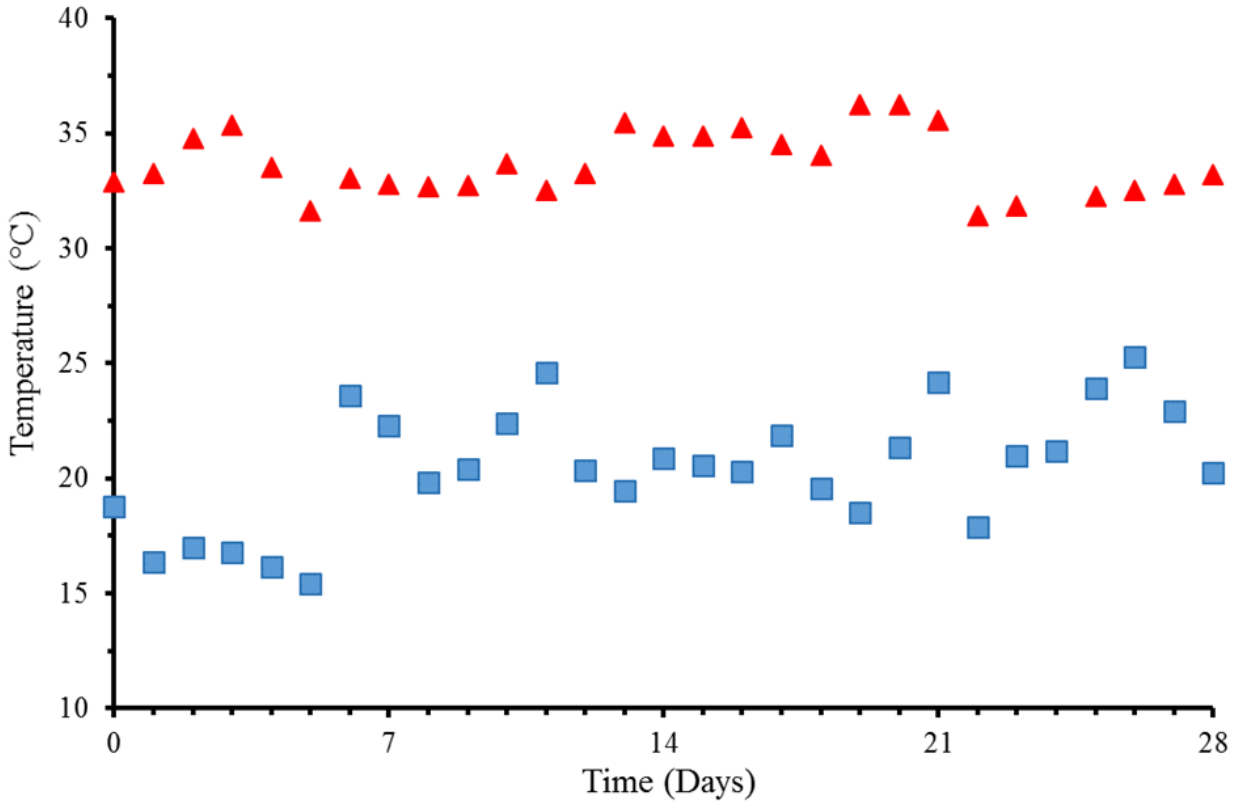


Figure 19. Daily Temperatures During the Survivability Study. The temperatures presented were during the cool (blue square) and warm (red triangle) temperatures in the greenhouse. This figure is reproduced from [158].

	Warm Season Trial	Cool Season Trial
Day length (hrs:min:sec)	From 11:29:58 To 12:35:58	From 14:38:40 To 14:06:32

Table 2. Day Length in Norfolk, VA Retrieved from the Time and Date Website Warm and Cool Temperature Study Period. The day length was retrieved from the website of Time and Date (https://www.timeanddate.com/sun/usa/norfolk) during the period of March 4th, 2019 to April 1st, 2019 and July 1st, 2019 to July 29th, 2019. This table is reproduced from [158].

Colony-Forming Unit (CFU) Determination

In addition to the survivability assay, the number of viable cells in each culture were determined via the colony-forming units. CFUs were measured for the GE *T. elongatus* BP1 liquid cultures; after day 1, week 2 and week 4 of greenhouse exposure, by plating a diluted sample of liquid culture on BG-11 agar plates with and without kanamycin 40µg/ml. The plates were then incubated in the Percival environmental chamber (~42.2 °C) for two weeks for colony formation. The colonies were then counted on each BG-11 agar plate and the colony-forming units were calculated as the viable cells/mL.

RESULTS AND DISCUSSION

***T. elongatus* BP1 Does Not Actively Grow in Temperatures Below 37 °C**

Greenhouse temperatures during the cool season trial ranged from 15 °C to 25 °C and day length varied from 11 hours 29 minutes to 12 hours 35 minutes (Figure 19; Table 2). The optical density (OD_{730}) for GE *T. elongatus* BP1 begins at 0.027 on day 0, slightly increased to 0.033 after one

week and then decreases to 0.016 by the end and from of the 4-week trial. Wild-type *T. elongatus* BP1 shows also shows an increase from 0.090 to 0.145 during the first three weeks and finally decreases to 0.100 at four weeks. The OD_{730} results suggests that there was an overall decrease in the average OD_{730} for both wild-type *T. elongatus* BP1 and GE *T. elongatus* BP1-pKA (Figure 20a). The greenhouse temperatures during the warm season trial ranged from 31 °C to 36 °C and day length varied from 14 hours 6 minutes to 14 hours 38 minutes (Figure 19; Table 2). The optical density (OD_{730}) was measured for GE *T. elongatus* BP1 was 0.093 on day 0 and ended at 0.089 by the end of week 4. Wild-type *T. elongatus* BP1 showed a similar trend, decreasing from 0.147 on day one to 0.120 (Figure 20b). Overall, the average OD_{730} during the warm temperature trial for both wild-type *T. elongatus* BP1 and GE *T. elongatus* BP1-pKA had a slight decrease but remained steady through the study. Although, both seasons had temperatures too low for active growth of *T. elongatus* BP1, the culture density seems to decrease more during the cool season trial than the warm season trial.

Survivability of *T. elongatus* BP1 Varies with Different Temperatures

The survivability assay tested the ability for *T. elongatus* BP1 to recuperate and grow after one, two, three and four weeks in greenhouse conditions during the cool and warm season trials. For the cool season trial, GE *T. elongatus* BP1-pKA was able to recuperate after one but not after two, three and four weeks in greenhouse conditions which is evident from the assay plate images and OD_{730} results (Figure 21). In great contrast, the survivability assay results for the warm temperature trial revealed that GE *T. elongatus* BP1-pKA was able to recuperate after all 4 weeks in greenhouse conditions, indicating they have a higher probability of surviving in the temperature during the warm season trial, even with non-continuous lighting (Figure 22).

Collectively, these results suggests that GE *T. elongatus* BP1 was not able to recover well when subjected to the cool season temperatures. Results for wild-type *T. elongatus* BP1 and *Synechocystis* PCC 6803 are provided in Appendix A and B for the cool and warm season, respectively.

Viability of *Thermosynechococcus elongatus* BP1 During Cool and Warm Season

A dilute sample of the cultures were plated on BG-11 agar plates with and without kanamycin 40µg/ml, placed in Percival environmental chamber for colony formation and the CFU was determined to measure number of viable cells after 1 day and 28 days in greenhouse conditions. The average CFU for GE *T. elongatus* BP1 in the cool season showed that after 1 day in greenhouse conditions, there were up to 6.01 x 10^7 cells/mL that were viable and decreased to 0 cell/mL after 28 days (Figure 23a). These results indicate that there are no viable *T. elongatus* BP1 cells and that these temperature conditions were detrimental to their survival. However, the results for warm season trial showed that there is a decrease in the number of viable cells from 5.54 x 10^7 cells/mL to 4.00 x 10^5 cells/mL (Figure 23b). Although these results show an overall decrease in the number of viable *T. elongatus* BP1 cells, they are not completely dead, and a small population of cells persists after 4 weeks in warmer temperatures.

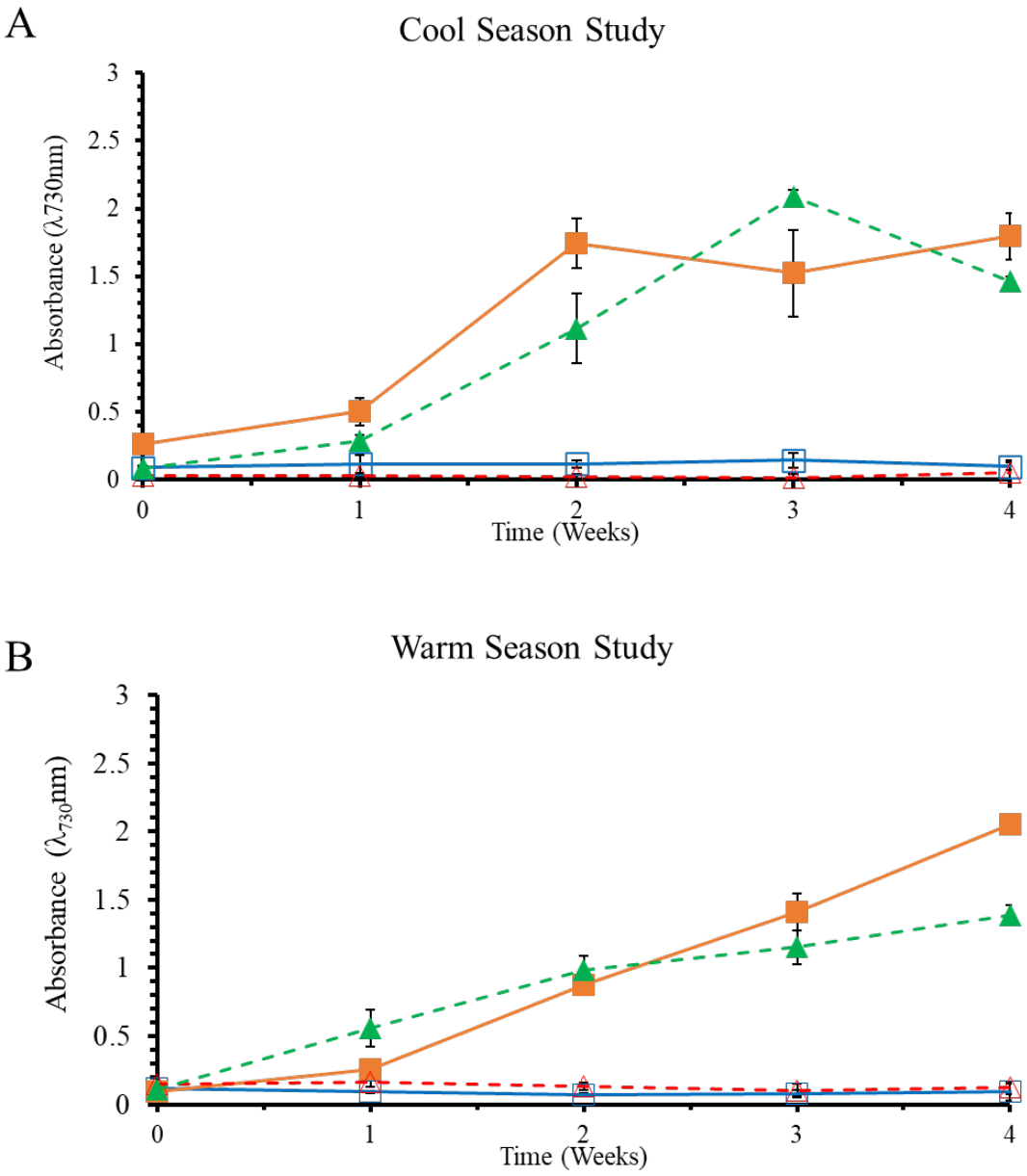


Figure 20. Growth Results for *T. elongatus* BP1 Cultures Starting at 1 x 10^7 cells/mL in Cool and Warm Season Temperatures. The OD_{730} of wild-type (solid line) and GE *T. elongatus* BP1-pKA (dashed line) over a 4-week period in laboratory (closed symbol) and greenhouse (open symbol) conditions. The colors are represented as followed: wild-type *T. elongatus* BP1 in Percival (orange), wild-type *T. elongatus* in greenhouse (blue), GE *T. elongatus* BP1 in Percival (green) and GE *T. elongatus* BP1 in greenhouse (red). This figure is adapted from [158].

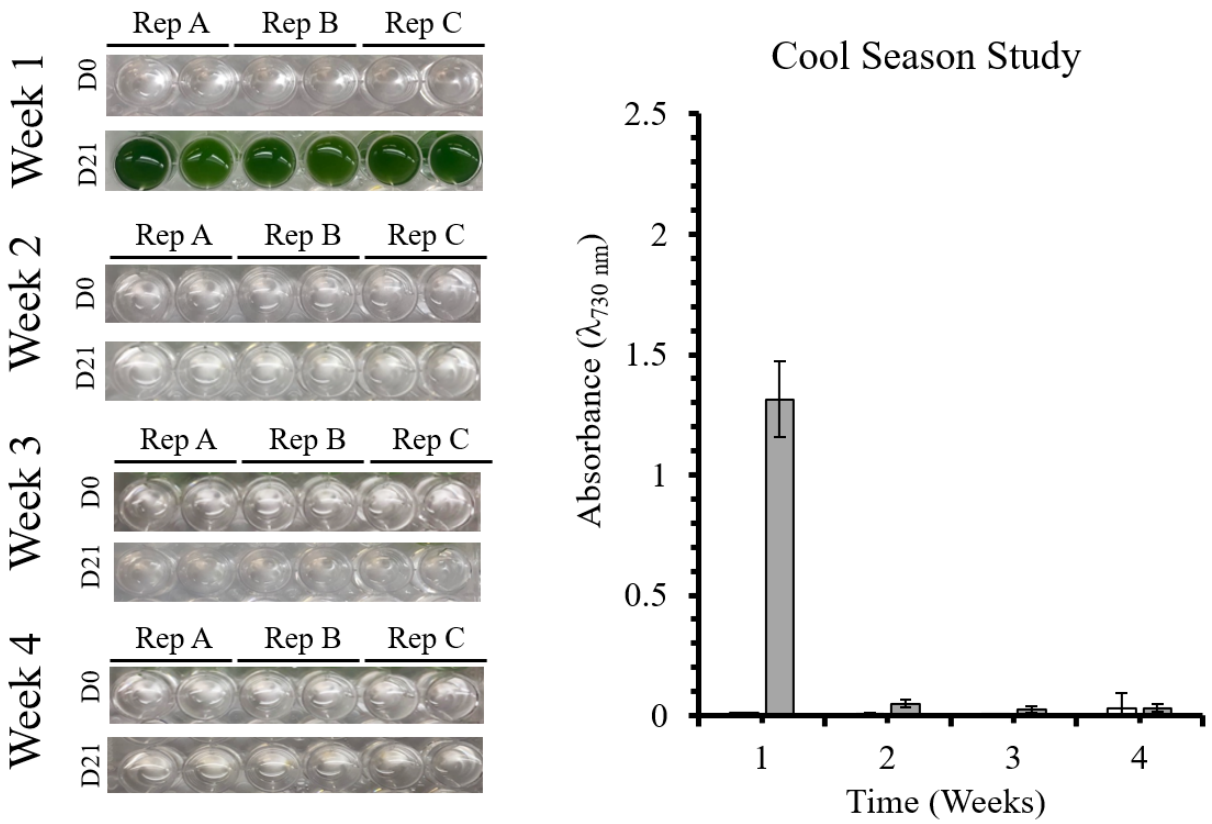


Figure 21. Survivability of *T. elongatus* BP1 Cultures Starting at 1 x 10^7 cells/mL in the Cool Season Study. The survivability assay for GE *T. elongatus* BP1-pKA was performed by adding a sample of culture incubated 1, 2, 3 and 4 weeks in greenhouse conditions to multi-well plates in fresh BG-11 media and incubated in the Percival for 21 days for signs of growth. The OD_{730} of the survivability plates for GE *T. elongatus* BP1-pKA cultures were measured weekly to monitor for growth. The white and gray bars represent the OD_{730} at day 0 and after 21 days of survivability assay was placed in the Percival, respectively. This figure is adapted from [158].

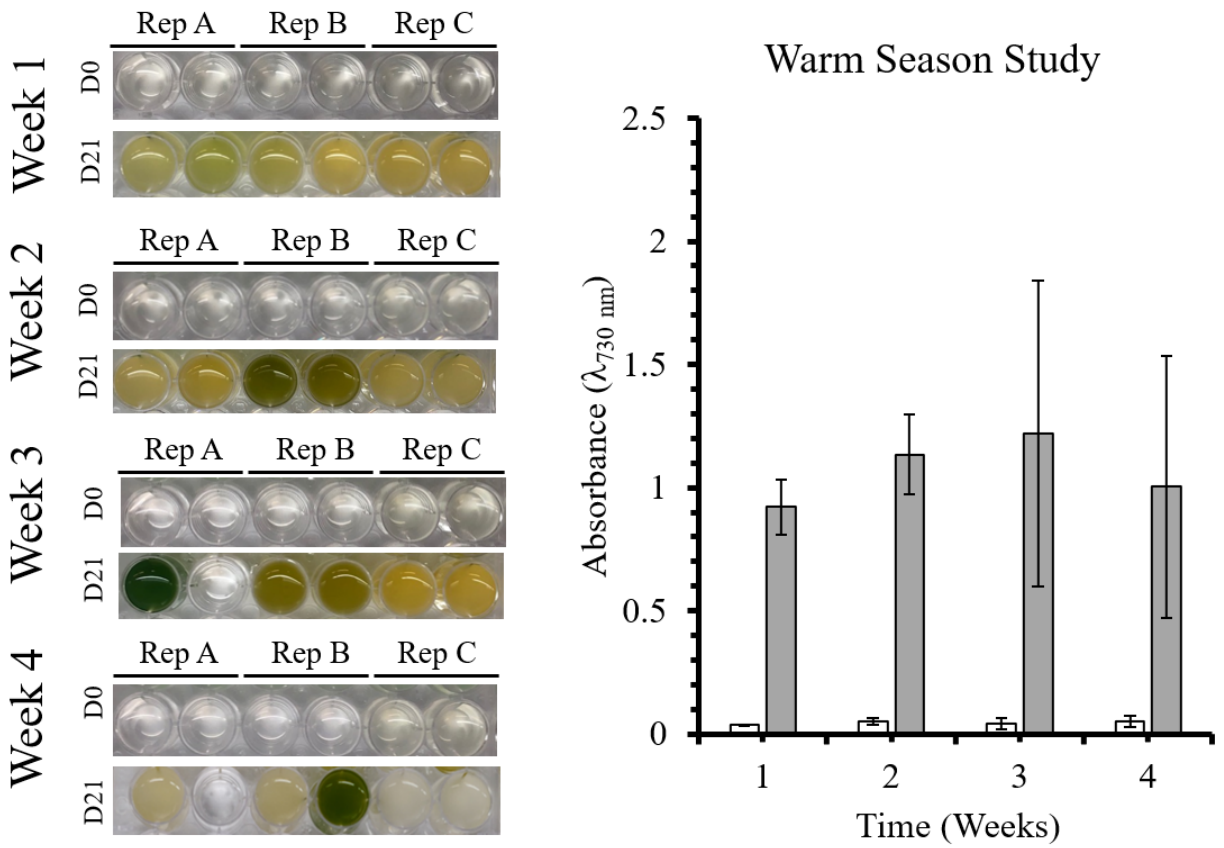


Figure 22. Survivability of *T. elongatus* BP1 Cultures Starting at 1 x 10^7 cells/mL in the Warm Season Study. The survivability assay for GE *T. elongatus* BP1-pKA was performed by adding a sample of culture incubated 1, 2, 3 and 4 weeks in greenhouse conditions to multi-well plates in fresh BG-11 media and incubated in the Percival for 21 days for signs of growth. The OD_{730} of the survivability plates for GE *T. elongatus* BP1-pKA cultures were measured weekly to monitor for growth. The white and gray bars in the graph represent the OD_{730} at day 0 and after 21 days of survivability in the Percival, respectively. This figure is adapted from [158].

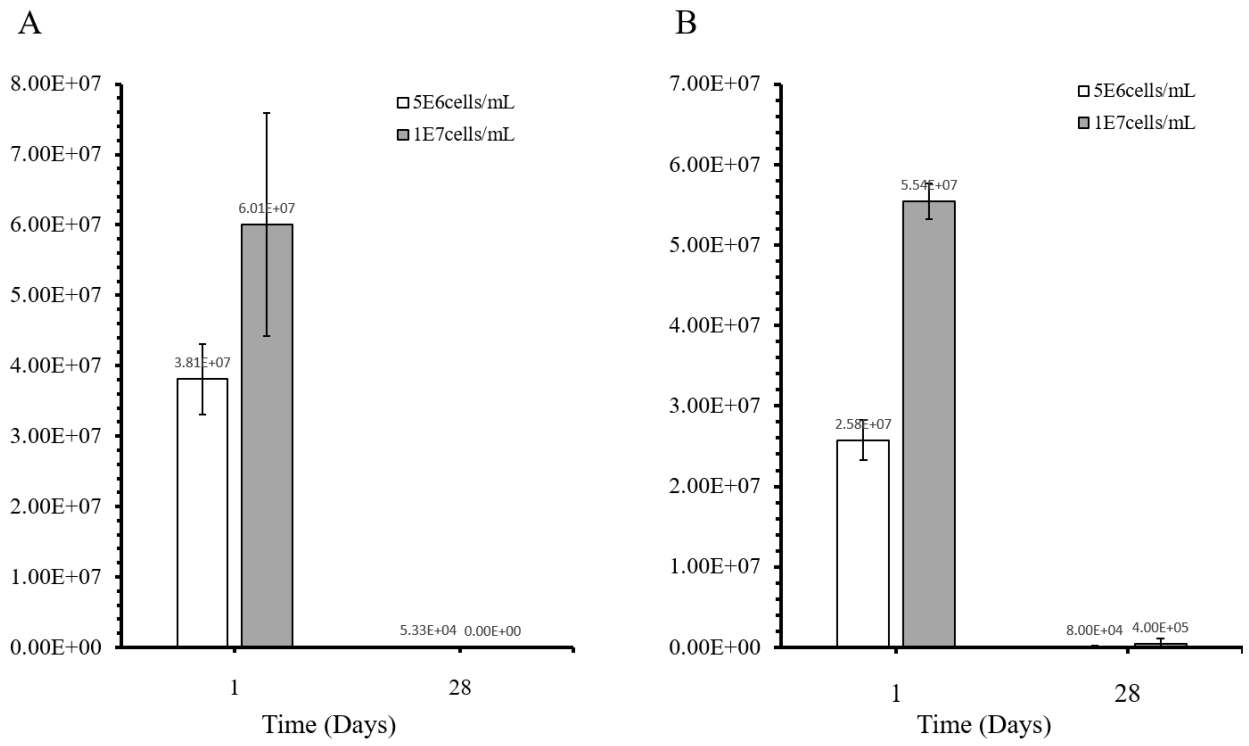


Figure 23. Viability of GE *T. elongatus* BP1-pKA Cultures After Four Weeks in Greenhouse Conditions During Cool and Warm Seasons. A diluted sample from cultures incubating in greenhouse conditions during (A) cool and (B) warm temperatures were plated on BG-11 solid media after 1 and 28 days and placed in the Percival incubator for 2 weeks. The colonies were counted, and the dilution factor applied to calculate concentration of viable cells (cells/mL). The white and gray bars represent cultures that started with 5 x 10^6 cells/mL and 1 x 10^7 cells/mL on day 0, respectively. This figure is adapted from [158].

Conclusion

Biocontainment of GE organisms is important to prevent their survival and persistence in natural environments in an effort to limit potential for bio-risk concern, such as horizontal gene transfer shown in the previous chapter [134]. Current biocontainment strategies explore ways to

target several mechanisms of transgene escape, such as HGT and environmental supplementation [159, 160]. However, additional genetic manipulation could take more time and labor which can increase the cost of developing the biocontainment method. Even more, some organisms have been shown to overcome these killing strategies through mechanisms of spontaneous mutations, HGT and leaky systems, which can still cause the GE organism to be a potential bio-risk in the ecosystem [161, 162].

Temperature can be a very straightforward and viable approach to containment of genetically engineered *T. elongatus* BP1. The growth and CFU results in this study validate the result seen from Yamaoka *et al* that show wild-type *T. elongauts* BP1 does not grow below 30 °C and the growth results were similar for their GE counterparts [60]. Therefore, there was no selective advantage allowing high growth rates due to the introduction of the transgene cassette. The temperatures in the warm season were slightly above this range, and although there were still viable GE cells after the four weeks in the warm season, the number was significantly decreased when compared to one day. These results indicate that the use of organisms that survive extreme conditions, such as thermophiles, could be used as a natural safety-guard to reduce its spread and bio-risk to ecosystem.

This work was completed and published as a co-first authorship with Oumar Sacko of Dr. James Lee's group [158]. Cherrelle Barnes performed all experiments for the pKA transformant, which is displayed in this dissertation. Oumar Sacko performed all the experiments for the BY20 transformant, which is provided in his dissertation [163]. The plasmids that were used to generate the pKA and BY20 transformants used in this study included the pUC57-pKA and the pUC-SP-YFP-ST-R-Lipase, respectively. The YFP-ST-R-Lipase plasmid includes a 1239 bp upstream and 1274 bp downstream recombination sites that promote recombination in a different

location of the *T. elongatus BP1* genome than the pKA plasmid. The genes within the plasmid also greatly differs from the pKA plasmid in that it possesses a yellow fluorescent protein molecular tag, a lipase gene from *Rhizopus oryzae* and a tetracycline resistance gene as a selection marker. The lipase gene in combination with others is intended to product biodiesel from free fatty acid, however there are no additional biofuel producing genes within the gene cassette. The regulatory component of the gene cassette (*nirA*, *cpc* and *slpA* promoters and terminator) is the same between the two plasmids.

When comparing the growth of both GE *T. elongatus* BP1 transformants, they both did not exhibit active growth in greenhouse conditions during the cool and warm seasons. The survivability assay revealed that both transformants were only able to recuperate after one week in greenhouse conditions during the cool season. However, in the warm season the BY20 transformant was only able to recuperate after three weeks in greenhouse condition during the warm season, while the pKA transformant was able to after all four weeks. Finally, there were no viable cells after four weeks during the cool season, which was shown for both pKA and BY20 transformants. During the warm season, there were still a small population of 4.00×10^5 cells/mL and 2.34×10^5 cells/mL for pKA and BY20, respectively. Although there was slight variability in some of the experimental studies, the overall results for both transformants indicate that GE *T. elongatus* BP1 cells do not survive during the cool season temperatures measured in this study and therefore their thermophilic nature could be used as a biocontainment method to limit their spread if released from laboratory containment.

CHAPTER IV

STABILITY OF TRANSGENES IN GENETICALLY ENGINEERED *THERMOSYNECHOCOCCUS ELONGATUS* BP1

OVERVIEW

Transgene stability can be defined as the persistent presence or expression of a foreign gene within a host. For commercial use of GMOs, the goal is for the organisms to possess and express their transgenes for the duration of their life and transgene stability has been studied in many GE organisms including bacteria, plants, and insects [164-166]. Transgene stability within GE organisms can be affected through gene silencing by epigenetic and transcriptional mechanisms or gene loss as a result of crossbreeding [167, 168]. From a bio-safety perspective, a concern about the development of GE organisms is the fate and stability of transgenes within the host, as it could lead to gene escape and allow transgenes to persist in unintended environments [169, 170]. It is important to investigate and understand long-term stability of transgenes, as it could have an impact on the environment. The third research aim focused on assessing the fate and stability of transgenes within genetically engineered cyanobacteria, which is of great concern if they escape containment. It is also of great interest to have a greater understanding of how long transgenes persist in GE organisms to address general mechanisms of microbial genetics.

To investigate the outcome and long-term stability of transgenes inserted into host chromosomes, cultures of cyanobacteria with a cassette of transgenes, which included the kan resistance gene as an antibiotic selection marker, were incubated with and without antibiotic for a two-year study. Monthly, genomic material was extracted from each culture and used to determine the presence and expression of the kanamycin resistance transgene. In the present

work, preliminary studies to investigate mutations that have been acquired over a one-year period was conducted by sequencing and performing a genome comparison analysis of cultures incubated with and without antibiotic pressure. Additionally, exploratory studies are underway to investigate the persistence of these transgenes when certain stressors, such as low nutrients, are applied to the cell cultures. The initial studies are presented in this chapter and further discussed in future work.

MATERIALS AND METHODS

Transgene Stability Study

A cassette of transgenes (Figure 10 and 11) was inserted into the chromosome of GE *T. elongatus* BP1-pKA (refer to chapter 2) and the GE organism was incubated in the presence and absence of antibiotic selection pressure and genetic testing was performed to monitor the inserted transgenes for up to 104 weeks (2 years). To prepare the cultures, GE *T. elongatus* BP1 cells were added to BG-11 liquid media. While one triplicate set of cultures was supplemented with kanamycin 40 μg/mL to represent the presence of selective pressure and will act as a control for this study, the other triplicate set did not contain any antibiotic to represent absence of selective pressure and will act as our experimental cultures. The flasks were then placed in the Percival environmental chamber at ~42 ℃ with continuous light at 30 μE m^{-2} s^{-1}. The cultures were re-inoculated bi-weekly into fresh BG-11 media (3 mL of culture into 75 mL of media), and 2 mL aliquots of each culture were collected and cryopreserved monthly. Two trials of the stability study were conducted: the first trial was for the duration of one year and the second trial has reached 2 years but is still ongoing.

Detection of Transgene Cassette by PCR

Genomic DNA was extracted and purified using the Qiagen QiaAMP DNA Mini Kit and 2 ng was used to amplify three genetic regions of interest: the insertion site, kan resistance gene and rspL gene via PCR (Table 3). For the insertion site, primers were designed to amplify from within the upstream recombination site of designer transgene cassette (homologous to *T. elongatus* BP1) to outside the downstream recombination site on the chromosome, which not part of transgene cassette (Figure 11). *T. elongatus* BP1 possess multiple copies of their chromosome, resulting in some copies that insert the transgenes while possibly others do not. Because of this, there are two expected band sizes for the PCR amplified insert and are located at 2.4 kb and 6.7 kb, which represents no integration and integration of the transgenes, respectively (Figure 24a). The kan resistance and the *rpsL* gene (the 30S ribosomal protein S12) which was used as a house-keeping gene, were also amplified and detectable as 0.7 kb and 0.4 kb bands, respectively (Figure 24b-c). Genomic DNA was tested monthly over the two-year period to monitor for any changes over time.

DNA Fragment	Size	Forward Primer	Reverse Primer
Insertion Site	2409 kb (wild-type) 6705 kb (integrated)	CCTTTGATCCTGTA AAGTTTGACCTCA	CCTCTGCGACACCTA CTACATCCAC
Kan Resistance Gene	762 bp	ATGAATGGCCCAA TAATAATGACTA	TCAAAATGGTATGCG TTTTGACACATC
***rpsL* Gene**	405 bp	ATGCCCACCATCC AGCAA	TTATTTTTTCTTACCG GTCGCAGC
Kan Resistance Gene Region	888 bp	GCAGAGGAAATGC GGCAA	CCCCTAACCCTTCAT TCAAC

Table 3. DNA Fragments Amplified for Genomic DNA PCR and Gene Sequencing. The primers sequences and the size of the amplified area is shown.

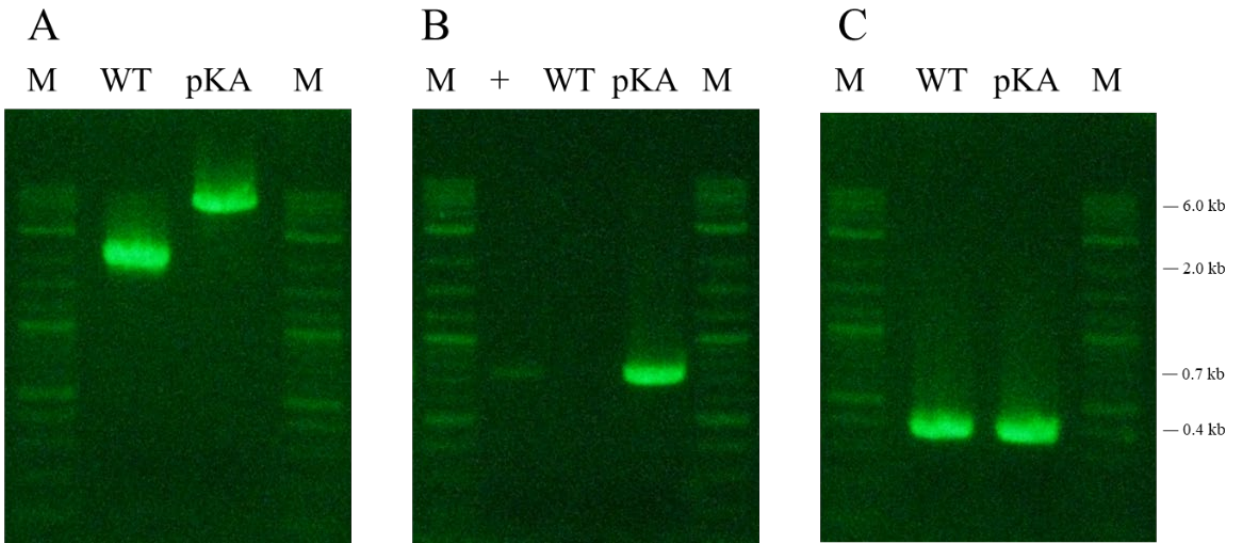


Figure 24. Genomic DNA PCR Results for Wild-Type and GE *T. elongatus* BP1 Stock Cultures. The (A) insert site, (B) kan resistance gene and (C) the *rpsL* gene were amplified and the expected band sizes are 2.4/6.7 kb, 0.7 kb and 0.4 kb respectively. The lanes are marked as follows: M = molecular weight marker, + = pUC57-pKA plasmid, WT = wild-type *T. elongatus* stock culture and pKA = GE *T. elongatus* BP1-pKA stock culture.

Expression of the Kan Resistance Gene

The expression of the kan resistance gene was also monitored monthly during this study by reverse-transcription PCR. Total RNA was extracted using the Invitrogen TRIzol™ Plus RNA Purification Kit and subsequently treated with DNase to rid of any residual genomic DNA. A sample of the RNA is used for PCR to ensure the absence of contaminating DNA and if the agarose gel is free of bands, it is considered safe for RT-PCR (Figure 25a). Reverse transcription takes place where select mRNA transcripts for the kan resistance gene or *rpsL* gene are converted into cDNA with the SuperScript III reverse transcriptase enzyme. The resulting cDNA is then used for conventional PCR, separated on a 1% agarose gel by electrophoresis and

visualized. The kan resistance and *rpsL* gene result in a 0.7 kb and 0.4 kb bands, respectively (Figure 25b-c).

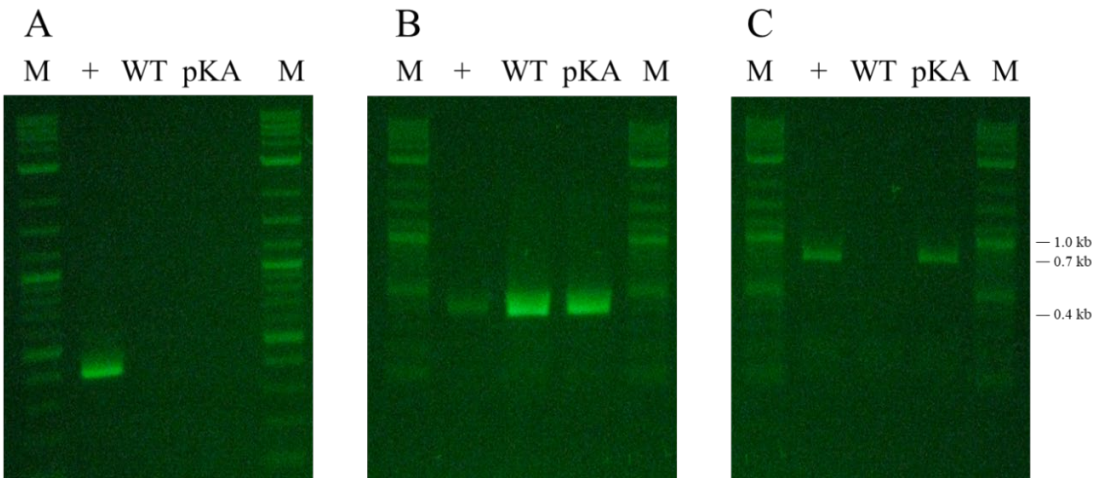


Figure 25. cDNA PCR of Wild-Type and GE *T. elongatus* BP1 Stock Cultures. Shown is the PCR result for (A) the negative control, (B) cDNA for the rspL gene and (C) cDNA for the kan resistance gene. The amplification resulted in no bands for wild-type and GE *T. elongatus* BP1 RNA samples in the negative control, 0.4 kb bands for wild-type and GE *T. elongatus* BP1 RNA samples for the rspL gene and 0.7 kb band for the GE *T. elongatus* BP1 RNA sample for the kan resistance gene. The lanes are marked as follows: M = molecular weight marker, + = pUC57-pKA plasmid, WT = wild-type *T. elongatus* BP1 cultures and pKA = GE *T. elongatus* BP1-pKA cultures.

Gene Sequencing and Comparison Analysis of the Kan Resistance Gene

Genomic DNA samples were obtained from cultures that were grown for in the presence and absence of antibiotic pressure (+/- kan) after one year using the Qiagen QiaAMP DNA Mini

Kit. There were 3 DNA samples prepared for the control (+ kan) and experimental (- kan) cultures. Primers were used to amplify an 888-bp region that included part of the *slpA* promoter, the entire kan resistance gene and part of the *rbcS* 3' UTR terminator (Figure 26a). The PCR product was run on a 1% agarose gel and the 888 bp bands were cut from the gel and subject to DNA extraction using the Invitrogen PureLink® Gel Extraction kit in preparation for sequencing at Eastern Virginia Medical School (EMVS) Molecular Core (Figure 26b). There was a total of 12 DNA sequences obtained, a forward and reverse sequence for each DNA sample, ranging from 211-880 bp in size.

First, the sequences were briefly imported into Sequence Scanner (Life Technologies Corporation) to determine the quality of the reads and determine low or uncalled bases at the 3' and 5' ends of the sequences that will need to be removed from the sequence (Figure 27a). The sequences were subsequently imported into CLC Genomics Workbench 12.0 to trim the ends of each sequence and the trimmed sequences were then assembled to a reference genome of *T. elongatus* BP1 where the transgene cassette from pUC57-pKA plasmid was integrated (refer to Figure 11) to produce a consensus sequence. A consensus sequence for the control (+ kan) and experimental (- kan) cultures was constructed by assembling all three forward and reverse sequences for each culture type (Figure 27b-c). The consensus sequence was furthered trimmed to only represent the protein coding region of the kan resistance gene (762 bp) and the trimmed consensus sequences were aligned to the reference sequence of the kan resistance gene from a. The consensus sequences were also translated to the amino acid sequence using the CLC Genomics Workbench (Ver. 12) and compared the amino acid sequence against the reference.

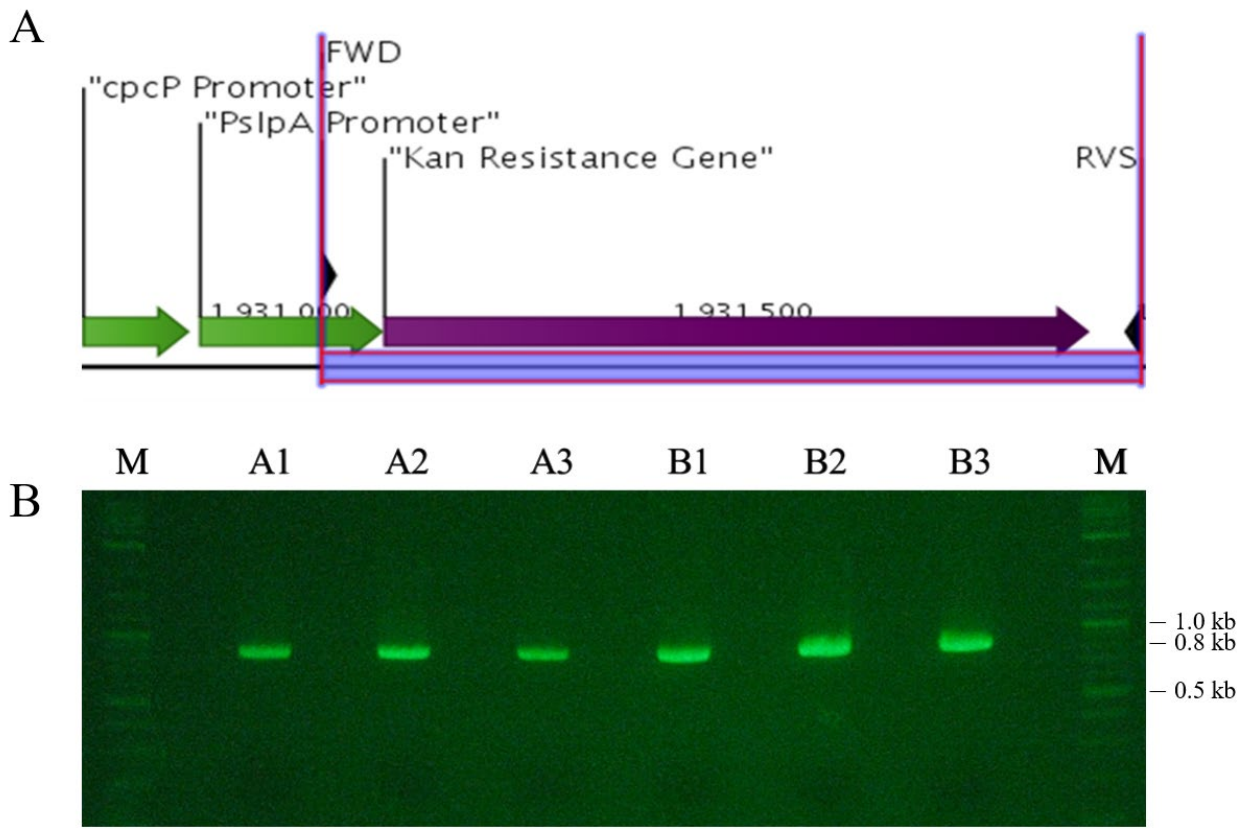


Figure 26. Amplification of Kan Resistance Gene for Gene Sequencing. (A) The schematic shows the primers (black arrows) used to amplify a 888 bp region including the kan resistance gene using genomic DNA from GE *T. elongatus* BP1-pKA at the one-year mark of the stability study. (B) The DNA agarose gel shows the amplification of the kan resistance gene region resulting with expected band between 0.8 and 0.9 kb. The lanes are marked as follows: M = molecular weight marker, A1-A3 = GE *T. elongatus* BP1 control (+ kan) cultures, B1-B3 = GE *T. elongatus* BP1 experimental (- kan) cultures.

A

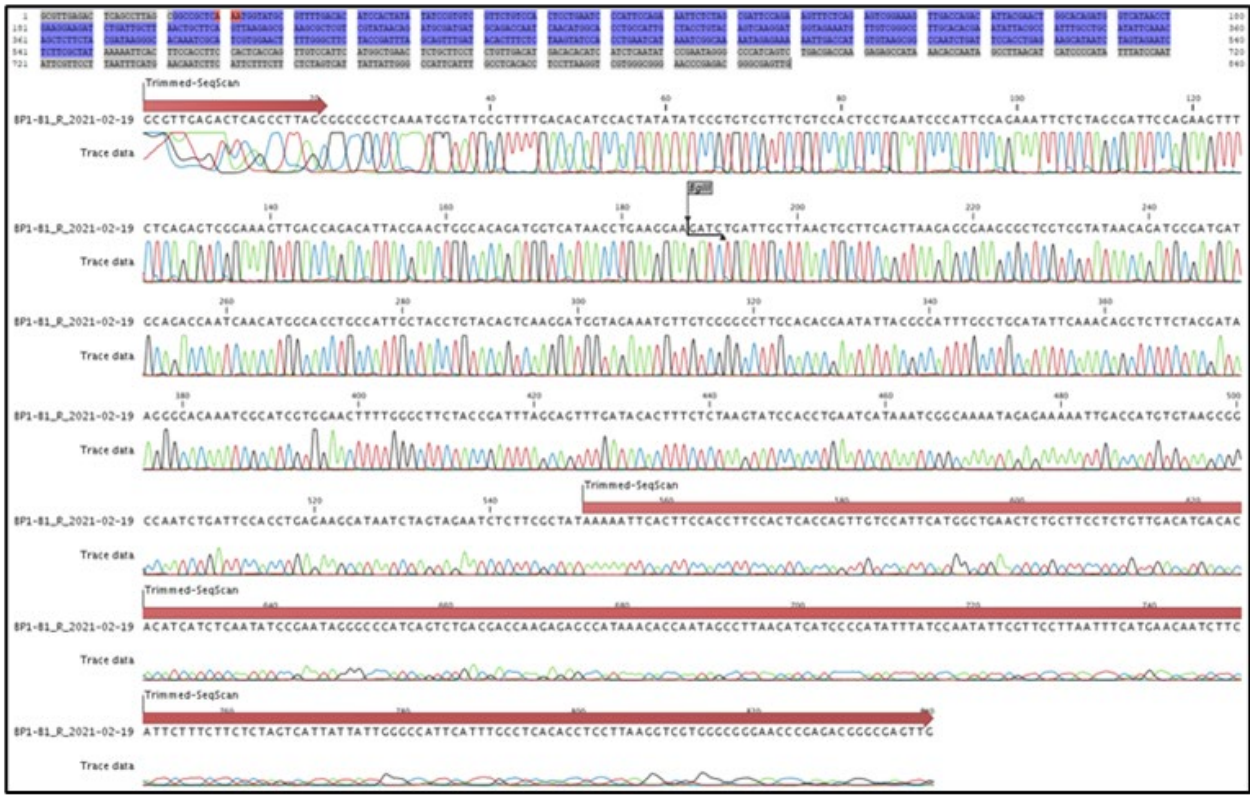


Figure 27. Generation of Control and Experimental Consensus Sequences for the Kan Resistance Gene. (A) Sequences were imported into Sequence Scanner to determine bases with low call quality (gray). These areas were highlighted and trimmed in CLC Genomics Workbench. The trimmed forward (green) and reverse (red) sequences for the control (B) and the experimental (C) DNA samples were assembled using the kan resistance gene sequence from the pUC57-pKA transgene cassette.

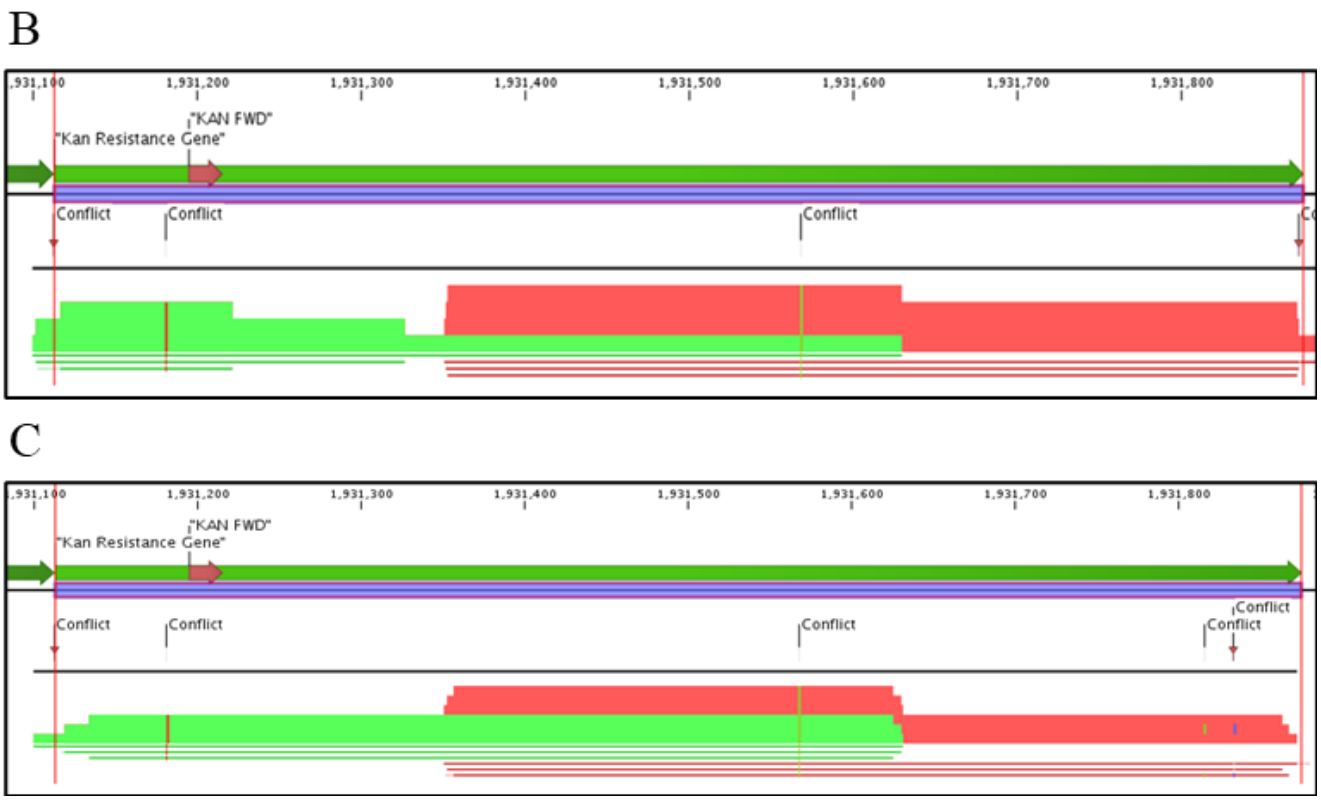


Figure 27 (Continued).

Whole-Genome Sequencing and Comparison of Gene Cassette Sequences

To determine if there were mutations acquired in other components of the transgene cassette, whole-genome sequencing was performed for GE *T. elongatus* BP1 experimental cultures in absence of antibiotic pressure. In preparation for whole-genome sequencing, genomic DNA for three experimental (- kan) cultures at the one-year mark was isolated using the Qiagen QIAamp DNA Mini Kit. Purity and DNA concentration was subsequently assessed using the Nanodrop (required to be ≥ 200 ng of DNA total and 260/280 = 1.8-2.0). WGS was performed by Novogene using Illumina-based technology and produce over 9.5 million (9,524,992) 150-bp raw reads. The assembly process was performed as folo short reads for the experimental (- kan)

sample were assembled using reference genome of *T. elongatus* BP1 where the transgene cassette from pUC57-pKA plasmid was integrated using the CLC Genomic Workbench 12.0. Once the consensus nucleotide sequence was produced, the nucleotide and/or amino acid sequence for several components of the transgene cassette were aligned and compared to the reference sequence for mutations. The components that were analyzed includes the promoters (*nirA*, *cpc* and *slpA*), the kan resistance gene and KIVD gene. The goal is to determine if there are any mutations that could possibly lead to a different protein sequence or point mutations that could disrupts the promoter regulatory sequence and affect the expression of the transgenes.

Exploratory Nutrient Deficiency Transgene Study

Preliminary studies were conducted to determine if the stability of the transgenes can be affected by the absence of certain key nutrients. In this study, *T. elongatus* BP1-pKA cultures were prepared with one of four different BG-11 media types deficient in one of the following sources: iron (-$C_6H_8FeNO_7$; ferric ammonium citrate), carbon (- Na_2CO_3; sodium carbonate) and phosphate (½ K_2HPO_4 and ¼ K_2HPO_4; dipotassium hydrogen sulfate). Wild-type and GE *T. elongatus* BP1 were placed these liquid media with no antibiotics added. The GE cultures were set up in triplicates within their respective medias. The cultures incubated in the Percival environmental chamber at ~42 ℃ and continuous light at 30 μE m^{-2} s^{-1}. The cultures were re-inoculated bi-weekly, and samples were collected and cryopreserved monthly. To determine how these cells grow in different media conditions, OD_{730} was measured for a 14-day period. Like the original transgene study, genomic DNA was isolated and used to amplify the insertion site and the kan resistance gene to determine its presence or absence of the transgenes over time. The pH of the media alone and cultures after 2 weeks of growth were recorded twice during this study.

RESULTS AND DISCUSSION

The Presence of the pUC57-pKA Cassette Slowly Decreases Over Time Without Antibiotic Pressure

Each monthly genomic DNA was isolated and PCR of the insertion site, the kan resistance gene and the *rpsL* gene was conducted to determine the presence of the integrated transgenes over a two-year period. The first PCR reaction amplifies the insert region for the transgenes, resulting in a 2.4 kb or 6.7 kb band suggesting chromosomes copies that have not integrated the transgene and those who have, respectively. Initially, the 2.4 kb band are much less intense than the 6.7 kb band among all cultures, with and without antibiotic selective pressure – indicating that more chromosomes within the culture have the integrated transgenes. This trend remained the same for cultures with antibiotic selective pressure the entire duration of the study. However, in the cultures without antibiotic selective pressure, the 2.4 kb band become more intense suggesting that a portion of the chromosomes with integrated transgenes decreases over time. This trend was evident in the one-year stability study and the on-going stability study that is past 2 years (Figure 28 and 29). The second and third PCR reaction amplifies the kan resistance gene and the *rpsL* gene, resulting in 0.7 kb and 0.4 bands, respectively. The kan resistance gene remained present within the genomic DNA of GE *T. elongatus* BP1-pKA for the entire year (Figure 28 and 30).

Component	BG-11 Control Stock Amount	BG-11 Control Final Molarity	M1	M2	M3a	M3b
1M TES	5 mL	5 mM	5 mL	5 mL	5 mL	5 mL
100x BG-11 stock	10 mL		10 mL	10 mL	10 mL	10 mL
NaNO3	149.58 g/L	17.6 mM				
MgSO4 x 7H2O	7.49 g/L	0.304 mM				
CaCl2 x 2H2O	3.60 g /L	0.245 mM				
NaEDTA	0.104 g/L	2.79×10^{-3} mM				
Citric Acid	0.66 g/L	3.14×10^{-2} mM				
Trace Minerals	100 mL					
H_3BO_3	1.430 g/L	2.31×10^{-2} mM				
$MnCl_2 \times 4H_2O$	0.905 g/L	4.57×10^{-3} mM				
$ZnSO_4 \times 7H2O$	0.111 g/L	3.86×10^{-4} mM				
$Na_2MoO_4 \times 2H_2O$	0.195 g/L	8.06×10^{-4} mM				
$CuSO_4 \times 5H_2O$	0.040 g/L	1.60×10^{-4} mM				
$Co(NO_3)_2 \times 6H_2O$	0.025 g/L	8.59×10^{-5} mM				
Micronutrients	1 mL					
$C_6H_8FeNO_7$	0.602 g/100 mL	2.1×10^{-2} mM	0 mL	1 mL	1 mL	1 mL
Na_2CO_3	4.0 g/100 mL	0.189 mM	1 mL	0 mL	1 mL	1 mL
K_2HPO_4	3.99 g/100 mL	0.223 mM	1 mL	1 mL	0.5 mL	0.25 mL

Table 4. Components of BG-11 Media for Nutrient Study. The amount of each component is shown as well as the final concentration in 1 liter of media. The BG-11 medias are labeled as followed: M1 (- $C_6H_8FeNO_7$), M2 (-Na_2CO_3), M3a (½ K_2HPO_4) and M3b (¼ K_2HPO_4).

A gradual decrease in the population of chromosomes that have the transgene cassette was observed over the two-year study with the genomic DNA. One explanation for the loss of genes over time could be genome streamlining. Bacteria, including cyanobacteria, have been known to reduce the size of the chromosomes as an evolutionary mechanism, resulting in the loss of genes not essential for survival of the organism [171-174]. Genomic streamlining has been demonstrated within *Prochlorococcus marinus* and *Pelagibacter ubique*, which both have genome sizes around or less than 2 Mb but still possesses essential genes for biosynthesis of all amino acids and complete metabolic network [172, 174]. Genomic studies have also revealed

evidence that several strains of the cyanobacterium *Phlanktothrix* lost the microcystin synthetase gene cluster through evolutionary deletion events, resulting in the strains becoming nontoxic [175]. Although the results in the genomic DNA PCR show there may be loss of the transgene cassette within the GE *T. elongatus* BP1 over time, further studies will need to occur to ascertain the mechanism of this gene loss.

The pH for the BG-11 media alone was 7.75. The average pH for all control and experimental cultures was 7.91 before incubation and 9.49 after incubation. The wild-type (non-GE) control cultures showed a similar trend with pH averaging at 7.89 and 9.53 before and after incubation, respectively.

The Kan Resistance Gene is Expressed up to One Year

The negative control did not show bands for the RNA samples and indicated that all RNA samples were absent of any DNA contamination (Figure 31a). Subsequently, the RNA was used as a template for cDNA synthesis of the kan resistance gene and a housekeeper (*rpsL* gene), which was then used for conventional PCR. The cDNA PCR for all control and experimental cultures showed that *rpsL* gene, the housekeeping gene, tested positive, indicating that the cDNA synthesis process is properly working (Figure 31b). The kan resistance gene was shown to be expressed in all the GE *T. elongatus* BP1-pKA cultures but showed negative for wild-type *T. elongatus* BP1, indicating that the kan resistance only expresses in the GE cells (Figure 31c). This also confirms the wild-type does not possess the kan resistance gene.

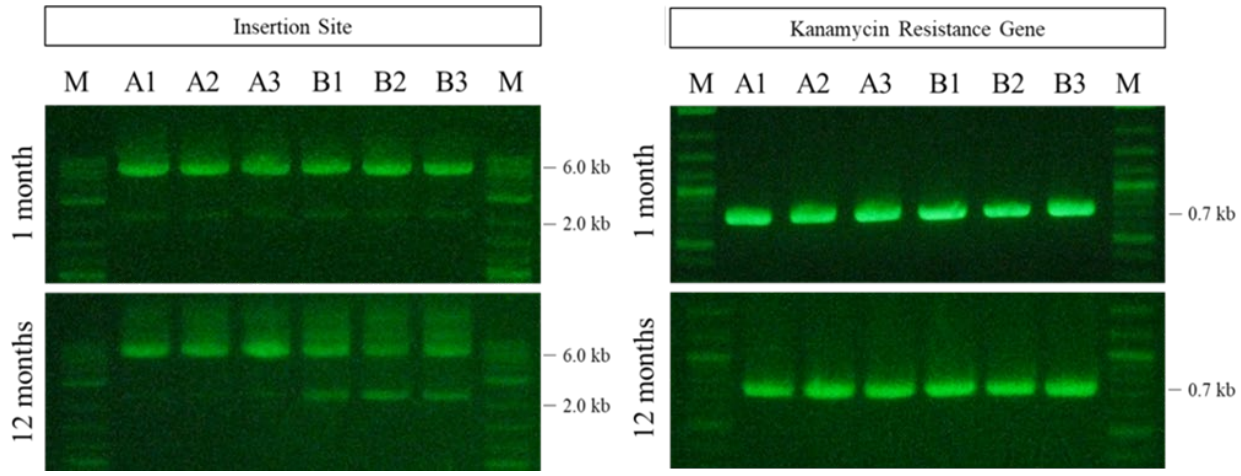


Figure 28. Genomic DNA PCR of GE *T. elongatus* BP1 for 12 Months. The following results are from the first trial of the stability study for one year. Primers were used to amplify the insertion site and the kan resistance gene after 1 and 12 months resulting in a 2.4 kb/6.7 kb and 0.7 kb bands, respectively. The lanes are marked as follows: M = molecular weight marker, A1-A3 = GE *T. elongatus* BP1 control (+ kan) cultures, B1-B3 = GE *T. elongatus* BP1 experimental (- kan) cultures.

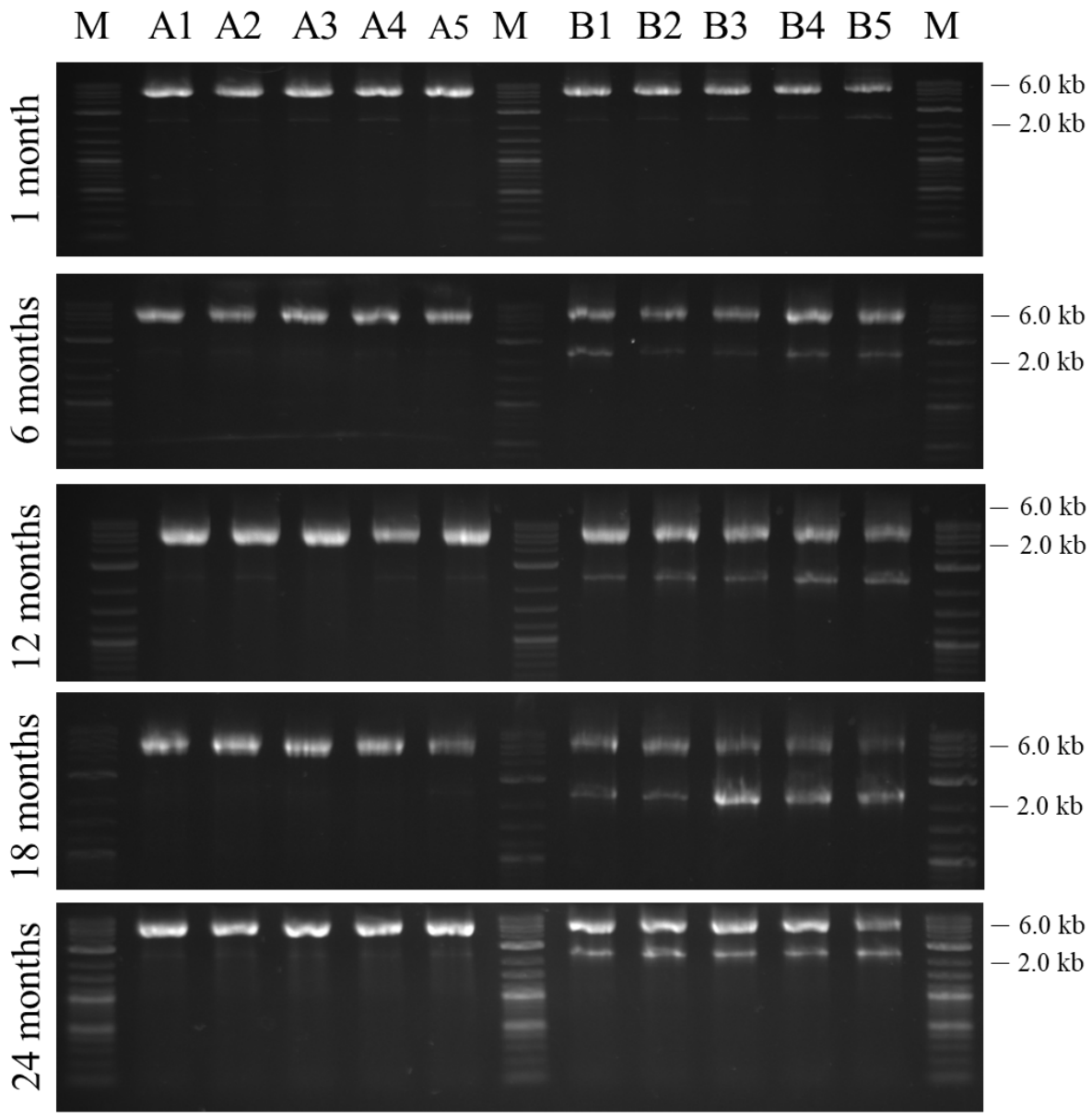


Figure 29. Presence of the Gene Cassette Within GE *T. elongatus* BP1 up to 24 Months. Primers were used to amplify the insertion site of *T. elongatus* BP1 genome containing the gene cassette, resulting in either a 2.4 kb band (wild-type size, no integration of genes) or 6.7 kb band (integration of genes) at 1, 6, 12, 18 and 24 months. The lanes are marked as followed: M = molecular weight marker, A1-5 = genomic DNA from the control GE *T. elongatus* BP1-pKA cultures (+ kan) and B1-5 = genomic DNA from the experimental GE *T. elongatus* BP1-pKA cultures (- kan).

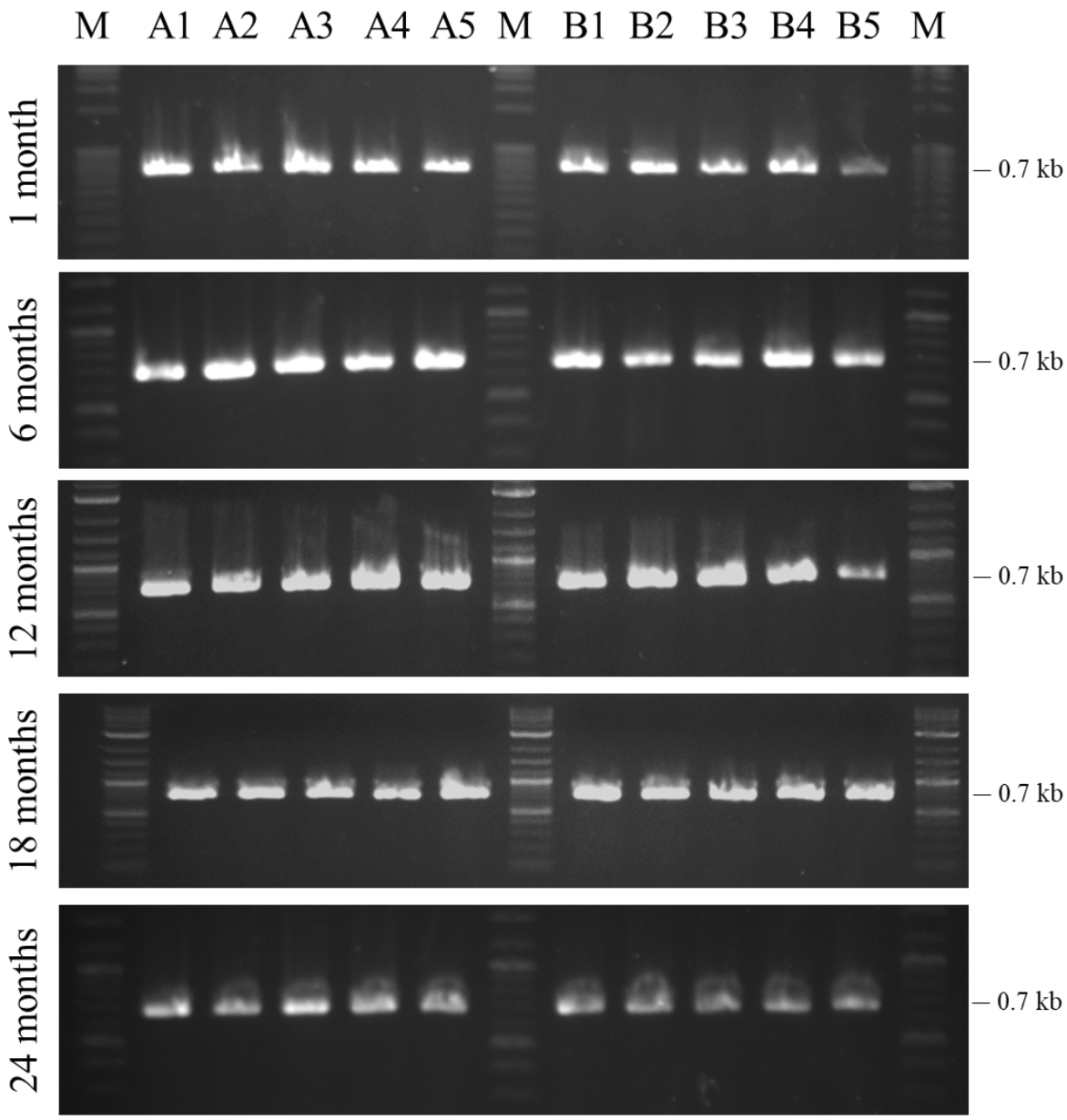


Figure 30. Presence of the Kan Resistance Gene Within GE *T. elongatus* BP1 up to 24 Months. Primers were used to amplify the kan resistance gene within the gene cassette, resulting in either a 0.7 kb band at 1, 6, 12, 18 and 24 months. The lanes are marked as followed: M = molecular weight marker, A1-5 = genomic DNA from the control GE *T. elongatus* BP1-pKA cultures (+ kan) and B1-5 = genomic DNA from the experimental GE *T. elongatus* BP1-pKA cultures (- kan).

Overall, these results suggest that the kan resistance gene is still expressed in the GE *T. elongatus* BP1 cells both after one year in the presence and absence of antibiotic pressure. In the gene cassette, the kan resistance gene is downstream of two continuous promoters, cpc from *T. elongatus* BP1 and splA from *Thermus thermophilis.* This could indicate why the kan resistance seems to still express after 1 year without antibiotic pressure. Quantifying the expression of the kan resistance gene by quantitative PCR would help validate the decrease in transgenes over time in the experimental *T. elongatus* BP1-pKA cultures as seen in the genomic DNA PCR of the insertion site. This is discussed in Chapter V in Future Directions.

Comparative Analysis of the Kan Resistance Gene

Genomic DNA was isolated from samples of 3 control (+ kan) and experimental (- kan) *T. elongatus* BP1-pKA cultures and used to amplify an 888-bp region of the gene cassette containing the kan resistance gene, which was isolated from the gel and sent to EVMS for sequencing (Figure 26). The trace score, which is the average base call quality, was high for all 12 sequences (Figure 32) and were assembled to the reference genome of *T. elongatus* BP1 where the genes are inserted to generate a consensus sequencing. The consensus sequence of the kan resistance gene was produced for the control (+ kan) cultures and the experimental (- kan) cultures. The reference sequence, control sequence and experimental sequenced were aligned for a comparative analysis and determine if the gene cassette acquired any mutations.

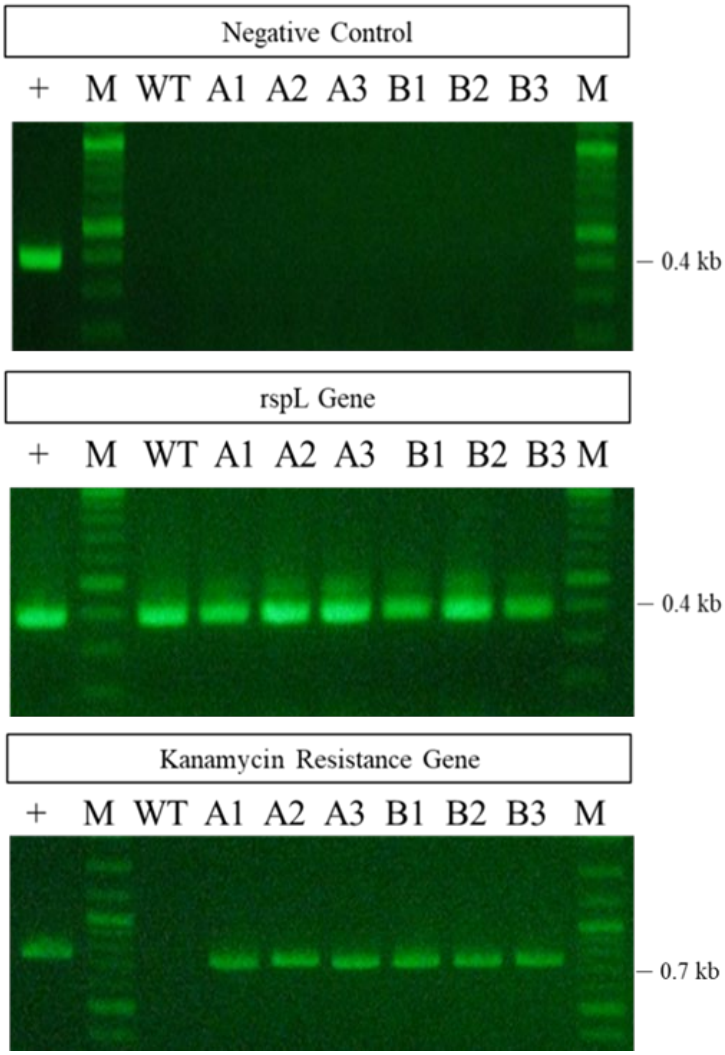
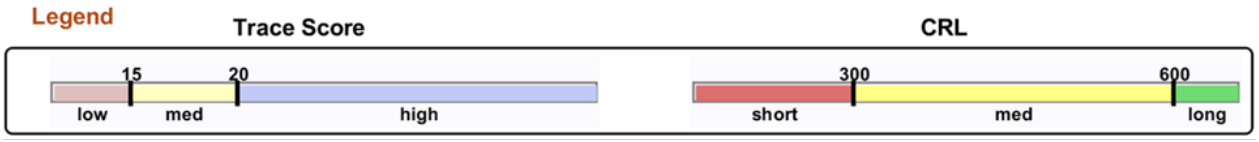


Figure 31. Expression of the Kan Resistance Gene in GE *T. elongatus* BP1 for 12 Months. Negative control where RNA is used as a template during PCR to ensure it is free of residual DNA. RNA is subsequently used for cDNA synthesis. cDNA PCR of the *rpsL* gene as a housekeeper with an expected band at 0.4 kb. cDNA PCR of the kan resistance gene with an expected band at 0.7 kb. The lanes are marked as follows: M = molecular weight marker, (+) = genomic DNA of wild-type *T. elongatus* (negative control and *rpsL* gene) or pUC57-pKA plasmid (kan resistance gene), WT = RNA or cDNA from wild-type *T. elongatus* BP1, A1-3 = RNA or cDNA from GE T. *elongatus* BP1-pKA control cultures (+ kan) and B1-3 = RNA or cDNA from GE *T. elongatus* BP1-pKA experimental cultures (- kan).



Trace File Name	Well#	Cap#	Trace Score	CRL	QV20+	Signal Intensity A	C	G	T
BP1-A1_F_2021-02-19.ab1	A8	1	43	69	127	242	167	321	219
BP1-A1_R_2021-02-19.ab1	B8	2	58	687	658	102	71	111	86
BP1-A2_F_2021-03-24.ab1	A12	1	51	235	236	113	59	176	82
BP1-A2_R_2021-03-24.ab1	B12	2	58	587	590	121	104	171	108
BP1-A3_F_2021-03-31.ab1	A1	1	58	602	644	268	146	427	210
BP1-A3_R_2021-03-31.ab1	B1	2	59	823	819	459	368	591	404
BP1-B1_F_2021-02-19.ab1	C8	3	59	768	791	216	77	267	168
BP1-B1_R_2021-02-19.ab1	D8	4	58	784	786	593	339	637	533
BP1-B2_F_2021-03-24.ab1	C12	3	60	814	804	839	423	1297	745
BP1-B2_R_2021-03-24.ab1	D12	4	53	819	757	1487	721	1865	1422
BP1-B3_F_2021-03-31.ab1	C1	3	54	576	551	562	226	773	470
BP1-B3_R_2021-03-31.ab1	D1	4	56	818	784	1375	765	1675	1305

Figure 32. Gene Sequencing Results for the Kan Resistance Gene. The forward (F) and reverse (R) sequences for the control (A1-A3) and experimental (B1-B3) cultures were sequences. The table also includes the trace score (average base call quality) and length for each sequence generated.

The nucleotide sequence analysis showed that there was high conservation between the control cultures and experimental cultures when compared to the kan resistance gene from the pUC57-pKA plasmid (Figure 33). There were some changes in base pairs when comparing the study cultures (control and experimental) with the reference sequence from the pUC57-pKA plasmid, where there were base pair changes from T to A and C to T at positions 69 and 456, respectively (Figure 33). After the nucleotide sequence was translated, it revealed that these were synonymous mutations as they did not lead to a change in the amino acid. It is also interesting to

note that while these two mutations are seen in the control (+ kan) and the experimental (- kan) samples, it is not found in the reference, which could be indicative of a natural mutation that has occurred. The nucleotide comparison analysis reveals that after one year the kan resistance gene does not seem to be mutated and suggests that the transgenes inserted are very robust with respect to mutations within the host genome. The nucleotide alignment using the whole-genome sequencing reads show the same differences between the study cultures and the reference (Appendix C), therefore validating the results we see from gene sequencing.

The nucleotide consensus sequence for the kan resistance the control (+ kan) and the experimental (- kan) were translated to produce the overall amino acid sequence for comparison in CLC Genomics Workbench. The consensus amino acid sequence for the control (+ kan) and experimental (- kan) were aligned to the reference sequence of the kan resistance gene to perform a comparative analysis. The alignment showed 100% conservation at each amino acid position for the entire kan resistance gene (Figure 34). Therefore, any mutations that were present at the nucleotide level were revealed to be synonymous mutations, meaning that the base pair change resulted in the same amino acid. There were no mutations observed when examining the amino acid alignment from the whole-genome sequencing results, again validating the results seen with the gene sequencing (Figure 35).

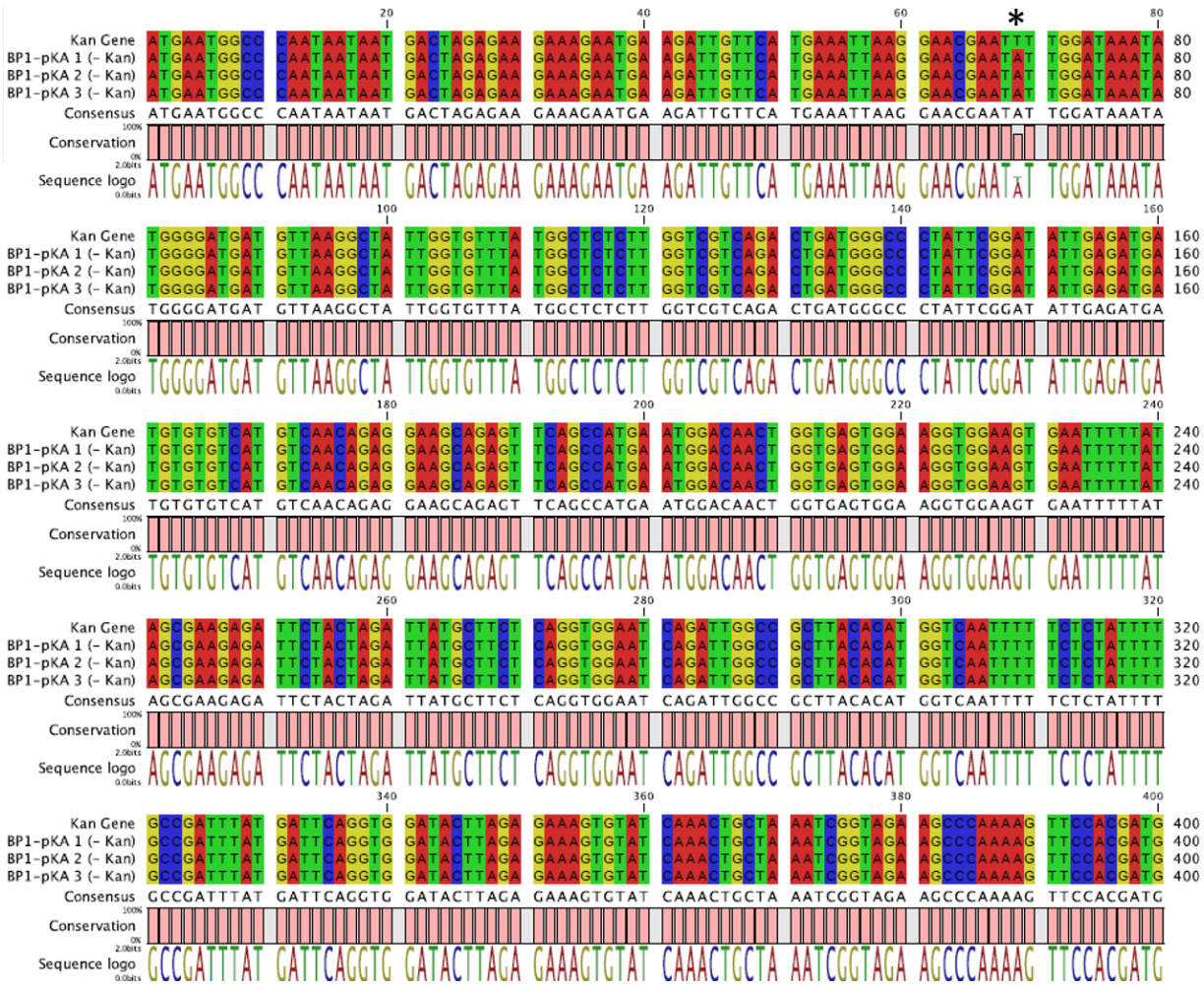


Figure 33. Nucleotide Mutations of the Kan Resistance Gene. The consensus sequences were aligned to each other and the kan resistance gene sequence from the pUS57-pKA plasmid. These are the control cultures (+ kan) which contained kanamycin in the media, the experimental cultures (- kan) which did not contain kanamycin in the media and the reference sequence (kan resistance gene from the pKA transgene cassette). The methodology for generating the consensus sequences is in materials and methods (Figure 27). Nucleotide mutations are indicated with an asterisk (*). The red bars display the level of conservation of the nucleotide base at each position. The sequence logo shows the frequency of residues at each position in the alignment. A = adenine (red), T = thymine (green), G = guanine (yellow), C = cytosine (blue), N = ambiguous base, - = gap in sequence

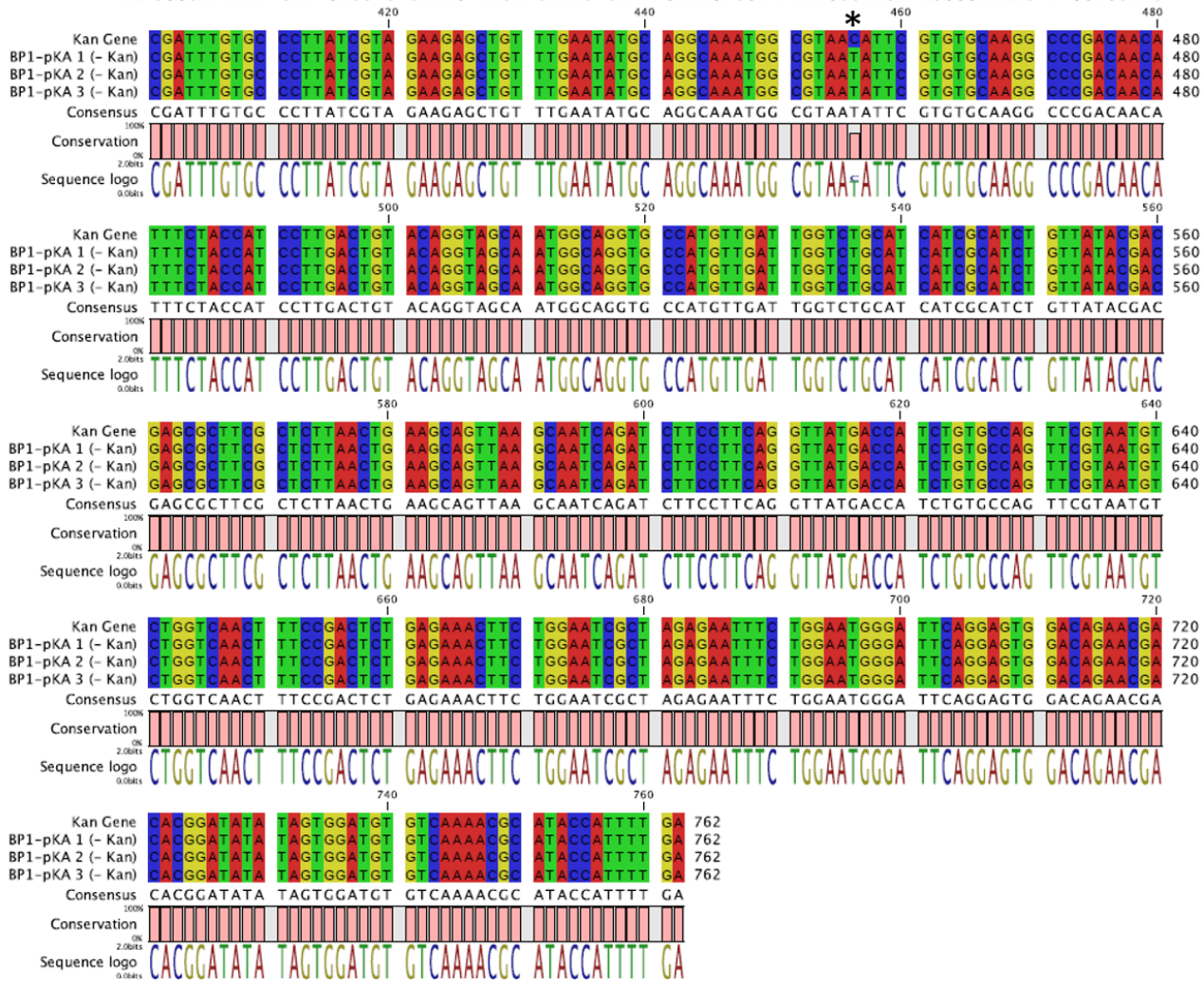


Figure 33 (Continued).

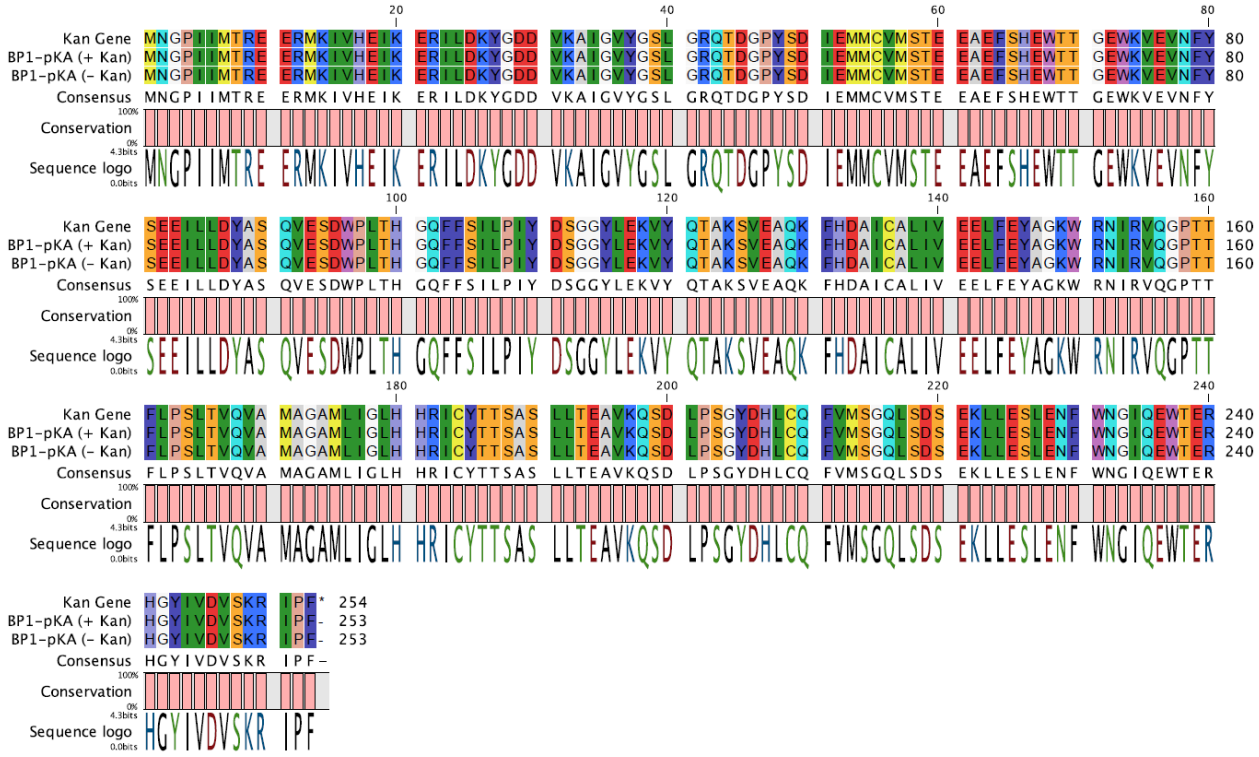


Figure 34. Amino Acid Sequence Alignment of the Kan Resistance Gene from Gene Sequencing. A consensus amino acid sequence was generated for an experimental (- kan) culture by assembling all forward and reverse sequences to the reference sequence for the reference protein sequence (kan resistance gene from the pKA transgene cassette). The consensus sequence for the experimental sample was then aligned to the reference. The red bars display the level of conservation of the amino acid base at each position.

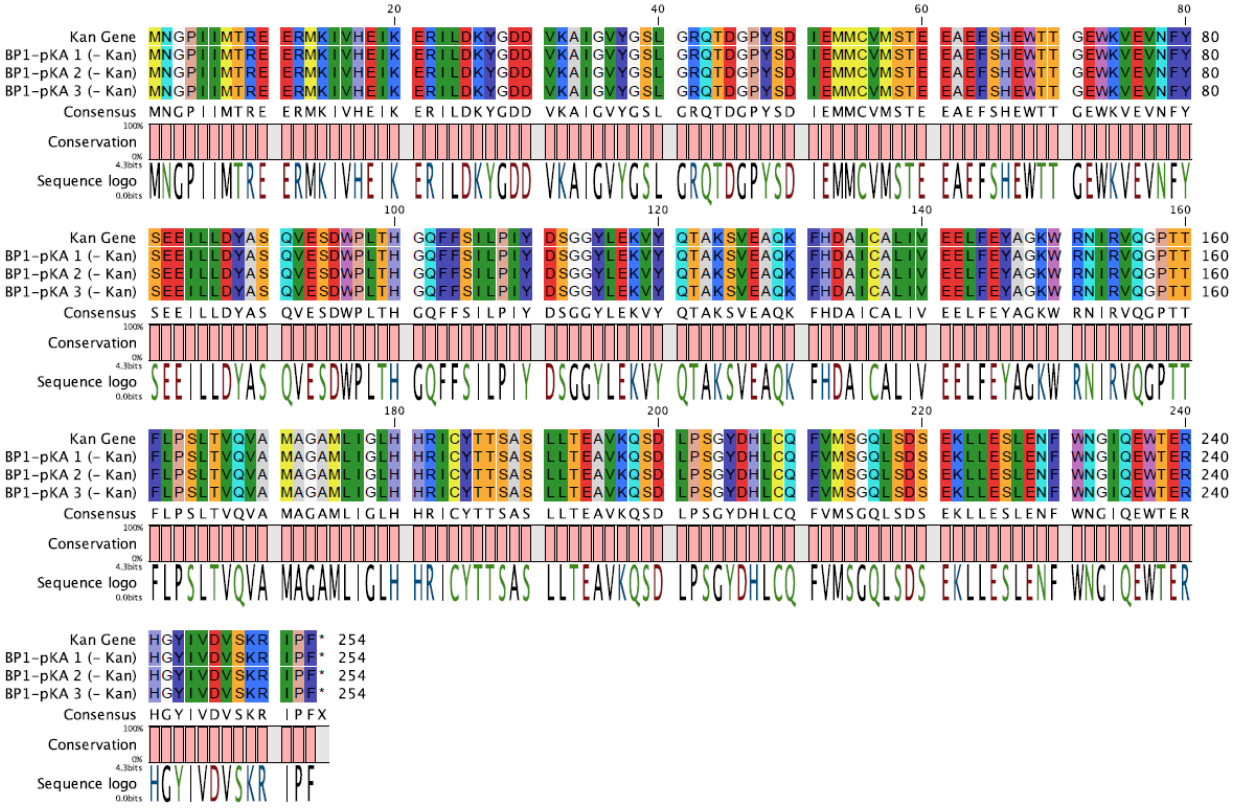


Figure 35. Amino Acid Sequence Alignment of the Kan Resistance Gene from Whole-Genome Sequencing. The consensus amino acid sequence for three experimental (- kan) cultures were aligned to each other and the reference protein sequence (kan resistance gene from the pKA transgene cassette). The red bars display the level of conservation of the amino acid at each position. The sequence logo shows the frequency of residues at each position in the alignment.

Figure 36. Alignment of Transgene Cassette Promoters in GE *T. elongatus* BP1 After One Year. The consensus nucleotide sequence for three experimental (- kan) cultures was aligned to the reference nucleotide sequence from the pKA transgene cassette for the nirA promoter (top red box), cpc promoter (middle blue box) and slpA promoter (bottom green box). The red bars display the level of conservation of the nucleotide base at each position. The sequence logo shows the frequency of residues at each position in the alignment. A = adenine (red), T = thymine (green), G = guanine (yellow), C = cytosine (blue), N = ambiguous base, - = gap in sequence.

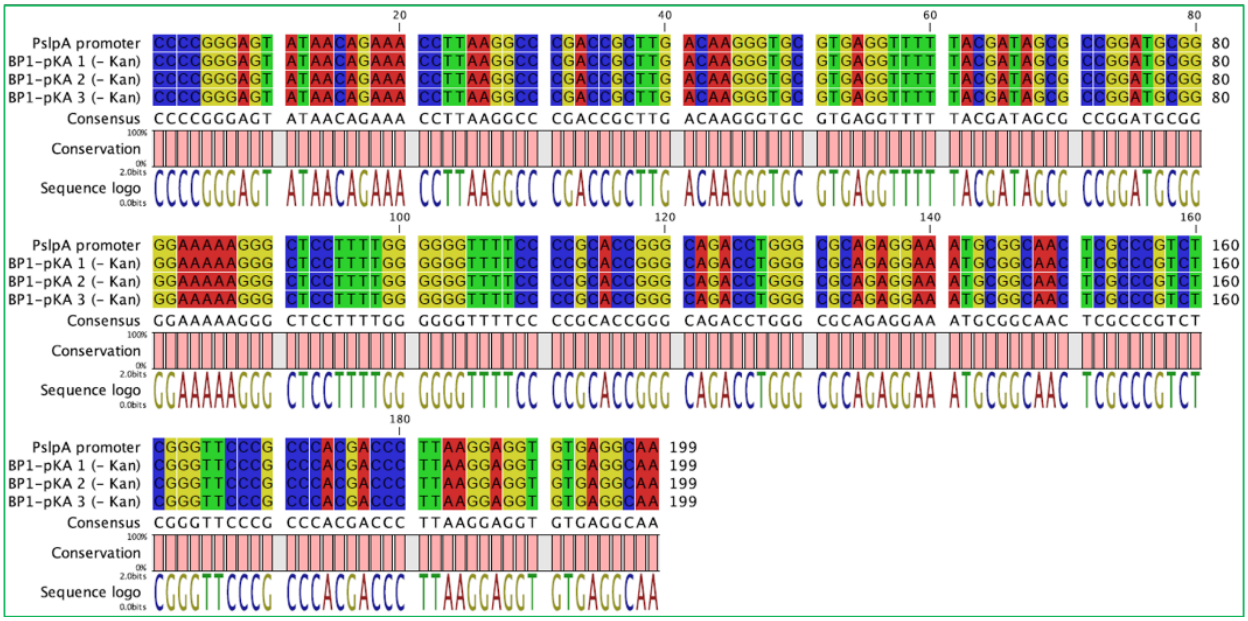


Figure 36 (Continued).

Comparative Analysis of Other Transgene Cassette Components

Whole-genome sequencing and assembly of *T. elongatus* BP1 the experimental cultures (-Kan) were performed and the resulting consensus sequence for the nirA promoter (231 bp), cpcP promoter (116 bp), pslpA promoter (199 bp), kan resistance gene (235 amino acids) and KIVD gene (547 amino acids) was aligned with the reference genome (Figure 36-37). The nucleotide sequence comparison of the promoter regions of the transgene cassette revealed that all three were 100% conserved across all positions in the sequence, suggesting that the regulator sequences are highly stable up to one year without antibiotic resistance (Figure 36). The comparison analysis of the kan resistance gene also proved to be 100% conserved on the amino acid level, which are similar to the gene sequencing results shown previously (Figure 35).

A nucleotide sequence alignment of the KIVD gene was performed between the reference and three experimental (- kan) samples and it was evident that there were many mutations

present (Appendix D). The nucleotide sequences were then translated to the amino acid sequence and aligned, and it was determined that there were many nucleotide changes which led to nonsynonymous mutations (Figure 37). A deeper analysis of the nonsynonymous mutations within one experiment sample was conducted and there was a total of 67 nonsynonymous mutation observed, which were annotated by the characteristics of their residues (ie. non-polar, polar, acidic, basic) and categorized according to their characteristic changes (Figure 38-39). Of these mutations, 37 resulted in a different residue with the same characteristic, 6 resulted in a change in charge, 13 resulted in a change in polarity, 15 resulted in a change in charge and polarity and 1 resulted in an additional residue instead of a stop codon (Figure 39). The amino acid alignment for the two additional experimental (- kan) samples were also analyzed and the amino acid sequence alignment showed that there were many more mutations that resulted in a number pf gaps in the alignment and several premature stop codons, which essentially produces a truncated protein (Appendix E). The first stop codon for these samples were at the amino acid position 228 and 224 for the BP1-pKA B1 and B2 samples, respectively. Although the alignment shows many amino acid changes downstream of these stop codons, realistically these would not be present in the actual protein product and therefore are seen as irrelevant. All mutations between the reference for the KIVD protein and all three samples are provided in Appendix F.

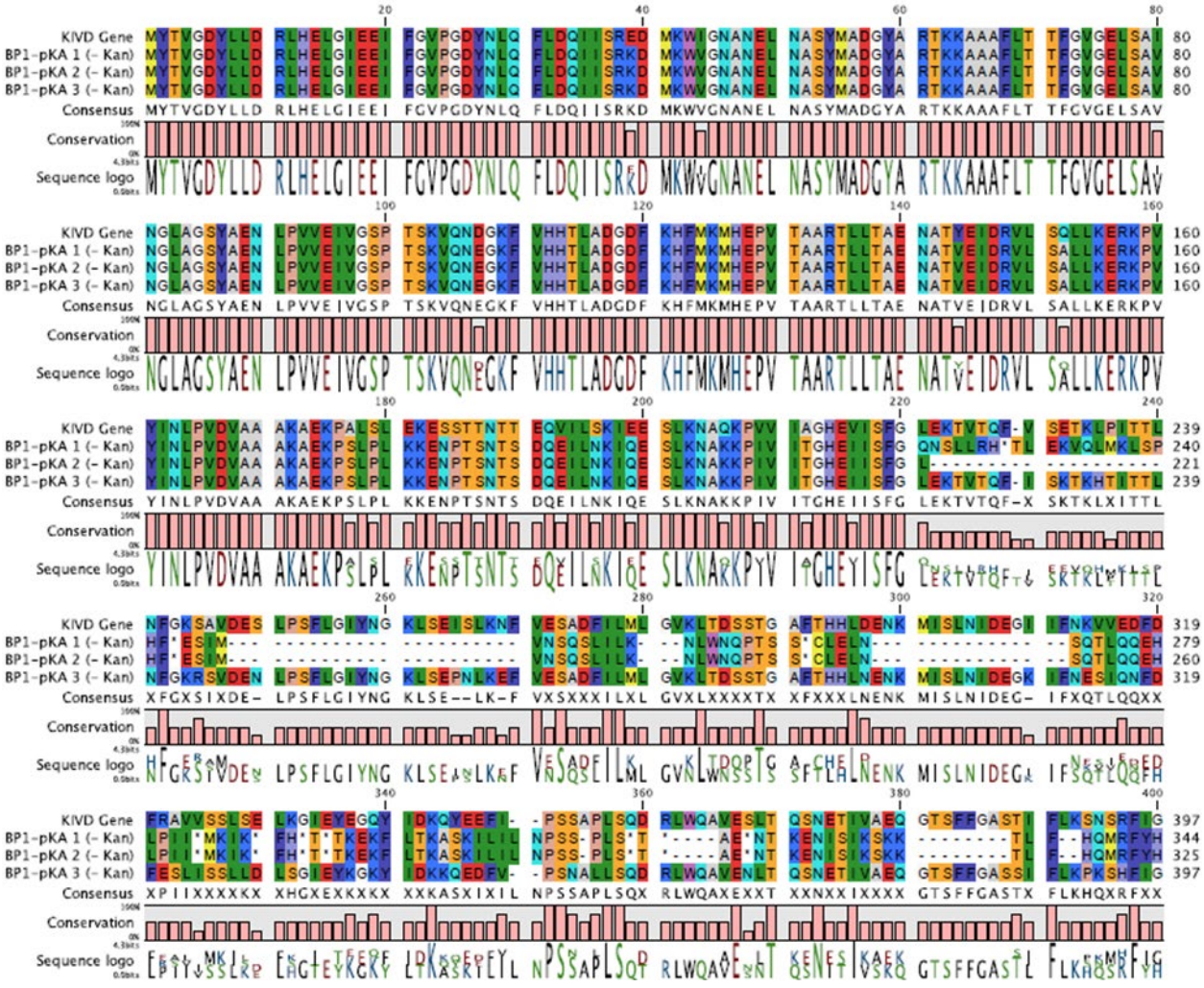


Figure 37. Amino Acid Sequence Alignment of the KIVD Gene. The consensus amino acid sequence for three experimental (- kan) cultures were aligned to each other and the reference protein sequence (from the pKA transgene cassette). The red bars display the level of conservation of the amino acid at each position. The sequence logo shows the frequency of residues at each position in the alignment.

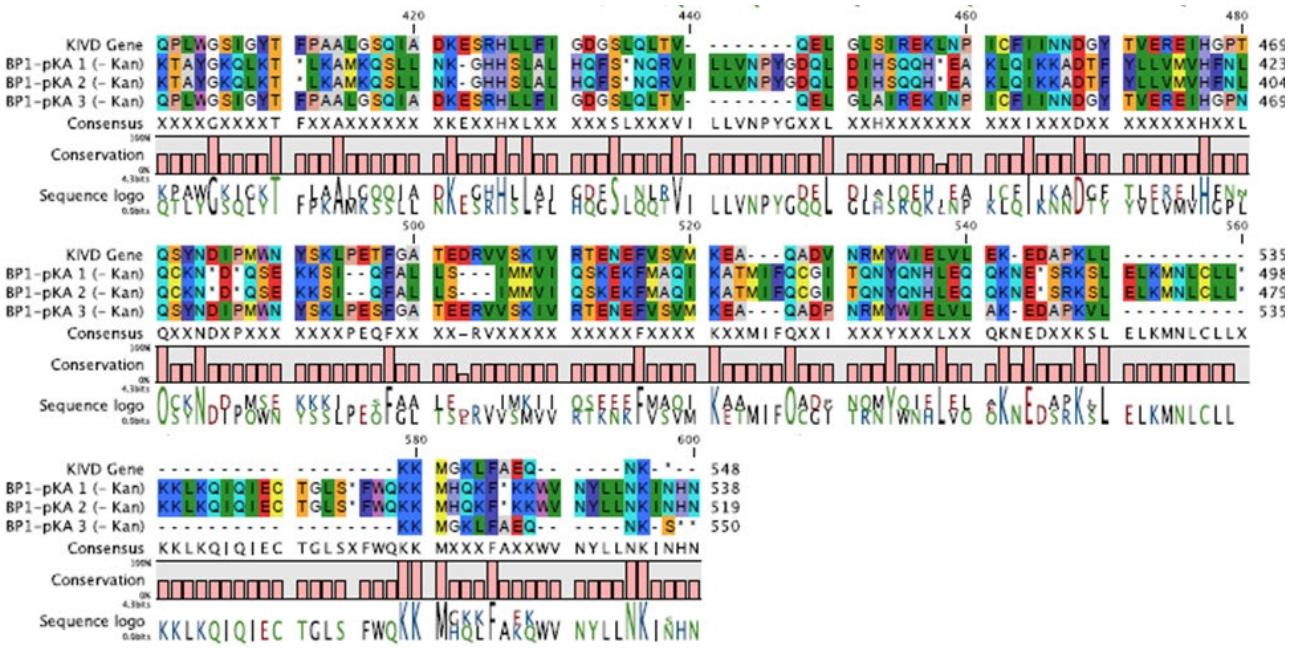


Figure 37 (Continued).

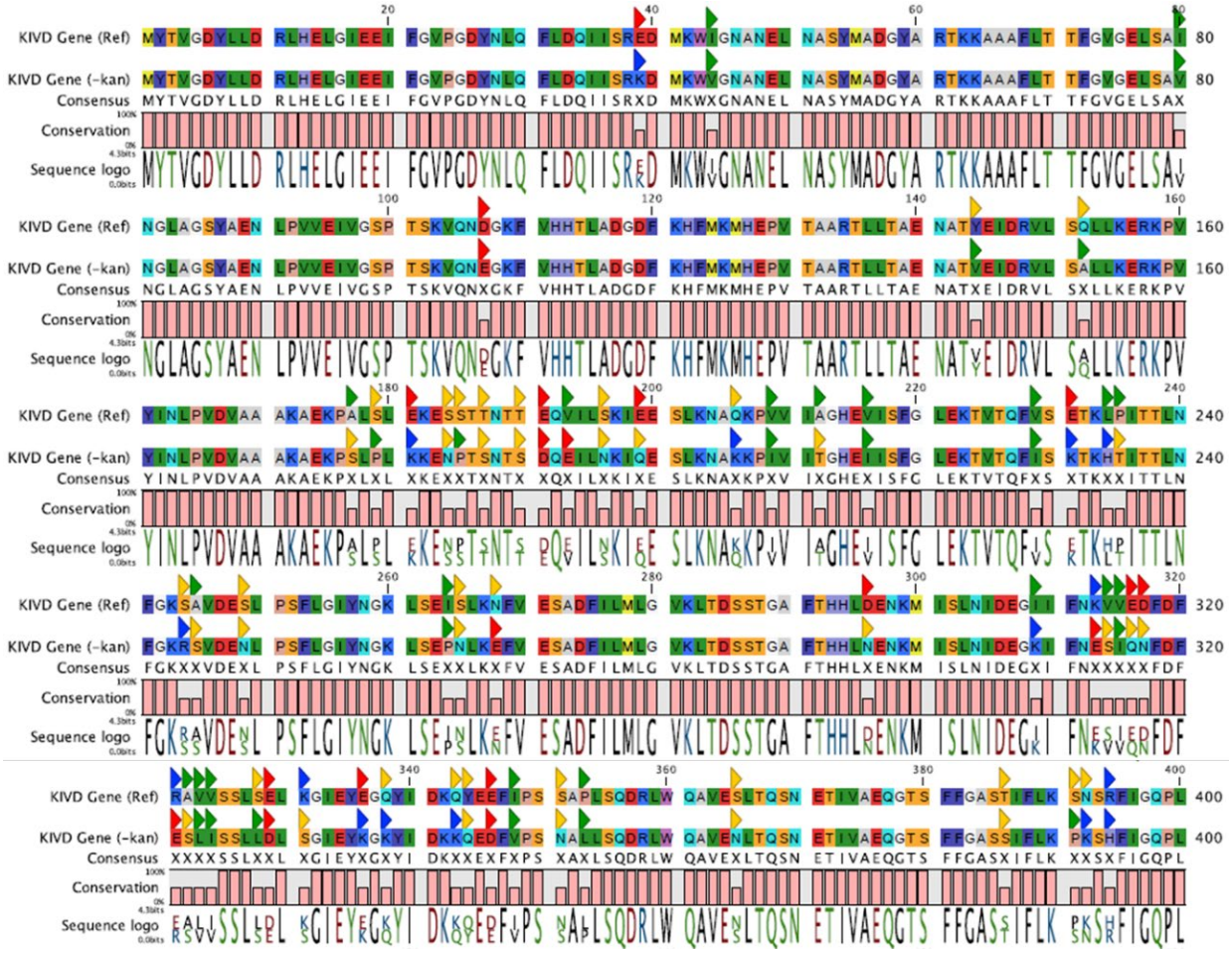


Figure 38. Annotation for Nonsynonymous Mutations within KIVD After One Year. The consensus nucleotide sequence for one experimental sample was translated to the amino acid sequence and aligned to the reference amino acid sequence (from the pKA transgene cassette). The residues associated with nonsynonymous mutations were annotated (arrows) according to their characteristics: non-polar (green), yellow (polar), acidic (red) and basic (blue). The red bars display the level of conservation of the amino acid base at each position. The sequence logo shows the frequency of residues at each position in the alignment.

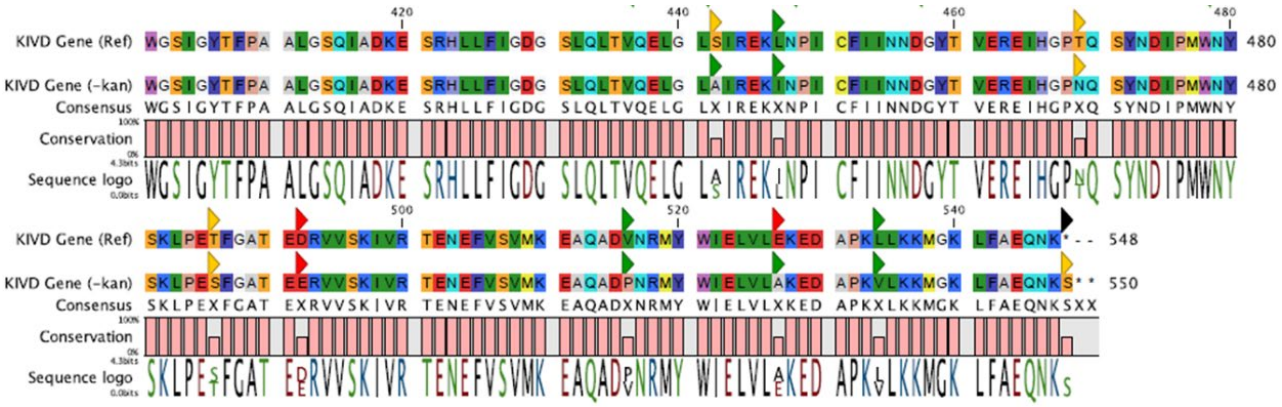


Figure 38 (Continued).

Growth and Preliminary PCR Results for the Exploratory Nutrient-Deficient Study

The transgene study PCR results indicated a steady increase in the chromosomes that no longer possess the transgene cassette. In an exploratory and preliminary effort to see if GE *T. elongatus* BP1 under nutritional stress will expediate the elimination of these transgenes, an exploratory nutrient-deficiency study was conducted. *T. elongatus* BP1 is normally grown in BG-11 media and a logical approach to select stressors was to reduce different components of the media. Before the experiment, the cells were tested in four different cultures missing or having a reduces component in the BG-11 media. One culture missing ferric ammonium citrate, one missing sodium carbonate, one with one half dipotassium hydrogen phosphate and one with one fourth of dipotassium hydrogen phosphate to ensure the conditions were not too harsh and inhibit cyanobacterial growth. The pH for the medias were recorded as follows; BG-11 control 7.75, BG-11 (no ferric ammonium citrate added) 7.77, BG-11 (no sodium carbonate added) 7.62, BG-11 (½ dipotassium hydrogen phosphate) 7.84 and BG-11 (¼ dipotassium hydrogen phosphate) 7.90. GE *T. elongatus* BP1 seems to grow best in BG-11 control media, followed by BG-11 medias with no sodium carbonate and decreased amounts of dipotassium hydrogen phosphate.

Finally, the GE *T. elongatus* BP1 cultures grew the slowest in BG-11 media lacking ferric ammonium citrate (Figure 40). Wild-type *T. elongatus* BP1 showed similar results (Appendix G). After two weeks, the pH of the BP1-pKA cultures in BG-11 control, BG-11 (no ferric ammonium citrate added), BG-11 (no sodium carbonate added), BG-11 (½ dipotassium hydrogen phosphate) and BG-11(¼ dipotassium hydrogen phosphate) were 9.62, 8.76, 9.75, 9.45 and 9.08 respectively.

Sodium carbonate is a component of BG-11 that provides a weak buffer system in the media and was not added in an attempt to gently stress the organism [176]. The cultures still grew fairly well. Dipotassium diphosphate is a source of phosphorus, which is a required element of several vital biomolecules including nucleic acids (DNA/RNA) and phospholipids and is essential for growth [176, 177]. Previously, media without any dipotassium hydrogen phosphate did not show signs of cyanobacterial growth (results not shown), therefore ½ and ¼ of the amount was used for this study. Ferric ammonium citrate is a source of iron for cyanobacteria, which is an essential mineral that plays in important role in several important processes, including photosynthesis, and has been shown to be a growth-limiting factor [176, 178, 179]. Although, additional steps to ensure trace iron on or in supplies used for this experiment were not taken, not including ferric ammonium citrate in the media still had significant effects on its growth when compared to the control. Collectively, wild-type and GE *T. elongatus* BP1 were able to grow in all medias and determined that the media conditions were suitable to carry out a long-term study.

Same Characteristic		
Position	Residue (ref)	Residue (-kan)
44	Isoleucine (I)	Valine (V)
80	Isoleucine (I)	Valine (V)
107	Aspartic Acid (D)	Glutamic Acid (E)
184	Serine (S)	Asparagine (N)
187	Threonine (T)	Serine (S)
190	Threonine (T)	Serine (S)
191	Glutamic Acid (E)	Aspartic Acid (D)
196	Serine (S)	Asparagine (N)
209	Valine (V)	Isoleucine (I)
216	Valine (V)	Isoleucine (I)
229	Valine (V)	Isoleucine (I)
249	Serine (S)	Asparagine (N)
264	Isoleucine (I)	Proline (P)
265	Serine (S)	Asparagine (N)
315	Valine (V)	Isoleucine (I)
323	Valine (V)	Leucine (L)
324	Valine (V)	Isoleucine (I)
329	Glutamic Acid (E)	Aspartic Acid (D)
344	Tyrosine (Y)	Glutamine (Q)
346	Glutamic Acid (E)	Aspartic Acid (D)
348	Isoleucine (I)	Valine (V)
351	Serine (S)	Asparagine (N)
353	Proline (P)	Leucine (L)
365	Serine (S)	Asparagine (N)
386	Threonine (T)	Serine (S)
394	Arginine (R)	Histidine (H)
447	Leucine (L)	Isoleucine (I)
469	Threonine (T)	Asparagine (N)
486	Threonine (T)	Serine (S)
492	Aspartic Acid (D)	Glutamic Acid (E)
516	Valine (V)	Proline (P)
534	Leucine (L)	Valine (V)

Change in Charge		
Position	Residue (ref)	Residue (-kan)
39	Glutamic Acid (E)	Lysine (K)
181	Glutamic Acid (E)	Lysine (K)
231	Glutamic Acid (E)	Lysine (K)
313	Lysine (K)	Glutamic Acid (E)
321	Arginine (R)	Glutamic Acid (E)
336	Glutamic Acid (E)	Lysine (K)
Change in Polarity		
Position	Residue (ref)	Residue (-kan)
144	Tyrosine (Y)	Valine (V)
152	Glutamine (Q)	Alanine (A)
177	Alanine (A)	Serine (S)
179	Serine (S)	Proline (P)
185	Serine (S)	Proline (P)
212	Alanine (A)	Threonine (T)
235	Proline (P)	Threonine (T)
245	Alanine (A)	Serine (S)
314	Valine (V)	Serine (S)
322	Alanine (A)	Serine (S)
328	Serine (S)	Leucine (L)
391	Serine (S)	Proline (P)
442	Serine (S)	Alanine (A)

Changes in Charge and Polarity		
	Non-polar - Acidic	
Position	Residue (ref)	Residue (-kan)
193	Valine (V)	Glutamic Acid (E)
527	Glutamic Acid (E)	Alanine (A)
	Polar - Acidic	
Position	Residue (ref)	Residue (-kan)
199	Glutamic Acid (E)	Glutamine (Q)
268	Asparagine (N)	Glutamic Acid (E)
296	Glutamic Acid (E)	Asparagine (N)
316	Glutamic Acid (E)	Glutamine (Q)
317	Aspartic Acid (D)	Asparagine (N)
	Non-polar - Basic	
Position	Residue (ref)	Residue (-kan)
234	Leucine (L)	Histidine (H)
309	Isoleucine (I)	Lysine (K)
	Polar to Basic	
Position	Residue (ref)	Residue (-kan)
206	Glutamine (Q)	Lysine (K)
244	Serine (S)	Arginine (R)
331	Lysine (K)	Serine (S)
338	Glutamine (Q)	Lysine (K)
343	Glutamine (Q)	Lysine (K)
392	Asparagine (N)	Lysine (K)
	Special	
Position	Residue (ref)	Residue (-kan)
548	STOP CODON	Serine (S)

Figure 39. Nonsynonymous Mutations Characterized by their Changes in Amino Acid Characteristics. The position, amino acid from the pUC57-pKA plasmid and amino acid for experimental sample #3 GE *T. elongatus* BP1 (- kan) were annotated and mutations were categorized based on their characteristic changes: same characteristic, change in charge (acidic vs basic), change in polarity (polar vs non-polar) and changes in both charge and polarity. The colors represent non-polar residues (green), polar residues (yellow), acidic residues (red) and basic residues (blue). Black indicated a mutation unique from the others that are annotated.

Similar to the transgene study, the cultures were prepared and placed in the Percival incubation chamber for growth. On a monthly basis, genomic DNA is isolated from these samples and used to amplify the transgene cassette insert region to determine its presence or absence over time. At

one month, GE *T. elongatus* BP1 cultures in all BG-11 media conditions showed a prominent 6.7 kb band and very faint 2.4 kb band, which is indicative of more chromosome copies possess the transgene cassette compared to those who do not (Figure 41). After six months, the 2.4 kb band tends to be brighter by eye compared to the one month for the cultures lacking sodium carbonate and ferric ammonium citrate in the media. These results suggests that the number of chromosomes copied without the transgene cassette is increasing – however it seems to be on the same time scale when compared to the control. For the cultures with decreased amounts of dipotassium hydrogen phosphate, there does not seem to be a difference between the one and six months when comparing the brightness of the 2.4 kb and 6.7 kb bands (Figure 41). However, when comparing the intensity of the 2.3 kb band at one month between these cultures and the others lacking sodium carbonate and ferric ammonium citrate, the 2.3 kb is much bright. This could indicate that within one month, more chromosome is present without the transgene cassette when there are reduced amounts of the phosphorus source in the media. This would seem sensible since phosphorus is an important building block of nucleic acids and perhaps the reduction of this source is the reason why there seems to be a higher loss after one month compared to the other nutrient-deficient medias.

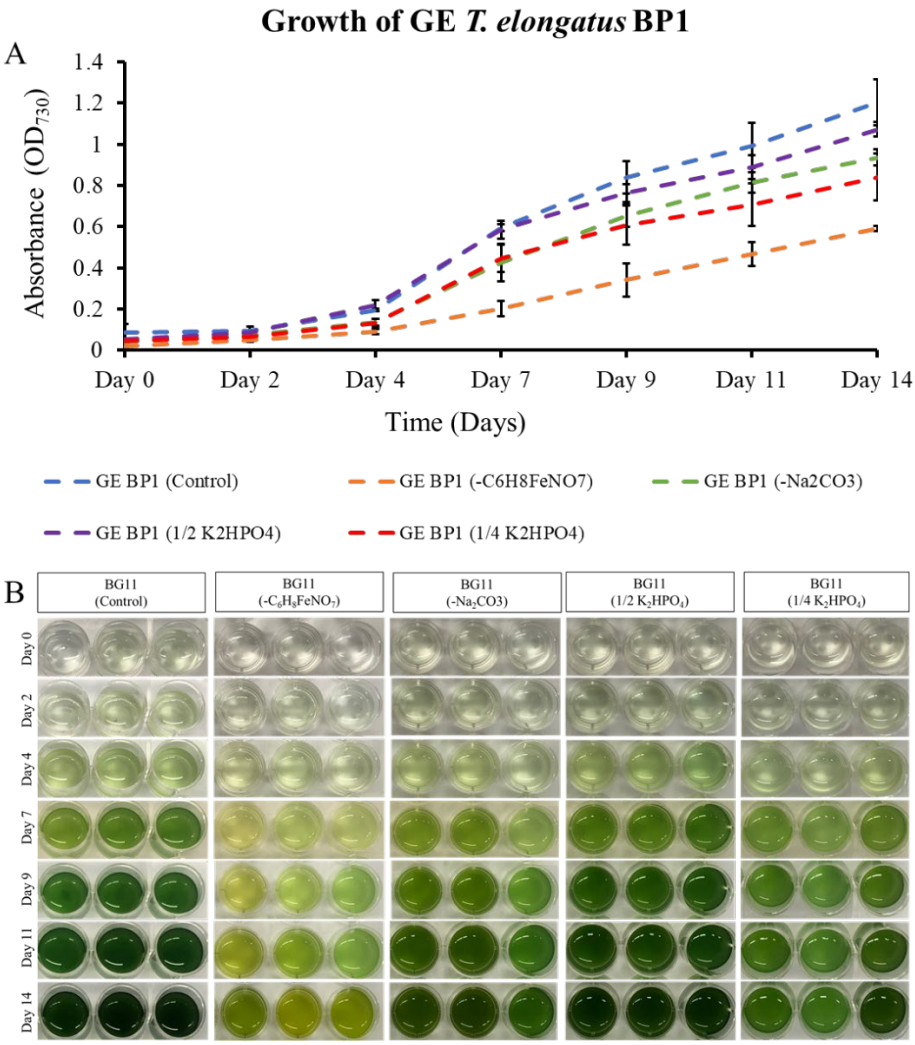


Figure 40. Growth of GE *T. elongatus* BP1 in Nutrient-Deficient BG-11 Media. (A) The growth is monitored by absorbance at OD_{730}. (B) Images of cultures in control BG-11, BG-11 without ferric ammonium citrate ($C_6H_8FeNO_7$) and sodium bicarbonate (Na_2CO_3), and BG-11 with ½ or ¼ of dipotassium phosphate (K_2HPO_4).

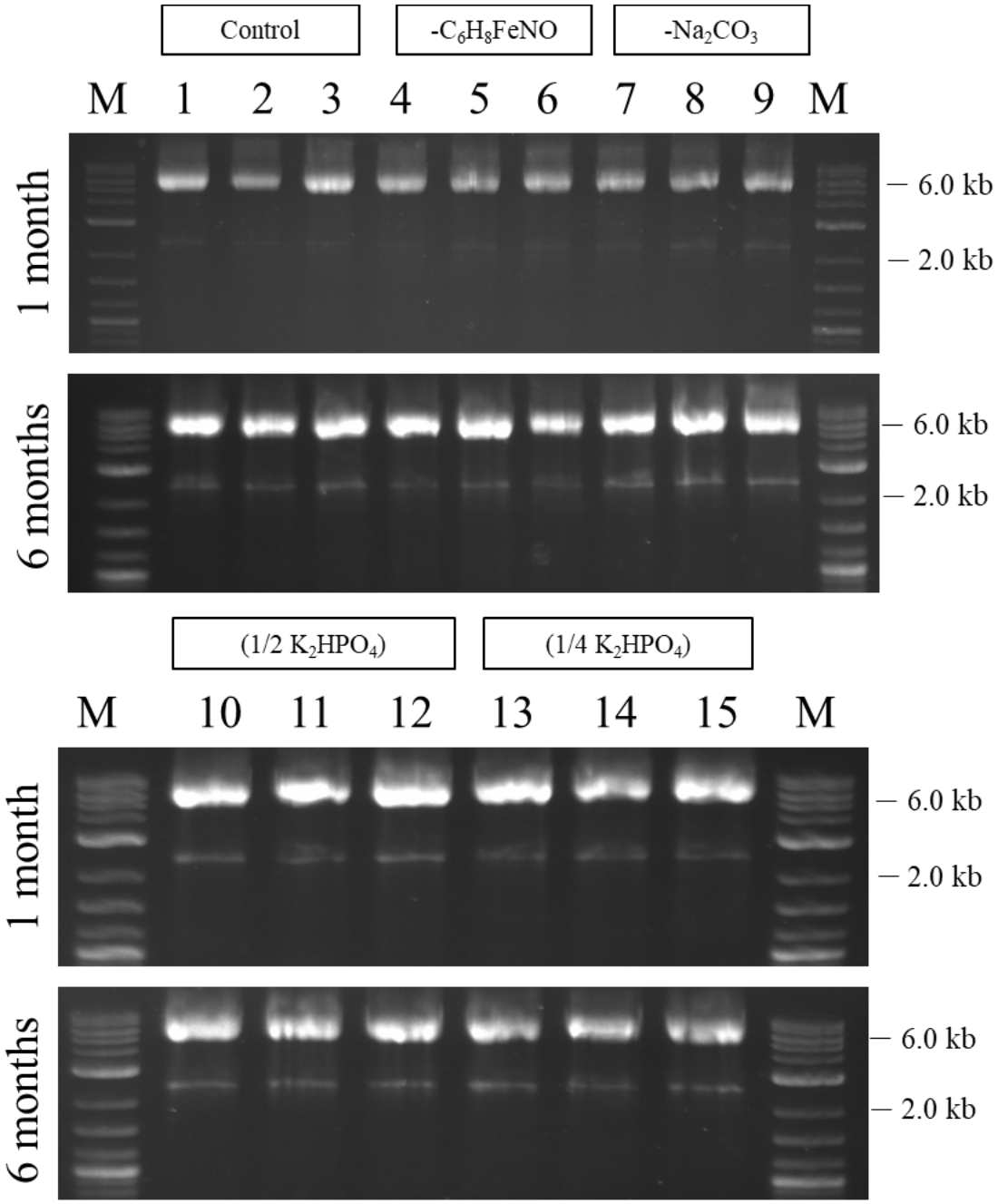


Figure 41. Genomic DNA PCR of the Nutrient-Deficient Cultures After 6 Months. The insertion site of the pKA transgene cassette was amplified for GE *T. elongatus* BP1 in BG-11 (control), BG-11 (-$C_6H_8FeNO_7$) and BG-11 (-Na_2SO_3) medias at 1 and 6 months. The lanes are marked as followed: M = molecular weight marker, 1-3 = GE *T. elongatus* BP1 (control), 4-6 = GE *T. elongatus* BP1 (-$C_6H_8FeNO_7$), 7-9 = GE *T. elongatus* BP1 (-Na_2CO_3).

Conclusion

Together, the results from the present work indicates that the transgene cassette within the genome of *T. elongatus* BP1 shows signs of long-term stability. The kan resistance gene was shown to still express after one year of the study, even in the absence of antibiotic pressure. One important aspect of stable transgene expression within GE organisms is the choice for the promoter. The transgene cassette was designed where the expression of the kan resistance gene was under the control of the *cpc* and *slpA* continuous promoters, which are known to be very strong [180, 181]. Also, the nucleotide sequence alignment showed that there were no mutations within these promoters that would result in the decrease or prevent the expression of the kan resistance gene.

The genomic stability of the transgene cassette is shown to be extremely stable up to two years within the chromosome of *T. elongatus* BP1, both in the presence and absence of antibiotic pressure. This finding was very surprising as we hypothesized the genes would be lost without the selective pressure of kanamycin in the media. There have been a few previous studies that investigated long-term transgene stability within plants and fungus [165, 182, 183]. One group investigated the long-term stability and expression of the *rolC* gene within GE aspen trees, which revealed that the transgene was still present within the genome and expression up to 18 years after transformation [165]. A different group examined the stability of transgenes within GE *Trichoderma virens* over a 250-day experiment and found that the *opd* gene was still present and express in the absence of antibiotic pressure [183]. Another group studied and expression of a chimeric gene, including an insecticidal peptide gene and the C-peptide of *Bt* gene, within *Betula platyphylla* for up to 15 subcultures and although the gene was silenced, it was still present within the chromosome for the duration of the study [182]. There have been no long-term studies

of transgene stability in cyanobacteria previously published based on extensive literature searches.

From a bio-risk perspective, the longer transgenes remain intact within the cyanobacterial chromosome, the more opportunity it has to transfer modified genes and persist in unintended environments thus leading to possible ecological and human health risks. For example, a study assessed the persistence of an escaped herbicide resistance gene from GE *Brassica rapa* to wild-type relatives through hybridization and the transgene was shown to be persistent in these hybrids for up to 6 years [169]. Another study demonstrated that antibiotic resistance genes of bacteria within beef cattle is shown to be transferred and persists for up to two years in feeding areas where they were housed [170]. If synthetic cyanobacteria are to be used for the commercial production of bioproducts, it is of paramount importance to understand the fate and stability of transgenes as an avenue to prevent any bio-risk concerns.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

SUMMARY

The bio-risk research conducted in this dissertation focused on three major questions: can transgenes horizontally transfer from cyanobacteria to *E. coli*; will temperature provide a safeguard to prevent spread of GE cyanobacteria; and what is the long-term fate and stability of transgenes in cyanobacteria.

In the first aim, we investigated the potential for HGT to occur between GE *T. elongatus* BP1 and wild-type *E. coli* DH5α. First, we were able to successfully transformed *T. elongatus* BP1 and integrated the designed gene cassette within its genome by homologous recombination. The *T. elongatus* BP1-pKA transformant was verified by PCR and used for the remainder of the studies in this dissertation. To assess HGT after co-incubation with GE *T. elongatus* BP1 and wild-type *E. coli* DH5α, we found that *E. coli* colonies appeared on the LB agar plates supplemented with kanamycin. We proposed that these colonies were able to grow in the presence of antibiotic because they had acquired the kan resistance gene. Rigorous controls reinforced that the transfer of the kan resistance gene to wild-type *E. coli* was due to interaction with GE *T. elongatus* BP1, rather than taking up the plasmid from liquid media. The outcomes in this study indicate that GE *T. elongatus* BP1 was able to transfer the transgenes to wild-type *E. coli* DH5α.

In the second aim, we studied in a greenhouse, the ability for wild-type and GE *T. elongatus* BP1 to grow and survive outside of laboratory conditions (continuous lighting and temperature is 42 - 45 °C). The focus sought to determine if the GE cyanobacteria can thrive

during cool and warm seasons. Although it was previously observed that *T. elongatus* BP1 has an optimum growth of 57 ℃ [60], we wanted to understand their growth and survivability in a variety of temperatures to determine if temperature can be a safeguard in case these GE cyanobacteria cells were to somehow break containment and enter the environment. The growth study showed that wild-type and GE *T. elongatus* BP1 do not actively grow in greenhouse conditions, as seen by the decrease of culture density over the 28-day period for the experiment. This is expected as the maximum temperature during the cool and warm trial does not surpass 57 ℃, which is optimal for active growth of *T. elongatus* BP1. However, the survivability revealed that wild-type and GE *T. elongatus* BP1 were able to recuperate better in the warm temperature trial than the cool temperature trial after incubation in greenhouse condition. In the warm trial, cells were able to grow back after 4 weeks, and the cool trial only showed growth after 3 weeks in greenhouse conditions. The same result can be concluded from the CFU assay – in the cool temperature trial there were no colony formation after 28 days in greenhouse conditions but there was colony formation for the warm temperature trial. Overall, the results suggest that the thermophilic nature of *T. elongatus* BP1 could possibly serve as a natural biosafety guard during cool temperature seasons to limit their spread if they were to break containment and escape to the outside environment.

In the third aim, we examined the fate and stability of transgenes that are integrated into the chromosome of *T. elongatus* BP1. PCR results of genomic DNA suggests that transgenes seem very stable for up to the 24 months in which the study was conducted. Over time there is a very slow loss of transgenes. This is most evident in the analysis of the presence of the transgenes at 1 month and 24 months where there is clear loss two years later. An interesting trend seen with the insertion site PCR shows the cultures in the absence of antibiotic pressure

showed a more prominent 2.4 kb. This trend strongly suggests that without antibiotic pressure, there are more chromosomes that do not have transgenes present over time. The cDNA PCR shows that the kan resistance gene is still expressed after one year. To determine if any mutations occurred after one year, sequencing of the kan resistance gene was performed for a comparison analysis. Overall, we observed that the nucleotide and amino acid sequence for the kan resistance gene remained virtually unchanged and there were no nonsynonymous mutations that altered the protein sequence.

Altogether, the results of these research aims shows the importance of studying and understanding the potential ramifications of GE organisms as they are more commonly used in research and biotechnology. These findings can be valuable to aid the scientific community safely produce such organisms and assist government agencies to make policies to regulate their safe use.

FUTURE DIRECTIONS

This novel work, while providing clear results from the three aims, sets a foundation for further investigation. There are three recommended avenues for specific studies to expand upon the transgene fate and stability within the chromosome of GE cyanobacteria. These studies include quantitative PCR analysis to validate the loss of transgenes over time, investigating the consequences of nonsynonymous mutations found in the KIVD protein from whole-genome sequencing results and expansion, as well as improvement, of the nutrient deficiency study.

Quantify the Expression of the Kan Resistance Gene for the Transgene Study

The results of the genomic DNA PCR for the insertion site revealed that the transgenes remain in the chromosome of *T. elongatus* BP1 for up to 24 months. However, the genomic DNA PCR results for the insertion site suggests that there is a decrease in the presence of chromosomes that have integrated the transgenes in *T. elongatus* BP1 cultures in the absence of antibiotics over time. To further validate these findings, the expression of the kan resistance gene will be determined using quantitative PCR (qPCR), a molecular biology technique used to quantify the up or down regulation of gene expression [184]. The first step is to isolate total RNA and subsequently treated with a DNase enzyme to ensure there is no contaminating genomic DNA. To measure the gene expression, a TaqMan assay can be conducted (Figure 40). A TaqMan assay employs a hydrolysis probe that consists of a 5' fluorophore, s small segment of DNA complementary to the target gene and 3' quencher. These probes are added to a reaction tube with template DNA and forward/reverse primers. When the probe is intact during the denature and annealing step, the fluorophore is quenched and there is no fluorescent signal. During the elongation step, the 5' to 3' exonuclease activity of the DNA polymerase degrades the TaqMan probe, releasing the fluorophore and a signal is detected as it is no longer in the proximity of the quencher. The fluorescence detected is proportional to the released, allowing gene expression to be quantified [185]. For this test, experimental cultures can be analyzed at 1-month, 6-month, 12-month, 18-month and 24-month and compared to determine if there is a stepwise decrease in the expression of the kan resistance gene. We propose that over time, as the number of chromosomes copies with the transgene cassette integrated decreases, the expression of the kan resistance gene will also decrease.

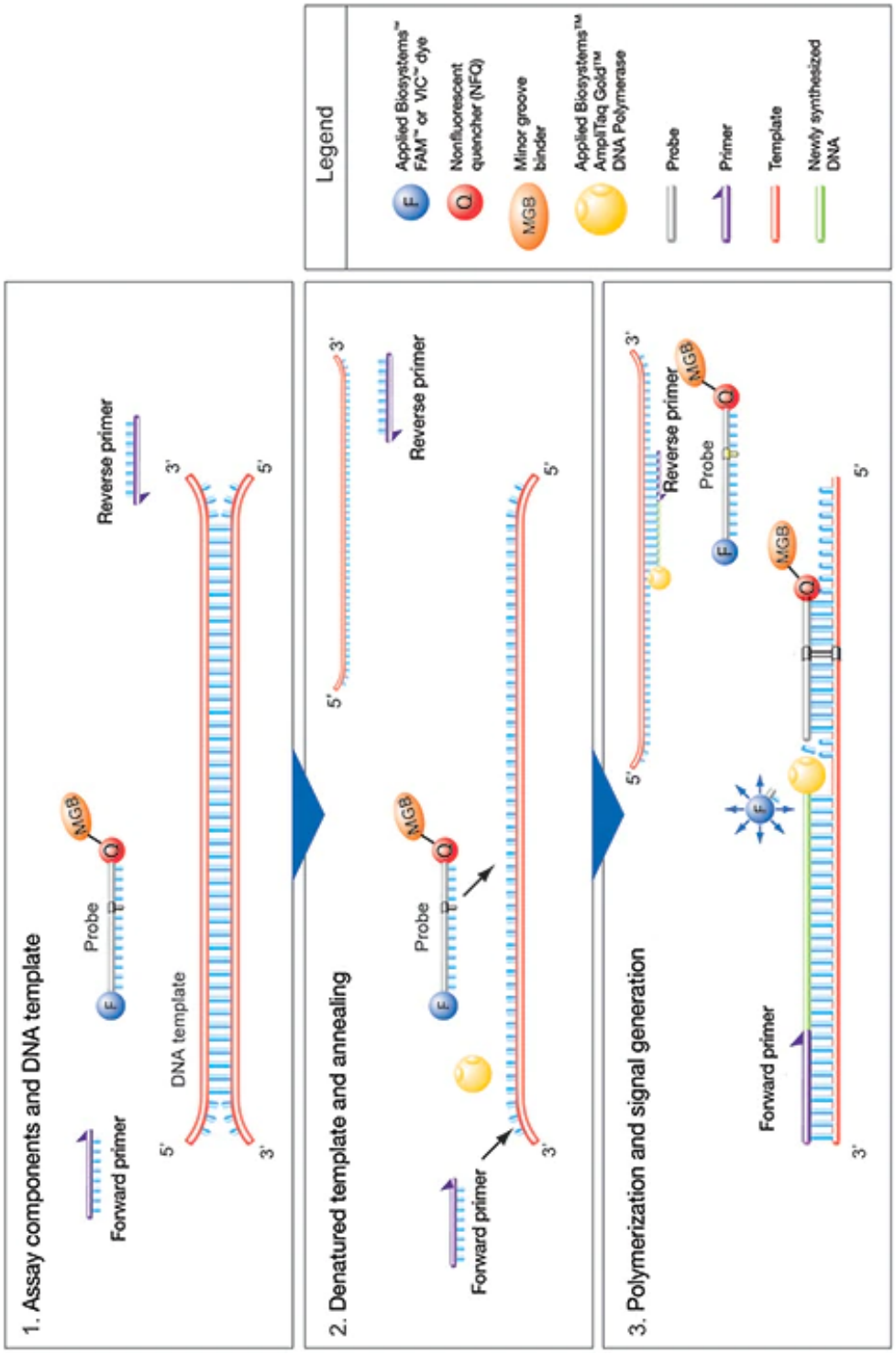


Figure 42. TaqMan Assay Schematic. This figure was reproduced from ThermoFisher Scientific (https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/real-time-pcr-basics/how-taqman-assays-work.html)

Explore Ramifications of Mutations on KIVD Protein

Current whole-genome sequencing results indicate that all the regulatory regions (nirA, cpc, slpA promoters) and the kan resistance gene within transgene cassette do not acquire any mutations after one year of incubation in absence of antibiotic pressure. However, it was interesting to find that the KIVD gene acquired many nonsynonymous mutations. To further investigate the occurrence of mutations within the transgene cassette, two additional GE *T. elongauts* BP1 experimental (- kan) samples were sent of Novogene for WGS. Using the same steps as the first sample (refer to Chapter IV), the sequence reads were assembled using the reference genome of *T. elongatus* BP1 with the transgene cassette inserted (Figure 11). Afterwards, an alignment of each component will be conducted to determine if any nonsynonymous mutations exist. The preliminary WGS results for the first GE *T. elongatus* BP1 revealed that after one year, it has obtained many mutations that cause the additional of a different amino acid. After annotating and compiling a list of nonsynonymous mutations from all three experimental samples, a protein visualization program (such as RasMol) can be used to determine if these mutations occur in hydrophobic regions, hydrophilic regions or loops to better identify mutation hotspots within the KIVD protein [186]. By understanding where these mutations are localized, we can speculate what sort of ramification the mutations have on the structure and function of the KIVD protein.

Genomic DNA PCR, Gene Expression, Whole-Genome Sequencing of Nutrient-Deficient Study Cultures

Current results for the nutrient study indicated that the transgenes are still present after 6 months of incubation within media with decreased iron, carbon, or phosphate sources. Although this

study shows a trend like that of the stability study, in that the 2.4 kb band representing chromosome without the transgene cassette seems to become brighter with time. However, the study must be continued past six months to determine if these conditions stress the cells to eliminate of the transgene cassette. To ascertain whether the kana resistance gene is expressed under these conditions, RT-PCR can be conducted. To determine whether these nutrient stresses encourage mutations within the transgene cassette, gene sequencing of the kan resistance gene and whole-genome sequencing can be conducted and subsequent comparison analysis at the primary and protein level.

There are a few ways the nutrient study can be improved upon. The exclusion of sodium carbonate in the media does not completely deplete source of carbon, as CO_2 from the air still can dissolve in the media. By limiting the availability of CO_2, the carbon source can be further reduced. Although, eliminating ferric ammonium citrate from the media was enough to decrease the growth of GE *T. elongatus* BP1, further steps can be taken to ensure the removal of trace amounts of iron by filtering BG-11 through Chelex columns and soaking flasks in EDTA [187]. To expand upon the nutrient-deficient study, other important nutrients, such as nitrogen and sulfur, can be reduced to investigate if it would expedite the loss of the transgene cassette over time. Nitrogen is an important component of biomolecules. including nucleic acids, amino acids and pigments [176, 188]. These nutrients are important for genomic material, so it is possible that reducing or eliminating these sources in the media could accelerate the loss of the transgene cassette over time.

REFERENCES

[1] Wang, G.H., Gamez, S., Raban, R.R., Marshall, J.M., Alphey, L., Li, M., et al., (2021). Combating mosquito-borne diseases using genetic control technologies. *Nat. Commun*., **12**, 4388.
[2] Zheng, X., Zhang, D., Li, Y., Yang, C., Wu, Y., Liang, X., et al., (2019). Incompatible and sterile insect techniques combined eliminate mosquitoes. *Nature*., **572**, 56-61.
[3] Häcker, I., Schetelig, M.F., (2018) Molecular tools to create new strains for mosquito sexing and vector control. *Parasit. Vectors*., **11**, 645.
[4] Zhang, X., Li, X., Yang, Z., Tao, K., Wang, Q., Dai, B., et al., (2019). A review of pig liver xenotransplantation: current problems and recent progress. *Xenotransplantation*., **26**, e12497.
[5] Cooper, D.K.C., Ezzelarab, M., Iwase, H., Hara, H., (2018). Perspectives on the optimal genetically engineered pig in 2018 for initial clinical trials of kidney or heart xenotransplantation. *Transplantation*., **102**, 1974-1982.
[6] Hamilton, T.L., Bryant, D.A., Macalady, J.L., (2016). The role of biology in planetary evolution: cyanobacterial primary production in low-oxygen Proterozoic oceans. *Environ. Microbiol*., **18**, 325-340.
[7] Sánchez-Baracaldo, P., Cardona, T., (2020). On the origin of oxygenic photosynthesis and cyanobacteria. *New. Phytol*., **225**, 1440-1446.
[8] Turmo, A., Gonzalez-Esquer, C.R., Kerfeld, C.A., (2017). Carboxysomes: metabolic modules for CO2 fixation. *FEMS. Microbiol. Let.,* **18**, 364.
[9] Dai, W., Chen, M., Myers, C., Ludtke, S.J., Pettitt, B.M., King, J.A., et al., (2018). Visualizing individual RuBisCO and its assembly into carboxysomes in marine cyanobacteria by cryo-electron tomography. *J. Mol. Biol*., **430**, 4156-4167.
[10] Zehr, J.P., (2011). Nitrogen fixation by marine cyanobacteria. *Trends. Microbiol*., **19**, 162-173.
[11] Wu, C., Fu, F.X., Sun, J., Thangaraj, S., Pujari, L., (2018). Nitrogen fixation by Trichodesmium and unicellular diazotrophs in the northern south China sea and the Kuroshio in summer. *Sci. Rep*., **8**, 2415.
[12] Chen, M.Y., Teng, W.K., Zhao, L., Hu, C.X., Zhou, Y.K., Han, B.P., et al., (2021). Comparative genomics reveals insights into cyanobacterial evolution and habitat adaptation. *ISME. J*., **15**, 211-227.
[13] Larsson, J., Nylander, J.A.A., Bergman, B., (2011). Genome fluctuations in cyanobacteria reflect evolutionary, developmental and adaptive traits. *BMC. Evol. Biol*., **11**, 187.
[14] Zhang, A., Carroll, A.L., Atsumi, S., (2017). Carbon recycling by cyanobacteria: improving CO2 fixation through chemical production. *FEMS. Microbiol. Lett*., **364**, 16.
[15] Mills, L.A., McCormick, A.J., Lea-Smith, D.J., (2020). Current knowledge and recent advances in understanding metabolism of the model cyanobacterium Synechocystis sp. PCC 6803. *Biosci. Rep*., **40**, 4.
[16] Bothe, H., Schmitz, O., Yates, M.G., Newton, W.E., (2010). Nitrogen fixation and hydrogen metabolism in cyanobacteria. *Microbiol. Mol. Biol. Rev*., **74**, 529-551.
[17] Ponce-Toledo, R.I., López-García, P., Moreira, D., (2019). Horizontal and endosymbiotic gene transfer in early plastid evolution. *New. Phytol*., **224**, 618-624.
[18] Jabbur , M.L., Johnson, C.H., (2022). Spectres of clock evolution: past, present, and yet to come. *Front. Physiol*., **12**, 815847.

[19] Vavitsas, K., Kugler, A., Satta, A., Hatzinikolaou, D.G., Lindblad, P., Fewer, D.P., et al., (2021). Doing synthetic biology with photosynthetic microorganisms. *Physiol. Plant*., **173**, 624-638.
[20] Khalifa, S.A.M., Shedid, E.S., Saied, E.M., Jassbi, A.R., Jamebozorgi, F.H., Rateb, M.E., et al., (2021). Cyanobacteria-from the oceans to the potential biotechnological and biomedical applications. *Mar. Drugs*., **19**, 5.
[21] Cameron, D.E., Bashor, C.J., Collins, J.J., (2014). A brief history of synthetic biology. *Nat. Rev. Microbiol*., **12**, 381-390.
[22] Bange, G., Waldminghaus, T., (2016). Editorial overview: synthetic biology – from understanding to engineering biology and back. *Curr. Opin. Chem. Biol*., **34**, A151-A153.
[23] Khan, S., Ullah, M.W., Siddique, R., Nabi, G., Manan, S., Yousaf, M., et al., (2016). Role of recombinant DNA technology to improve life. *Int J Genomics*., **2016**, 2405954.
[24] Niraula, P.M., Fondong, V.N.. (2021). Development and adoption of genetically engineered plants for virus resistance: advances, opportunities and challenges. *Plants (Basel)*., **10**, 2339.
[25] Yin, T., Diao, Z., Blum, N.T., Qiu, L., Ma, A., Huang, P., (2021). Engineering bacteria and bionic bacterial derivatives with nanoparticles for cancer therapy. *Small*., **18**, e2104643.
[26] Upadhyay, A., Feltman, N.R., Sychla, A., Janzen, A., Das, S.R., Maselko, M., et al., (2022). Genetically engineered insects with sex-selection and genetic incompatibility enable population suppression. *eLife*., **11**, e71230.
[27] Maksimenko, O.G., Deykin, A.V., Khodarovich, Y.M., Georgiev, P.G., (2013). Use of transgenic animals in biotechnology: prospects and problems. *Acta. naturae*., **5**, 33-46.
[28] Shokravi, H., Shokravi, Z., Heidarrezaei, M., Ong, H.C., Rahimian-Koloor, S.S., Petrů, M., et al., (2021). Fourth generation biofuel from genetically modified algal biomass: challenges and future directions. *Chemosphere*., **285**, 131535.
[29] Aro, E.M., (2016). From first generation biofuels to advanced solar biofuels. *Ambio*., **45**, S24-S31.
[30] Berla, B.M., Saha, R., Immethun, C.M., Maranas, C.D., Moon, T.S., Pakrasi, H.B., (2013). Synthetic biology of cyanobacteria: unique challenges and opportunities. *Front. Microbiol*., **4**, 246.
[31] Ahmad, A.L., Yasin, N.H.M., Derek, C.J.C., Lim, J.K., (2011). Microalgae as a sustainable energy source for biodiesel production: a review. *Renew. Sust. Energ. Rev*., **15**, 584-593.
[32] Gressel, J., (2008). Transgenics are imperative for biofuel crops. *Plant. Sci*., **174**, 246-263.
[33] Patel, A., Matsakas, L., Rova, U., Christakopoulos, P., (2019). A perspective on biotechnological applications of thermophilic microalgae and cyanobacteria. *Bioresour. Technol*., **278**, 424-434.
[34] Sarsekeyeva, F., Zayadan, B.K., Usserbaeva, A., Bedbenov, V.S., Sinetova, M.A., Los, D.A., (2015). Cyanofuels: biofuels from cyanobacteria. Reality and perspectives. *Photosynth. Res*., **125**, 329-340.
[35] Wijffels, R.H., Kruse, O., Hellingwerf, K.J., (2013). Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae. *Curr. Opin. Biotechnol*., **24**, 405-413.
[36] Hitchcock, A., Hunter, C.N., Canniffe, D.P., (2020). Progress and challenges in engineering cyanobacteria as chassis for light-driven biotechnology. *Microb. Biotechnol*., **13**, 363-367.
[37] Ruffing, A.M., (2011). Engineered cyanobacteria: teaching an old bug new tricks. *Bioeng. Bugs*., **2**, 136-149.

[38] Nguyen, V.H., Kim, H.S., Ha, J.M., Hong, Y., Choy, H.E., Min, J.J., (2010). Genetically engineered salmonella typhimurium as an imageable therapeutic probe for cancer. *Cancer. Res*., **70**, 18-23.
[39] González-Ponce, K.S., Casados-Vázquez, L.E., Salcedo-Hernández, R., Bideshi, D.K., del Rincón-Castro, M.C., Barboza-Corona, J.E., (2017). Recombinant Bacillus thuringiensis subsp. kurstaki HD73 strain that synthesizes cry1Ac and chimeric chiA74Δsp chitinase inclusions. *Arch. Microbiol*., **199**, 627-633.
[40] Ferreira, S.A., Pitz, K.Y., Manshardt, R., Zee, F., Fitch, M., Gonsalves, D., (2002). Virus coat protein transgenic papaya provides practical control of papaya ringspot virus in Hawaii. *Plant. Dis*., **86**, 101-105.
[41] Bonfim, K., Faria, J.C., Nogueira, E.O., Mendes, E.A., Aragão, F.J., (2007). RNAi-mediated resistance to bean golden mosaic virus in genetically engineered common bean (Phaseolus vulgaris). *Mol. Plant. Microbe. Interact*., **20**, 717-726.
[42] Concha, C., Palavesam, A., Guerrero, F.D., Sagel, A., Li, F., Osborne, J.A., et al., (2016). A transgenic male-only strain of the new world screwworm for an improved control program using the sterile insect technique. *BMC. Biol*., **14**, 72.
[43] Otsuki, R., Yamamoto, M., Matsumoto, E., Iwamoto, S.I., Sezutsu, H., Suzui, M., et al., (2017). Bioengineered silkworms with butterfly cytotoxin-modified silk glands produce sericin cocoons with a utility for a new biomaterial. *Proc. Natl. Acad. Sci. USA*., **114**, 6740-6745.
[44] Kolber-Simonds, D., Lai, L., Watt, S.R., Denaro, M., Arn, S., Augenstein, M.L., et al., (2004). Production of alpha-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. *Proc. Natl. Acad. Sci. USA*., **101**, 7335-7340.
[45] Lai, L., Kolber-Simonds, D., Park, K.W., Cheong, H.T., Greenstein, J.L., Im, G.S., et al., (2002). Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science*., **295**, 1089-1092.
[46] Mukae, T., Okumura, S., Watanobe, T., Yoshii, K., Tagami, T., Oishi, I., (2020). Production of recombinant monoclonal antibodies in the egg white of gene-targeted transgenic chickens. *Genes*., **12**, 38.
[47] Kopka, J., Schmidt, S., Dethloff, F., Pade, N., Berendt, S., Schottkowski, M., et al., (2017). Systems analysis of ethanol production in the genetically engineered cyanobacterium Synechococcus sp PCC 7002. *Biotechnol. Biofuels*., **10**, 56.
[48] Lan, E.I., Ro, S.Y., Liao, J.C. (2013). Oxygen-tolerant coenzyme A-acylating aldehyde dehydrogenase facilitates efficient photosynthetic n-butanol biosynthesis in cyanobacteria. *Energy Environ Sci*. **6**, 2672-2681.
[49] Machado, I.M.P., Atsumi, S., (2012). Cyanobacterial biofuel production. *J Biotechnol*., **162**, 50-56.
[50] Miao, R., Xie, H., Ho, F.M. and Lindblad, P. (2018). Protein engineering of alpha-ketoisovalerate decarboxylase for improved isobutanol production in Synechocystis PCC 6803. *Metab Eng*. **47**, 42-48.
[51] Miao, R., Xie, H., Lindblad, P., (2018). Enhancement of photosynthetic isobutanol production in engineered cells of Synechocystis PCC 6803. *Biotechnol. Biofuels*., **11**, 267.
[52] Case, A.E, Atsumi, S., (2016). Cyanobacterial chemical production. *J. Biotechnol*., **231**, 106-114.
[53] Gao, Z., Zhao, H., Li, Z., Tan, X., Lu, X., (2012). Photosynthetic production of ethanol from carbon dioxide in genetically engineered cyanobacteria. *Energy. Environ. Sci*., **5**, 9857-9865.

[54] Angermayr, S.A., van der Woude, A.D., Correddu, D., Vreugdenhil, A., Verrone, V., Hellingwerf, K.J., (2014). Exploring metabolic engineering design principles for the photosynthetic production of lactic acid by Synechocystis sp. PCC6803. *Biotechnol. Biofuels*., **7**, 99.
[55] Atsumi, S., Higashide, W., Liao J.C., (2002). Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Nat. Biotechnol*., **27**, 1177-1180.
[56] Hirokawa, Y., Maki, Y., Hanai, T., (2017). Improvement of 1,3-propanediol production using an engineered cyanobacterium, Synechococcus elongatus by optimization of the gene expression level of a synthetic metabolic pathway and production conditions. *Metab. Eng*., **39**, 192-199.
[57] Koksharova, O.A, Wolk, C.P., (2002). Genetic tools for cyanobacteria. *Appl. Microbiol. Biotechnol*., **58**, 123-137.
[58] Sun, T., Li, S., Song, X., Diao, J., Chen, L., Zhang, W., (2018). Toolboxes for cyanobacteria: recent advances and future direction. *Biotechnol. Adv*., **36**, 1293-1307.
[59] Yadav, I., Rautela, A., Kumar, S., (2021). Approaches in the photosynthetic production of sustainable fuels by cyanobacteria using tools of synthetic biology. *World. J. Microbiol. Biotechnol*., **37**, 201.
[60] Yamaoka, T., Satoh, K., Katoh, S., (1978). Photosynthetic activities of a thermophilic blue-green alga. *Plant. Cell. Physiol*., **19**, 943-954.
[61] Nakamura, Y., Kaneko, T., Sato, S., Ikeuchi, M., Katoh, H., Sasamoto, S., et al., (2002). Complete genome structure of the thermophilic cyanobacterium Thermosynechococcus elongatus BP-1. *DNA. Res*., **9**, 123-130.
[62] Lambertz, J., Liauw, P., Whitelegge, J.P., Nowaczyk, M.M., (2021). Mass spectrometry analysis of the photosystem II assembly factor Psb27 revealed variations in its lipid modification. *Photosynth. Res*.
[63] Zabret, J., Bohn, S., Schuller, S.K., Arnolds, O., Möller, M., Meier-Credo, J., et al., (2021). Structural insights into photosystem II assembly. *Nat. Plants*., **7**, 524-538.
[64] Çoruh, O., Frank, A., Tanaka, H., Kawamoto, A., El-Mohsnawy, E., Kato, T., et al., (2021). Cryo-EM structure of a functional monomeric Photosystem I from Thermosynechococcus elongatus reveals red chlorophyll cluster. *Commun. Biol*., **4**, 304.
[65] Karalis, D.T., Karalis, T., Karalis, S., Kleisiari, A.S., (2020). Genetically modified products, perspectives and challenges. *Cureus*., **12**, e7306.
[66] Trump, B.D., Galaitsi, S.E., Appleton, E., Bleijs, D.A., Florin, M.V., Gollihar, J.D., et al., (2020). Building biosecurity for synthetic biology. *Mol. Syst. Biol*., **16**, e9723.
[67] Gressel, J., van der Vlugt, C.J.B., Bergmans, H.E.N., (2013). Environmental risks of large scale cultivation of microalgae: mitigation of spills. *Algal. Res*., **2**, 286-298.
[68] Hails, R.S., Morley, K., (2005). Genes invading new populations: a risk assessment perspective. *Trends. Ecol. Evol*., **20**, 245-252.
[69] Henley, W.J., Litaker, R.W., Novoveská, L., Duke, C.S., Quemada, H.D., Sayre, R.T., (2013). Initial risk assessment of genetically modified (GM) microalgae for commodity-scale biofuel cultivation. *Algal. Res*., **2**, 66-77.
[70] Sjostrand, J., Tofigh, A., Daubin, V., Arvestad, L., Sennblad, B., Lagergren, J., (2014). A Bayesian method for analyzing lateral gene transfer. *Syst. Biol*., **63**, 409-420.
[71] Snow, A.A., Smith, V.H., (2012). Genetically engineered algae for biofuels: a key role for ecologists. *Bioscience*., **62**, 765-768.

[72] Yoshida, N., Sato, M., (2009). Plasmid uptake by bacteria: a comparison of methods and efficiencies. *Appl. Microbiol. Biotechnol*., **83**, 791-798.
[73] Lorenz, M.G., Wackernagel, W., (1994). Bacterial gene transfer by natural genetic transformation in the environment. *Microbiol. Rev*., **58**, 563-602.
[74] Hanahan, D., Jessee, J., Bloom, F.R., (1991). Plasmid transformation of Escherichia coli and other bacteria. *Meth. Enzymol*., **204**, 63-113.
[75] Gehl, J., (2003). Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research. *Acta. Physiol. Scand*., **177**, 437-447.
[76] Koksharova, O., Wolk, C., (2002). Genetic tools for cyanobacteria. *Appl. Microbiol. Biotechnol*., **58**, 123-137.
[77] Thiel, T., Poo, H., (1989). Transformation of a filamentous cyanobacterium by electroporation. *J. Bacteriol*., **171**, 5743-5746.
[78] Zang, X., Liu, B., Liu, S., Arunakumara, K.K., Zhang, X., (2007). Optimum conditions for transformation of Synechocystis sp. PCC 6803. *J. Microbiol*., **45**, 241-245.
[79] Iwai, M., Katoh, H., Katayama, M. and Ikeuchi, M. (2004). Improved genetic transformation of the thermophilic cyanobacterium, Thermosynechococcus elongatus BP-1. *Plant Cell Physiol*. **45**,171-175.
[80] Katoh, H., Itoh, S., Shen, J.R., Ikeuchi, M., (2001). Functional analysis of psbV and a novel c-type cytochrome gene psbV2 of the thermophilic cyanobacterium Thermosynechococcus elongatus strain BP-1. *Plant. Cell. Physiol*., **42**, 599-607.
[81] Mühlenhoff, U., Chauvat, F., (1996). Gene transfer and manipulation in the thermophilic cyanobacterium Synechococcus elongatus. *Mol. Gen. Genet*., **252**, 93-100.
[82] Lorenz, T.C., (2012). Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *J. Vis. Exp*., **63**, 3998.
[83] Garibyan, L, Avashia, N., (2013). Polymerase chain reaction. *J. Invest. Dermatol*., **133**, 1-4.
[84] Zhu, H., Zhang, H., Xu, Y., Laššáková, S., Korabečná, M., Neužil, P., (2020). PCR past, present and future. *BioTechniques*., **69**, 317-325.
[85] Green, M.R., Sambrook, J., (2019). Polymerase chain reaction. *Cold. Spring. Harb. Protoc*., **6**.
[86] Weier, H.U., Gray, J.W., (1988). A programmable system to perform the polymerase chain reaction. *DNA*., **7**, 441-447.
[87] Cavanaugh, S.E., Bathrick, A.S., (2018). Direct PCR amplification of forensic touch and other challenging DNA samples: a review. *Forensic. Sci. Int. Genet*., **32**, 40-49.
[88] Hoorfar, J., (2011). Rapid detection, characterization, and enumeration of foodborne pathogens. *APMIS. Suppl*., **133**, 1-24.
[89] Liu, H., Whitehouse, C.A., Li, B., (2018). Presence and persistence of Salmonella in water: the impact on microbial quality of water and food safety. *Front. Public. Health*., **6**, 159.
[90] Mahanama, A., Wilson-Davies, E., (2021). Insight into PCR testing for surgeons. *Surgery (Oxf)*., **39**, 759-768.
[91] Krüger, J., Schleinitz, D., (2017). Genetic fingerprinting using microsatellite markers in a multiplex PCR reaction: a compilation of methodological approaches from primer design to detection systems. *Methods. Mol. Biol*., **1492**, 1-15.
[92] Pearson, L.A., D'Agostino, P.M., Neilan, B.A., (2021). Recent developments in quantitative PCR for monitoring harmful marine microalgae. *Harmful Algae*., **108**, 102096.
[93] Bachman, J., (2013). Reverse-transcription PCR (RT-PCR). *Meth. Enzymol*., **530**, 67-74.

[94] Sauer, N.J., Mozoruk, J., Miller, R.B., Warburg, Z.J., Walker, K.A., Beetham, P.R., et al., (2016). Oligonucleotide-directed mutagenesis for precision gene editing. *Plant. Biotechnol. J.*, **14**, 496-502.
[95] Mo, Y., Wan, R., Zhang, Q., (2012). Application of reverse transcription-PCR and real-time PCR in nanotoxicity research. *Methods. Mol. Biol*., **926**, 99-112.
[96] Bastard, J.P., Chambert, S., Ceppa, F., Coude, M., Grapez, E., Loric, S., et al., (2008). RNA isolation and purification methods. *Ann. Biol. Clin. (Paris)*., **60**, 513-523.
[97] Tzertzinis, G., Tabor, S., Nichols, N.M., (2008) RNA-dependent DNA polymerases. *Curr. Protoc. Mol. Biol*., **84**, 3.7.1-3.7.4.
[98] Tajadini, M., Panjehpour, M., Javanmard, S.H., (2014). Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. *Adv. Biomed. Res*., **3**, 85.
[99] Sanger, F., Nicklen, S., Coulson, A.R., (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*., **74**, 5463-5467.
[100] Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., et al., (2001). Initial sequencing and analysis of the human genome. *Nature*., **409**, 860-921.
[101] Schloss, J.A., (2008). How to get genomes at one ten-thousandth the cost. *Nat. Biotechnol*., **26**, 1113-1115.
[102] Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., et al., (2001). The sequence of the human genome. *Science*., **291**, 1304-1351.
[103] Reuter, J.A., Spacek, D.V., Snyder, M.P., (2015). High-throughput sequencing technologies. *Mol. Cell*., **58**, 586-597.
[104] Slatko, B.E., Gardner, A.F., Ausubel, F.M., (2018). Overview of next-generation sequencing technologies. *Curr. Protoc. Mol. Biol*., **122**, e59.
[105] Heather, J.M., Chain, B., (2016). The sequence of sequencers: the history of sequencing DNA. *Genomics*., **107**, 1-8.
[106] Bunnik, E., Le Roch, K., (2013). An introduction to functional genomics and systems biology. *Adv. Wound. Care*., **2**, 490-498.
[107] Kyriakidou, M., Tai, H.H., Anglin, N.L., Ellis, D., Strömvik, M.V., (2018). Current strategies of polyploid plant genome sequence assembly. *Front. Plant. Sci*., **9**, 1660.
[108] Khan, A.R., Pervez, M.T., Babar, M.E., Naveed, N., Shoaib, M., (2018). A comprehensive study of de novo genome assemblers: current challenges and future prospective. *Evol. Bioinform. Online*., **14**, 1176934318758650.
[109] Miller, J.R., Koren, S., Sutton, G., (2010). Assembly algorithms for next-generation sequencing data. *Genomics*., **95**, 315-327.
[110] Ghurye, J.S., Cepeda-Espinoza, V., Pop, M., (2016). Metagenomic assembly: overview, challenges and applications. *Yale. J. Biol. Med*., **89**, 353-362.
[111] Li, Z., Chen, Y., Mu, D., Yuan, J., Shi, Y., Zhang, H., et al., (2011). Comparison of the two major classes of assembly algorithms: overlap–layout–consensus and de-bruijn-graph. *Brief. Funct. Genomics*., **11**, 25-37.
[112] Sutton, G.G., White, O., Adams, M.D., Kerlavage, A.R., (1995). TIGR assembler: a new tool for assembling large shotgun sequencing projects. *Genome. Sci. Technol*., **1**, 9-19.
[113] Jeck, W.R., Reinhardt, J.A., Baltrus, D.A., Hickenbotham, M.T., Magrini, V., Mardis, E.R., et al., (2007). Extending assembly of short DNA sequences to handle error. *Bioinformatics*., **23**, 2942-2944.

[114] Myers, E.W., Sutton, G.G., Delcher, A.L., Dew, I.M., Fasulo, D.P., Flanigan, M.J., et al., (2000). A whole-genome assembly of Drosophila. *Science*., **287**, 2196-2204.
[115] Pevzner, P.A., Tang, H., Waterman, M.S., (2001). An eulerian path approach to DNA fragment assembly. *Proc. Natl. Acad. Sci. USA*., **98**, 9748-9753.
[116] Simpson, J.T., Wong, K., Jackman, S.D., Schein, J.E., Jones, S.J.M., Birol, I., (2009). ABySS: a parallel assembler for short read sequence data. *Genome. Res*., **19**, 1117-1123.
[117] Butler, J., MacCallum, I., Kleber, M., Shlyakhter, I.A., Belmonte, M.K., Lander, E.S., et al., (2008). ALLPATHS: de novo assembly of whole-genome shotgun microreads. *Genome. Res*., **18**, 810-820.
[118] Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., et al., (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol*., **9**, 455-477.
[119] Klinthong, W., Yang, Y.H., Huang, C.H., Tan, C.S., (2015). A review: microalgae and their applications in CO2 capture and renewable energy. *Aerosol. Air. Qual. Res*., **15**, 712-742.
[120] Singh, V., Chaudhary, D.K., Mani, I., Dhar, P.K., (2016). Recent advances and challenges of the use of cyanobacteria towards the production of biofuels. *Renew. Sust. Energ. Rev*., **60**, 1-10.
[121] Gao, X., Sun, T., Pei, G., Chen, L., Zhang, W., (2016). Cyanobacterial chassis engineering for enhancing production of biofuels and chemicals. *Appl. Microbiol. Biotechnol*., **100**, 3401-3413.
[122] Kopka, J., Schmidt, S., Dethloff, F., Pade, N., Berendt, S., Schottkowski, M., et al., (2017). Systems analysis of ethanol production in the genetically engineered cyanobacterium Synechococcus sp. PCC 7002. *Biotechnol. Biofuels*., **10**, 56.
[123] Oliver, N.J., Rabinovitch-Deere, C.A., Carroll, A.L., Nozzi, N.E., Case, A.E., Atsumi, S., (2016). Cyanobacterial metabolic engineering for biofuel and chemical production. *Curr. Opin. Chem. Biol*., **35**, 43-50.
[124] Couso, I., Vila, M., Rodriguez, H., Vargas, M.A., León, R., (2011). Overexpression of an exogenous phytoene synthase gene in the unicellular alga Chlamydomonas reinhardtii leads to an increase in the content of carotenoids. *Biotechnol. Prog*., **27**, 54-60.
[125] Farrokh, P., Sheikhpour, M., Kasaeian, A., Asadi, H., Bavandi, R., (2019). Cyanobacteria as an eco-friendly resource for biofuel production: a critical review. *Biotechnol. Prog*., **35**, e2835.
[126] Gimpel, J.A., Specht, E.A., Georgianna, D.R., Mayfield, S.P., (2013). Advances in microalgae engineering and synthetic biology applications for biofuel production. *Curr. Opin. Chem. Biol*., **17**, 489-495.
[127] Lan, E.I., Liao, J.C., (2011). Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide. *Metab. Eng*., **13**, 353-363.
[128] Radakovits, R., Eduafo, P.M., Posewitz, M.C., (2011). Genetic engineering of fatty acid chain length in Phaeodactylum tricornutum. *Metab. Eng*., **13**, 89-95.
[129] Robertson, D.E., Jacobson, S.A., Morgan, F., Berry, D., Church, G.M., Afeyan, N.B., (2011). A new dawn for industrial photosynthesis. *Photosynth. Res*., **107**, 269-277.
[130] Shuba Eyasu, S., Kifle, D., (2018). Microalgae to biofuels: 'promising' alternative and renewable energy, review. *Renew. Sust. Energ. Rev*., **81**, 743-755.
[131] Jain, R., Rivera, M.C., Moore, J.E., Lake, J.A., (2002). Horizontal gene transfer in microbial genome evolution. *Theor. Popul. Biol*., **61**, 489-495.

[132] Soucy, S.M., Huang, J., Gogarten, J.P., (2015). Horizontal gene transfer: building the web of life. *Nat. Rev. Genet*., **16**, 472-482.
[133] Gyles, C., Boerlin, P., (2014). Horizontally transferred genetic elements and their role in pathogenesis of bacterial disease. *Vet. Pathol*., **51**, 328-340.
[134] Nguyen, T.H., Barnes, C.L., Agola, J.P., Sherazi, S., Greene, L.H., Lee, J.W., (2019). Demonstration of horizontal gene transfer from genetically engineered Thermosynechococcus elongatus BP1 to wild-type E. coli DH5α. *Gene*., **704**, 49-58.
[135] Ventola, C.L., (2015). The antibiotic resistance crisis: part 1: causes and threats. *P T*., **40**, 277-83.
[136] Juhas, M., (2015). Horizontal gene transfer in human pathogens. *Crit. Rev. Microbiol*., **41**, 101-108.
[137] Centers for Disease Control and Prevention. (2019). Antibiotic resistance threats in the United States.
[138] Leffler, D.A., Lamont, J.T., (2015). Clostridium difficile infection. *N. Engl. J. Med*., **372**, 1539-1548.
[139] Lessa, F.C., Mu, Y., Bamberg, W.M., Beldavs, Z.G., Dumyati, G.K., Dunn, J.R., et al., (2015). Burden of Clostridium difficile infection in the United States. *N. Engl. J. Med*., **372**, 825-834.
[140] McGlone, S.M., Bailey, R.R., Zimmer, S.M., Popovich, M.J., Tian, Y., Ufberg, P., et al., (2012). The economic burden of Clostridium difficile. *Clin. Microbiol. Infect*., **18**, 282-289.
[141] Spigaglia, P., (2016). Recent advances in the understanding of antibiotic resistance in Clostridium difficile infection. *Ther. Adv. Infect. Dis*., **3**, 23-42.
[142] Jasni, A.S., Mullany, P., Hussain, H., Roberts, A.P., (2010). Demonstration of conjugative transposon Tn5397-mediated horizontal gene transfer between Clostridium difficile and Enterococcus faecalis. *Antimicrob. Agents. Chemother*., **54**, 4924-4926.
[143] Lodise, T.P., Jr. McKinnon, P.S., (2007). Burden of methicillin-resistant Staphylococcus aureus: focus on clinical and economic outcomes. *Pharmacotherapy*., **27**, 1001-1012.
[144] Guo, Y., Song, G., Sun, M., Wang, J., Wang, Y., (2020). Prevalence and therapies of antibiotic-resistance in Staphylococcus aureus. *Front. Cell. Infect. Microbiol*., **10**, 107.
[145] Craft, K.M., Nguyen, J.M., Berg, L.J., Townsend, S.D., (2019). Methicillin-resistant Staphylococcus aureus (MRSA): antibiotic-resistance and the biofilm phenotype. *Medchemcomm*., **10**, 1231-1241.
[146] Encinas, D., Garcillán-Barcia, M.P., Santos-Merino, M., Delaye, L., Moya, A., de la Cruz, F., (2014). Plasmid conjugation from proteobacteria as evidence for the origin of xenologous genes in cyanobacteria. *J. Bacteriol*., **196**, 1551-1559.
[147] Brahamsha, B., (1996). A genetic manipulation system for oceanic cyanobacteria of the genus Synechococcus. *Appl. Environ. Microbiol*., **62**, 1747-1751.
[148] Thiel, T., Wolk, C.P., (1987). Conjugal transfer of plasmids to cyanobacteria. *Meth Enzymol*. **153**, 232-243.
[149] Sode, K., Tatara, M., Ogawa, S., Matsunaga, T., (1992). Maintenance of broad host range vector pKT230 in marine unicellular cyanobacteria. *FEMS. Microbiol. Lett*., **99**, 73-78.
[150] Kreps, S., Ferino, F., Mosrin, C., Gerits, J., Mergeay, M., Thuriaux, P., (2004). Conjugative transfer and autonomous replication of a promiscuous IncQ plasmid in the cyanobacterium Synechocystis PCC 6803. *Mol. Gen. Genet*., **221**, 129-133.
[151] Nguyen-Jones, T.H., (2021). Genetically engineered Thermosynechococcus elongatus BP1: assessment of potential biorisks and biofuel production. Old Dominion University.

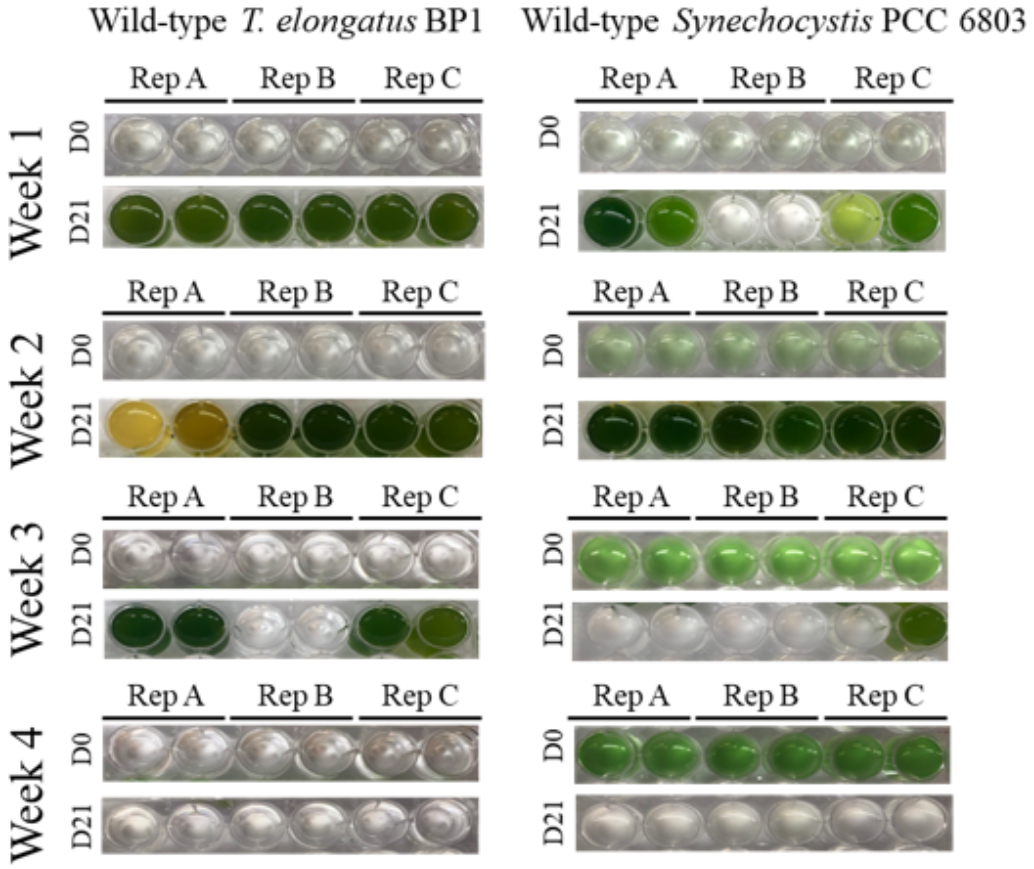
[152] Zhou, Y., Sun, T., Chen, Z., Song, X., Chen, L., Zhang, W., (2019). Development of a new biocontainment strategy in model cyanobacterium Synechococcus strains. *ACS. Synth. Biol*., **8**, 2576-2584.
[153] Mandell, D.J., Lajoie, M.J., Mee, M.T., Takeuchi, R., Kuznetsov, G., Norville, J.E., et al., (2015). Biocontainment of genetically modified organisms by synthetic protein design. *Nature*., **518**, 55.
[154] Clark, R.L., Gordon, G.C., Bennett, N.R., Lyu, H., Root, T.W., Pfleger, B.F., (2018). High CO2 requirement as a mechanism for the containment of genetically modified cyanobacteria. *ACS. Synth. Biol*., **7**, 384-391.
[155] Čelešnik, H., Tanšek, A., Tahirović, A., Vižintin, A., Mustar, J., Vidmar, V., et al., (2016). Biosafety of biotechnologically important microalgae: intrinsic suicide switch implementation in cyanobacterium Synechocystis sp. PCC 6803. *Biol. Open*., **5**, 519-528.
[156] García, J.L., Díaz, E. (2014). Plasmids as tools for containment. *Microbiol. Spectr*., **2**, 5.
[157] Torres, B., Jaenecke, S., Timmis, K.N., García, J.L., Díaz, E., (2003). A dual lethal system to enhance containment of recombinant micro-organisms. *Microbiology*., **149**, 3595-3601.
[158] Sacko, O., Barnes, C.L., Greene, L.H., Lee, J.W., (2020). Survivability of wild-type and genetically engineered Thermosynechococcus elongatus BP1 with different temperature conditions. *Appl. Biosaf*., **25**, 104-117.
[159] Mandell, D.J., Lajoie, M.J., Mee, M.T., Takeuchi, R., Kuznetsov, G., Norville, J.E., et al., (2015). Biocontainment of genetically modified organisms by synthetic protein design. *Nature*., **518**, 55-60.
[160] Gallagher, R.R., Patel, J.R., Interiano, A.L., Rovner, A.J., Isaacs, F.J., (2015). Multilayered genetic safeguards limit growth of microorganisms to defined environments. *Nucleic. Acids. Res*., **43**, 1945-1954.
[161] Cai, Y., Agmon, N., Choi, W.J., Ubide, A., Stracquadanio, G., Caravelli, K., et al., (2015). Intrinsic biocontainment: multiplex genome safeguards combine transcriptional and recombinational control of essential yeast genes. *Proc. Natl. Acad. Sci. USA*., **112**, 1803-1808.
[162] Wright, O., Delmans, M., Stan, G.B., Ellis, T., (2015). GeneGuard: A modular plasmid system designed for biosafety. *ACS. Synth. Biol*., **4**, 307-316.
[163] Sacko, O., (2021). Improvement of biochar through ozonization and biosafety of genetically engineered cyanobacteria. Old Dominion University.
[164] Handler, A.M., (2004). Understanding and improving transgene stability and expression in insects for SIT and conditional lethal release programs. *Insect. Biochem. Mol. Biol*., **34**,121-130.
[165] Li, J., Brunner, A.M., Meilan, R., Strauss, S.H., (2009). Stability of transgenes in trees: expression of two reporter genes in poplar over three field seasons. *Tree. Physiol*., **29**, 299-312.
[166] Abidin, A.A.Z., Othman, N.A., Yusoff, F.M., Yusof, Z.N.B., (2021). Determination of transgene stability in Nannochloropsis sp. transformed with immunogenic peptide for oral vaccination against vibriosis. *Aquac. Int*., **29**, 477-486.
[167] Dietz-Pfeilstetter, A., (2010). Stability of transgene expression as a challenge for genetic engineering. *Plant. Sci*., **179**, 164-167.
[168] Finnegan, J., McElroy, D., (1994) Transgene Inactivation: Plants Fight Back. *Bio. Technol*., **12**, 883-888.
[169] Warwick, S.I., Légère, A., Simard, M.J., James, T., (2008). Do escaped transgenes persist in nature? The case of an herbicide resistance transgene in a weedy Brassica rapa population. *Mol. Ecol*., **17**, 1387-1395.

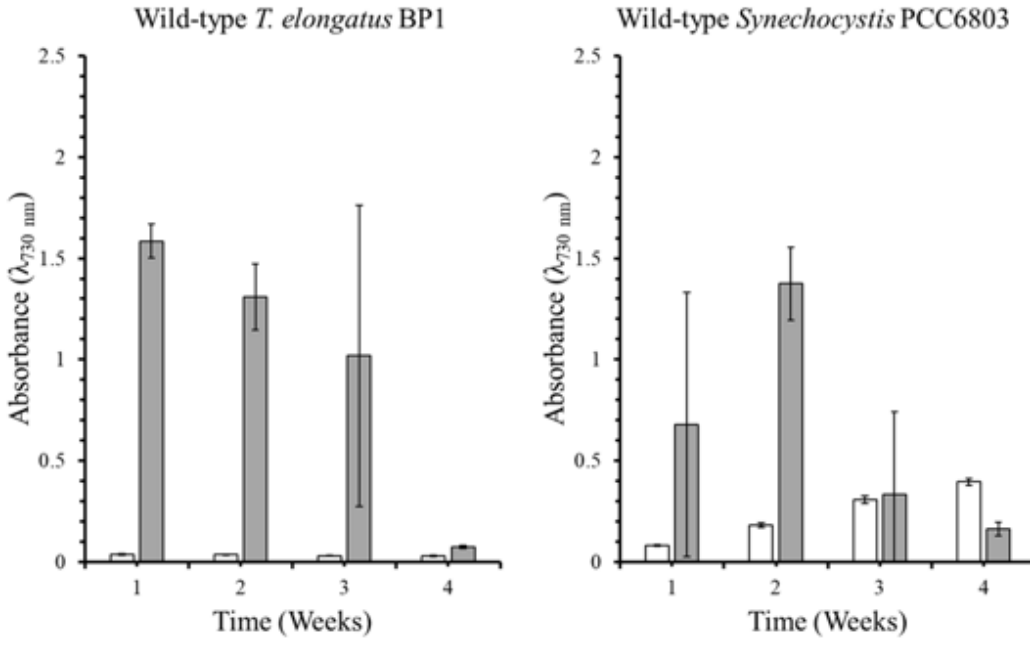
[170] Agga, G.E., Cook, K.L., Netthisinghe, A.M.P., Gilfillen, R.A., Woosley, P.B., Sistani, K.R., (2019). Persistence of antibiotic resistance genes in beef cattle backgrounding environment over two years after cessation of operation. *PLOS. ONE*., **14**, e0212510.
[171] Yus, E., Maier, T., Michalodimitrakis, K., van Noort, V., Yamada, T., Chen, W.H., et al., (2009). Impact of genome reduction on bacterial metabolism and its regulation. *Science*. **326**, 1263-1268.
[172] Moya, A., Gil, R., Latorre, A., Peretó, J., Pilar Garcillán-Barcia, M., De La Cruz, F., (2009). Toward minimal bacterial cells: evolution vs. design. *FEMS. Microbiol. Rev*., **33**, 225-235.
[173] Marais, G.A., Calteau, A., Tenaillon, O., (2008). Mutation rate and genome reduction in endosymbiotic and free-living bacteria. *Genetica*., **134**, 205-210.
[174] Giovannoni, S.J., Tripp, H.J., Givan, S., Podar, M., Vergin, K.L., Baptista, D., et al., (2005). Genome streamlining in a cosmopolitan oceanic bacterium. *Science*., **309**, 1242-1245.
[175] Christiansen, G., Molitor, C., Philmus, B., Kurmayer, R., (2008). Nontoxic strains of cyanobacteria are the result of major gene deletion events induced by a transposable element. *Mol. Biol. Evol*., **25**, 1695-1704.
[176] Markou, G., Vandamme, D., Muylaert, K., (2014). Microalgal and cyanobacterial cultivation: the supply of nutrients. *Water. Res*., **65**, 186-202.
[177] Rao, N.N., Gómez-García, M.R., Kornberg, A., (2009). Inorganic polyphosphate: essential for growth and survival. *Annu. Rev. Biochem*., **78**, 605-647.
[178] Hunnestad, A.V., Vogel, A.I.M., Armstrong, E., Digernes, M.G., Ardelan, M.V., Hohmann-Marriott, M.F., (2020). From the ocean to the lab-assessing iron limitation in cyanobacteria: an interface paper. *Microorganisms*., **8**, 1889.
[179] Cunningham, B.R., John, S.G., (2017). The effect of iron limitation on cyanobacteria major nutrient and trace element stoichiometry. *Limnol. Oceanogr*., **62**, 846-858.
[180] Zhou, J., Zhang, H., Meng, H., Zhu, Y., Bao, G., Zhang, Y., et al., (2014). Discovery of a super-strong promoter enables efficient production of heterologous proteins in cyanobacteria. *Sci. Rep*., **4**, 4500.
[181] Hynönen, U., Avall-Jääskeläinen, S., Palva, A. (2010). Characterization and separate activities of the two promoters of the Lactobacillus brevis S-layer protein gene. *Appl. Microbiol. Biotechnol*., **87**, 657-668.
[182] Zeng, F., Qian, J., Luo, W., Zhan, Y., Xin, Y., Yang, C., (2009). Stability of transgenes in long-term micropropagation of plants of transgenic birch (Betula platyphylla). *Biotechnol. Lett*., **32**, 151.
[183] Weaver, M., Vedenyapina, E., Kenerley, C.M., (2005). Fitness, persistence, and responsiveness of a genetically engineered strain of Trichoderma virens in soil mesocosms. *Appl Soil Ecol*. **29**, 125-134.
[184] Ginzinger, D.G., (2002). Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp. Hematol*., **30**, 503-512.
[185] Navarro, E., Serrano-Heras, G., Castaño, M.J., Solera, J., (2015). Real-time PCR detection chemistry. *Clin. Chim. Acta*., **439**, 231-250.
[186] Martz, E., (2002). Protein explorer: easy yet powerful macromolecular visualization. *Trends. Biochem. Sci*., **27**, 107-109.
[187] Ivanov, A.G., Krol, M., Sveshnikov, D., Selstam, E., Sandström, S., Koochek, M., et al., (2006). Iron deficiency in cyanobacteria causes monomerization of photosystem I trimers and

reduces the capacity for state transitions and the effective absorption cross section of photosystem I in vivo. *Plant. Physiol*., **141**, 1436-1445.
[188] de Loura, I.C., Dubacq, J.P., Thomas, J.C., (1987). The effects of nitrogen deficiency on pigments and lipids of cyanobacteria. *J. Plant. Physiol*., 83, 838-843.

APPENDIX A

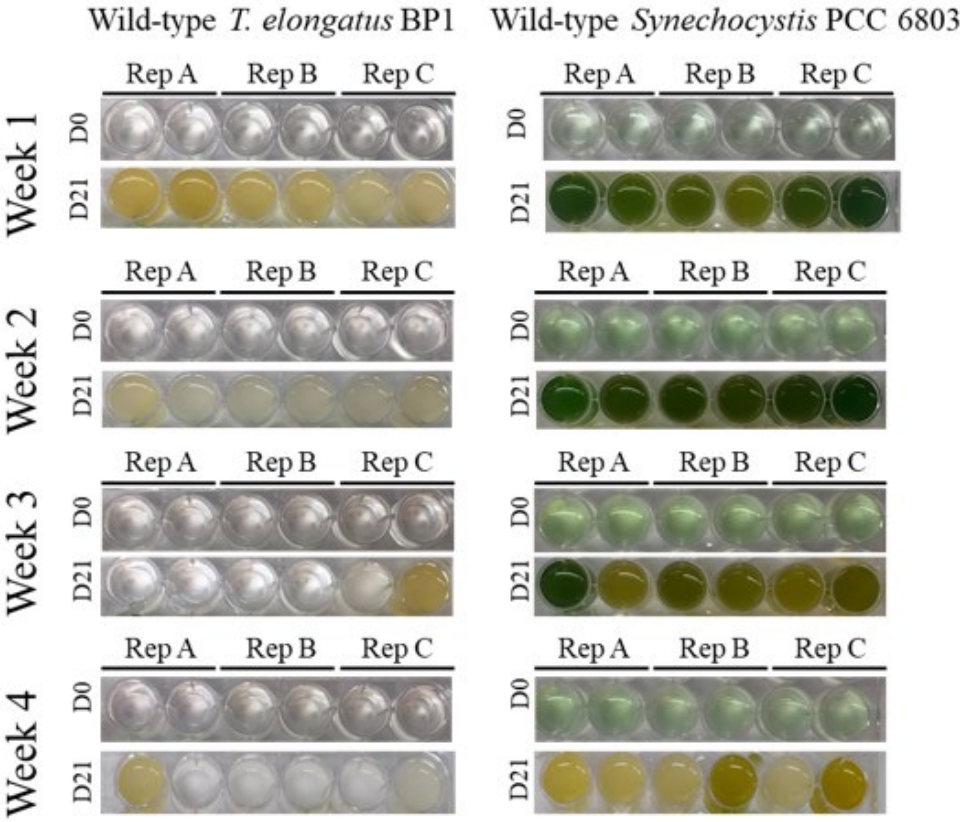
SURVIVABILITY OF WT BP1 AND 6803 IN GREENHOUSE DURING COOL SEASON

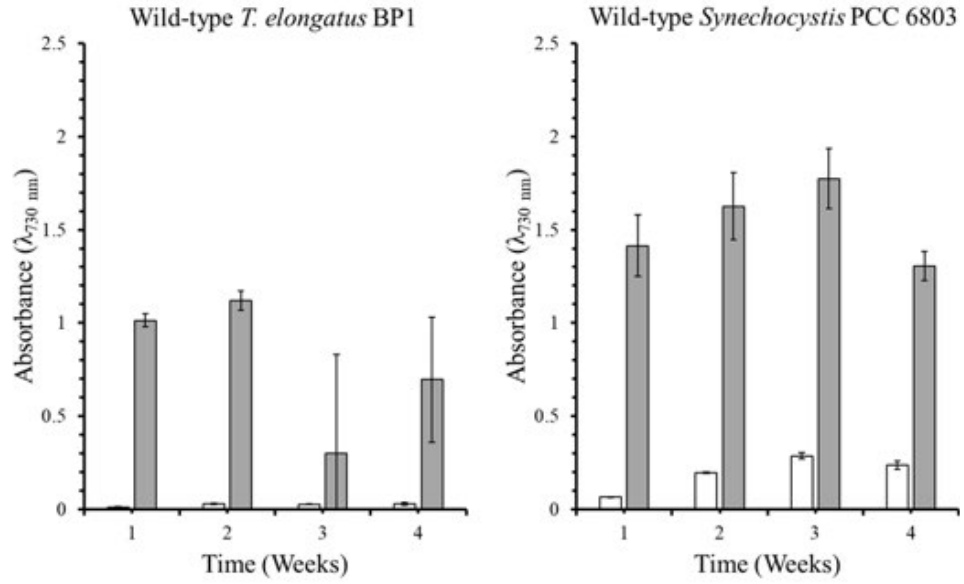




APPENDIX B

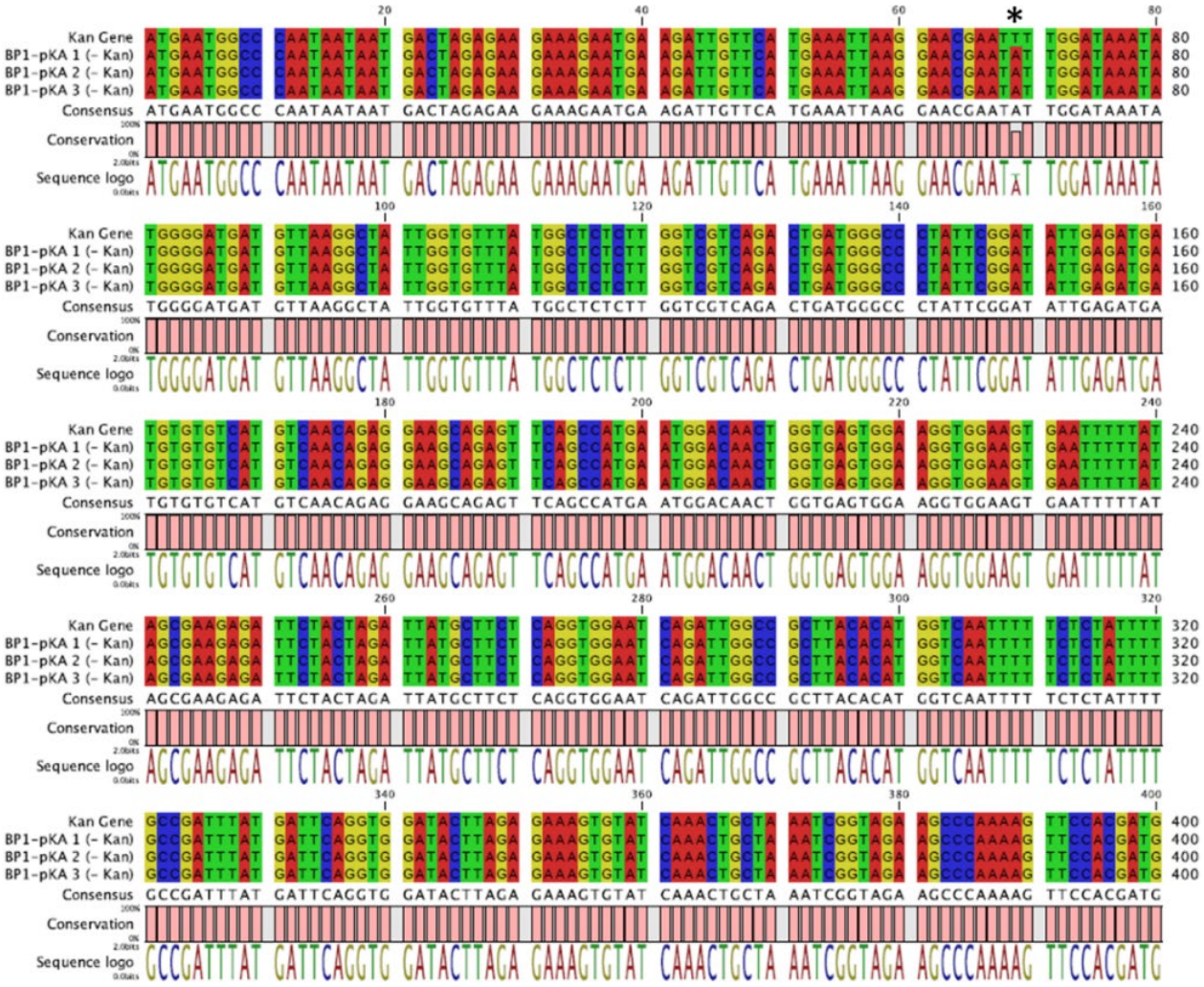
SURVIVABILITY OF WT BP1 AND 6803 IN GREENHOUSE DURING WARM SEASON

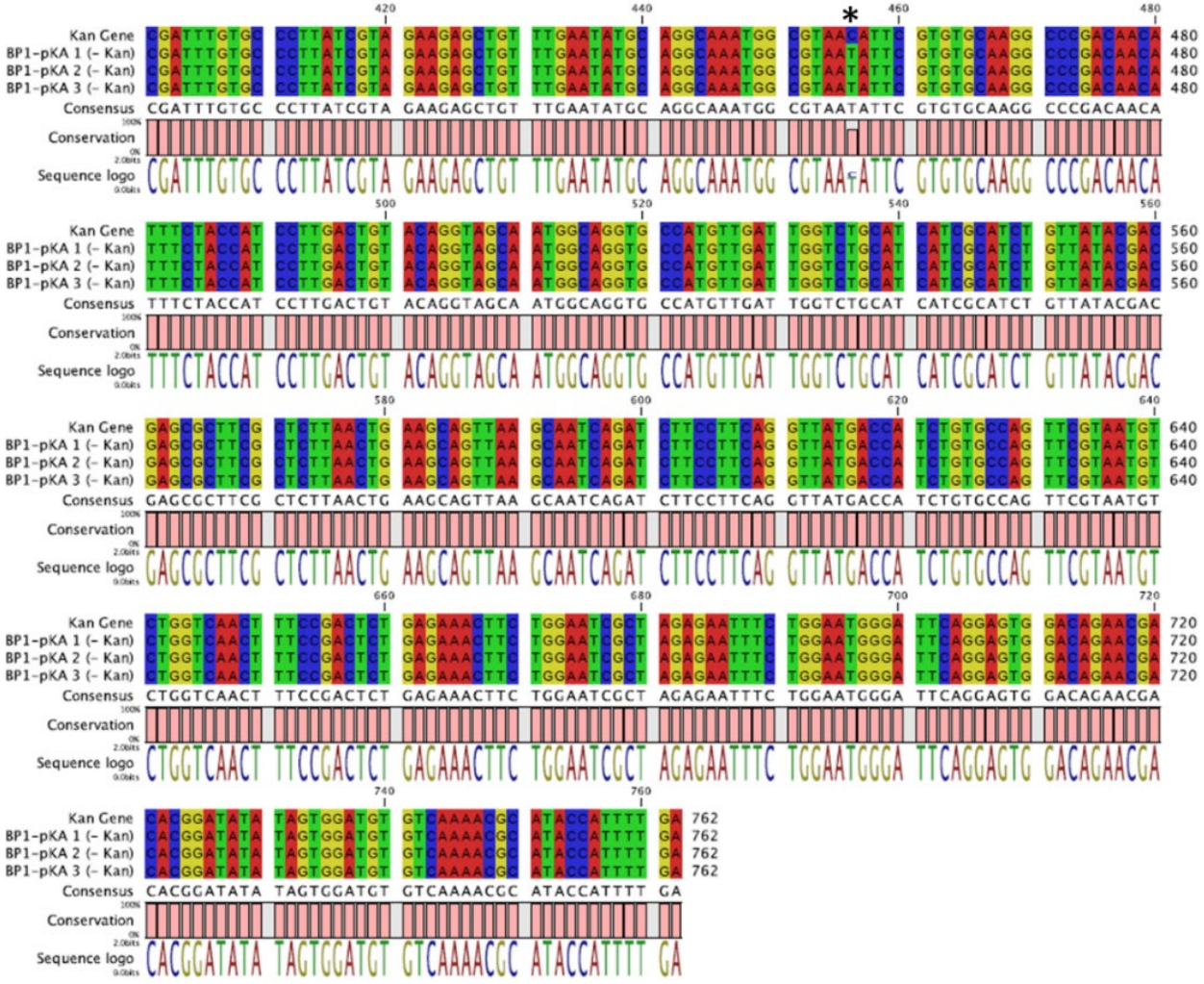




APPENDIX C

NUCLEOTIDE ALIGNMENT OF THE KAN RESISTANCE GENE FROM WHOLE-GENOME SEQUENCING




Kan Gene
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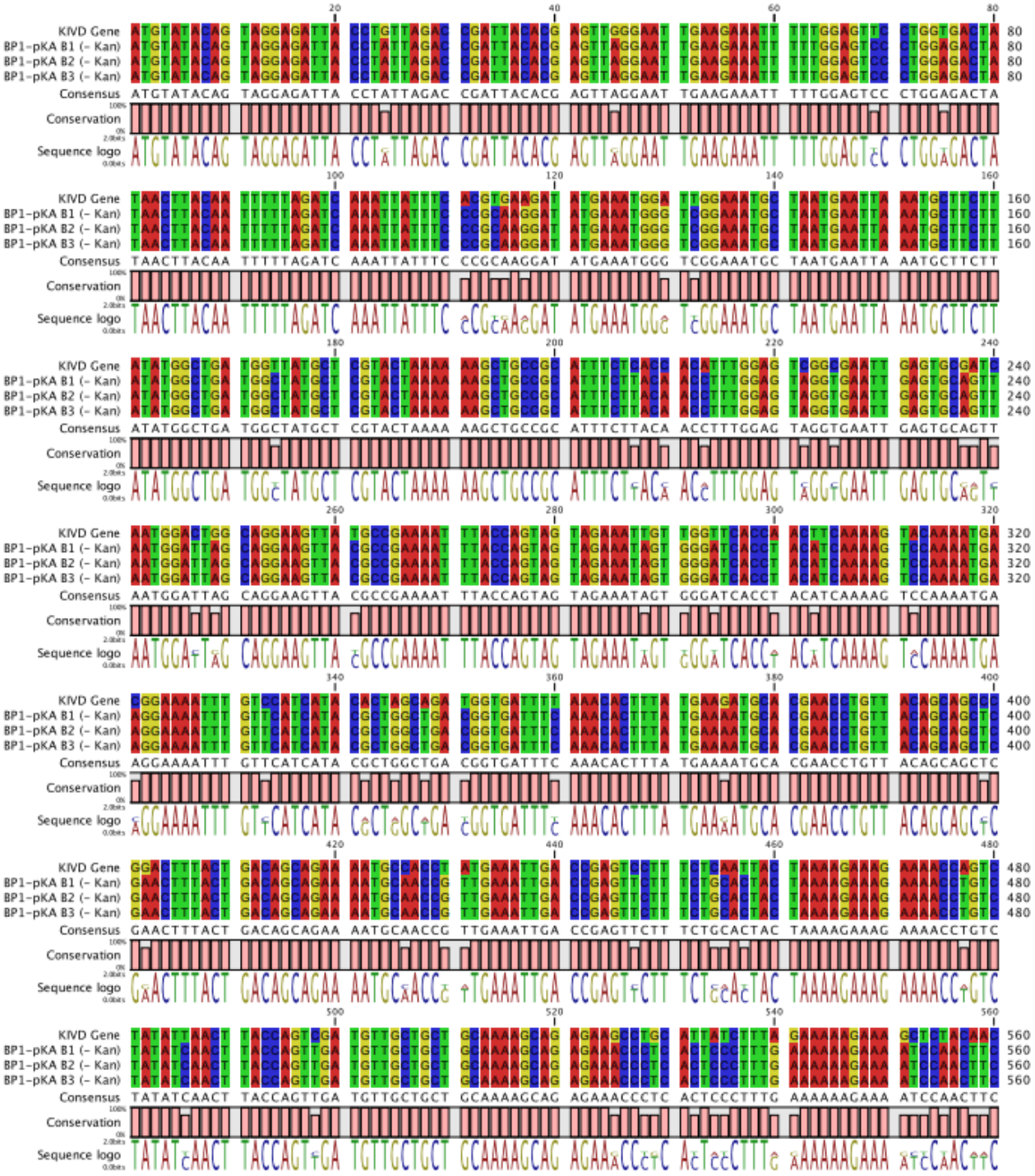
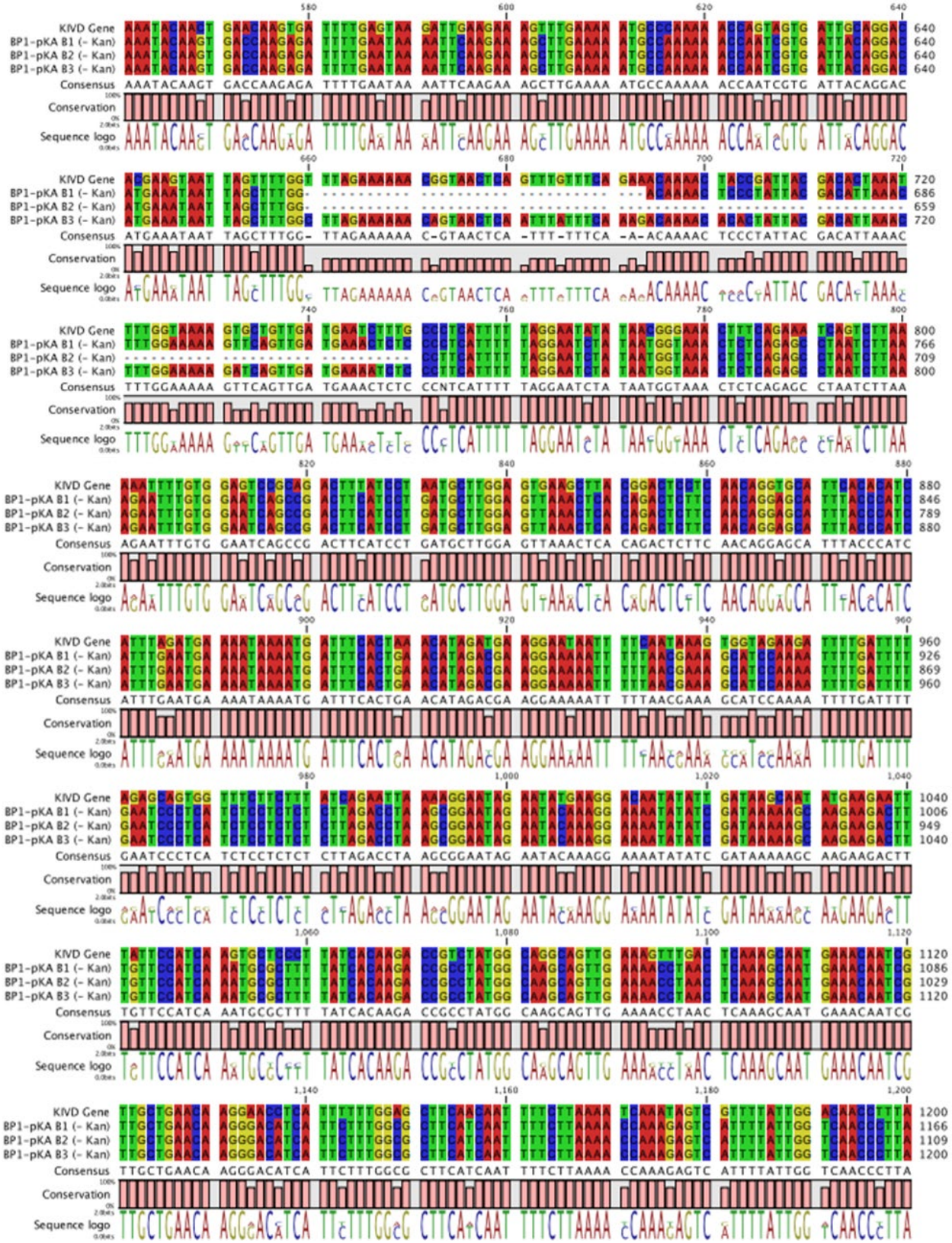
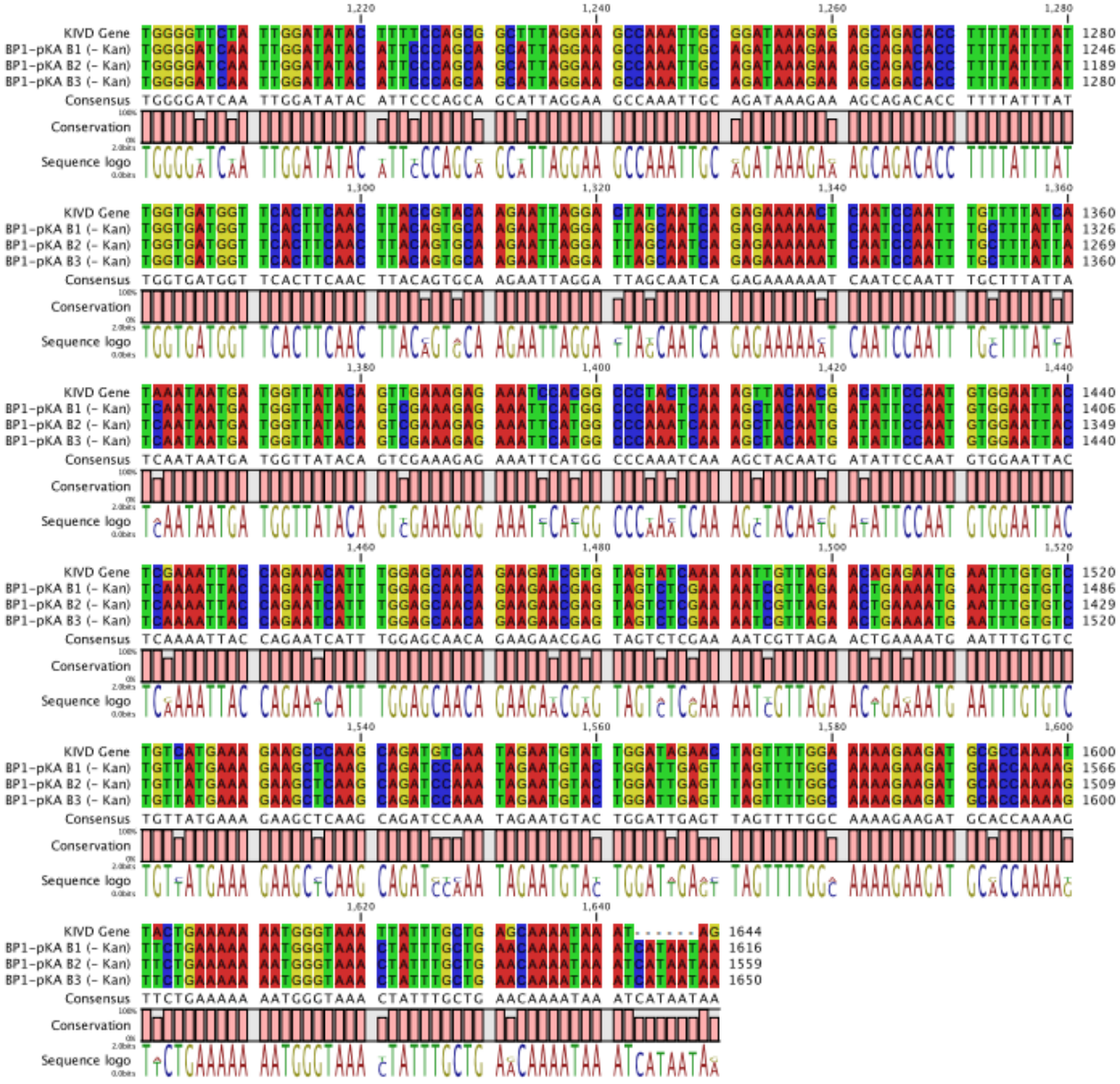
BP1-pKA 2 (- Kan)
BP1-pKA 3 (- Kan)
Consensus
Conservation
Sequence logo

APPENDIX D

NUCLEOTIDE ALIGNMENT OF THE KIVD GENE FROM WHOLE-GENOME SEQUENCING




KIVD Gene
BP1-pKA B1 (- Kan)
BP1-pKA B2 (- Kan)
BP1-pKA B3 (- Kan)
Consensus
Conservation
Sequence logo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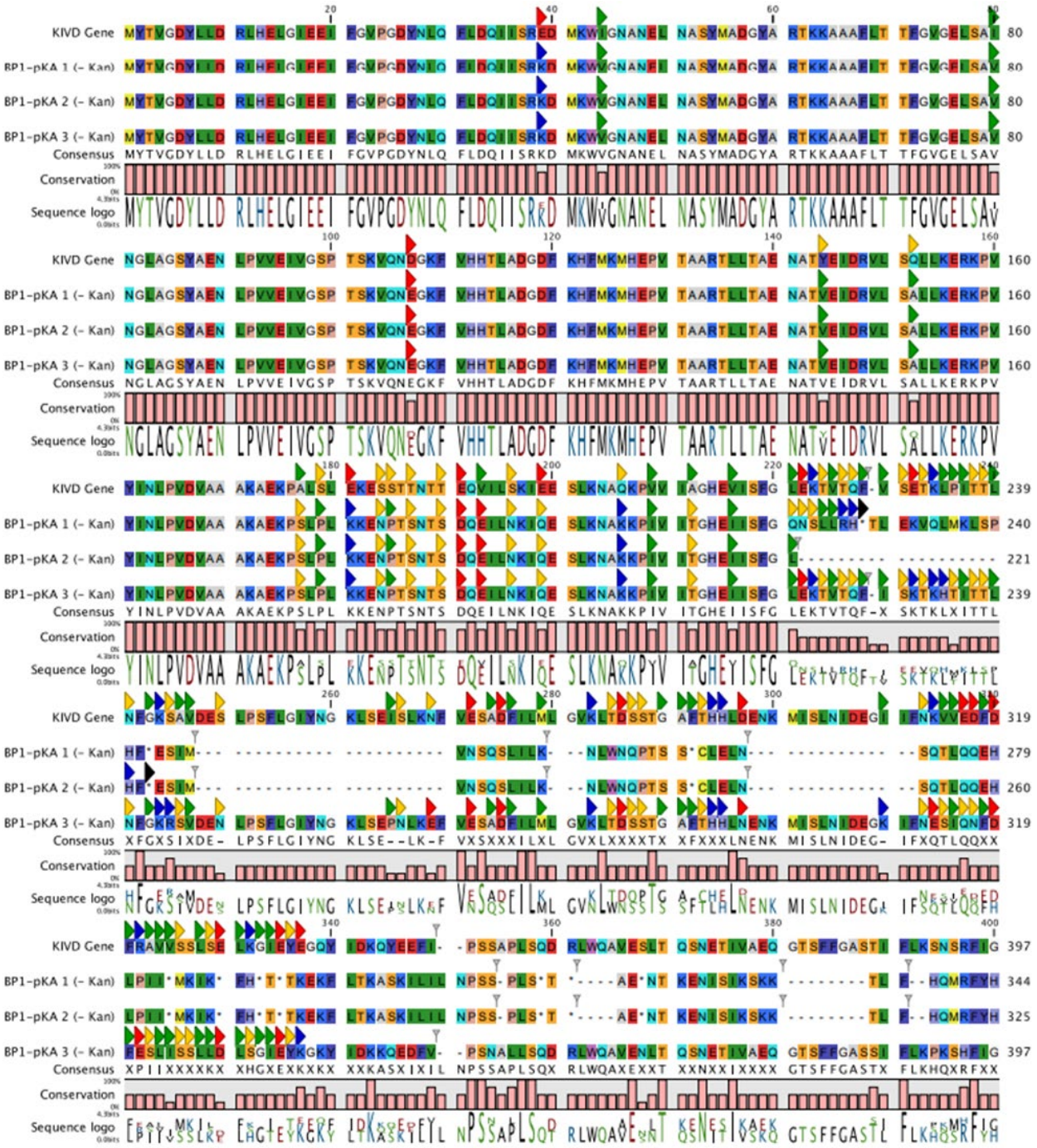
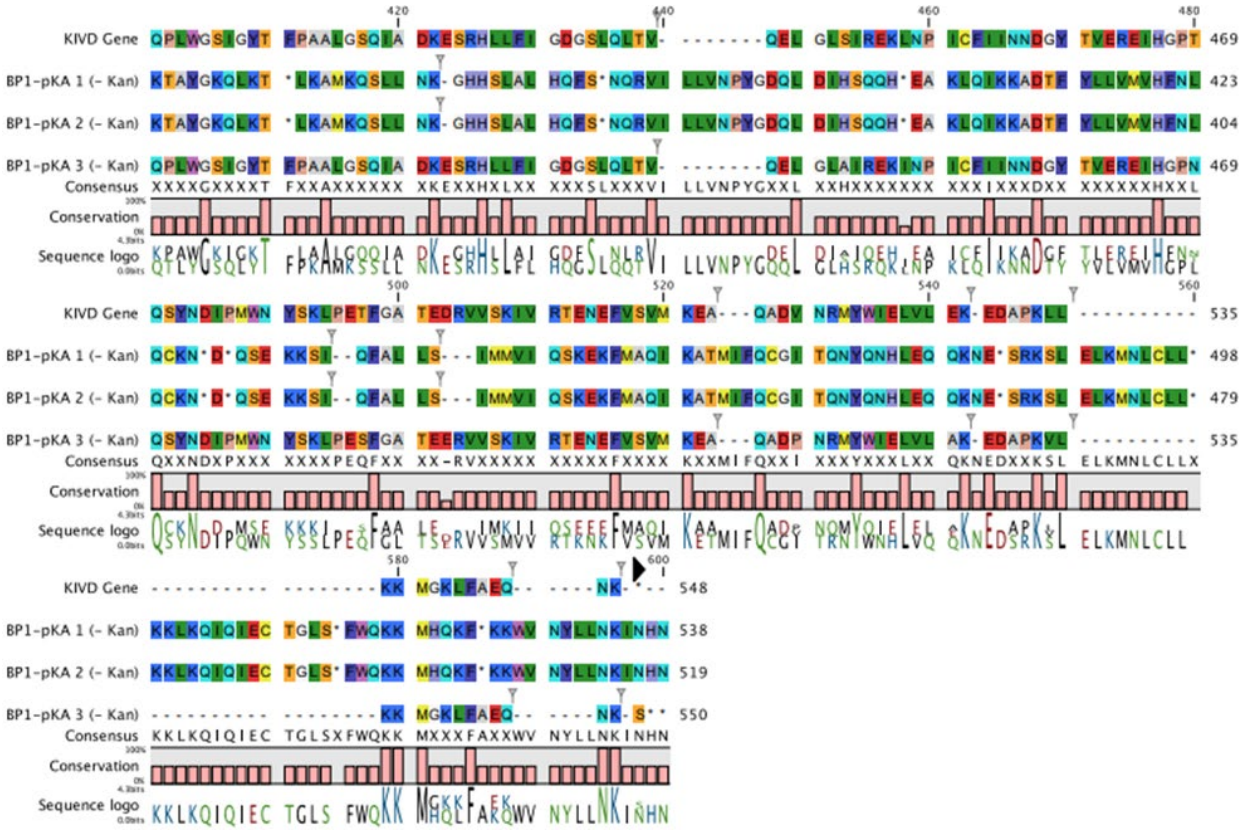
Conservation
Sequence logo

APPENDIX E

ANNOTATION FOR NONSYNONYMOUS MUTATIONS WITHIN KIVD FOR ALL EXPERIMENTAL SAMPLES




KIVD Gene
BP1-pKA 1 (- Kan)
BP1-pKA 2 (- Kan)
BP1-pKA 3 (- Kan)
Consensus
Conservation
Sequence logo

APPENDIX F

KIVD NONSYNONYMOUS MUTATIONS ANNOTATED BY CHARACTERISTICS

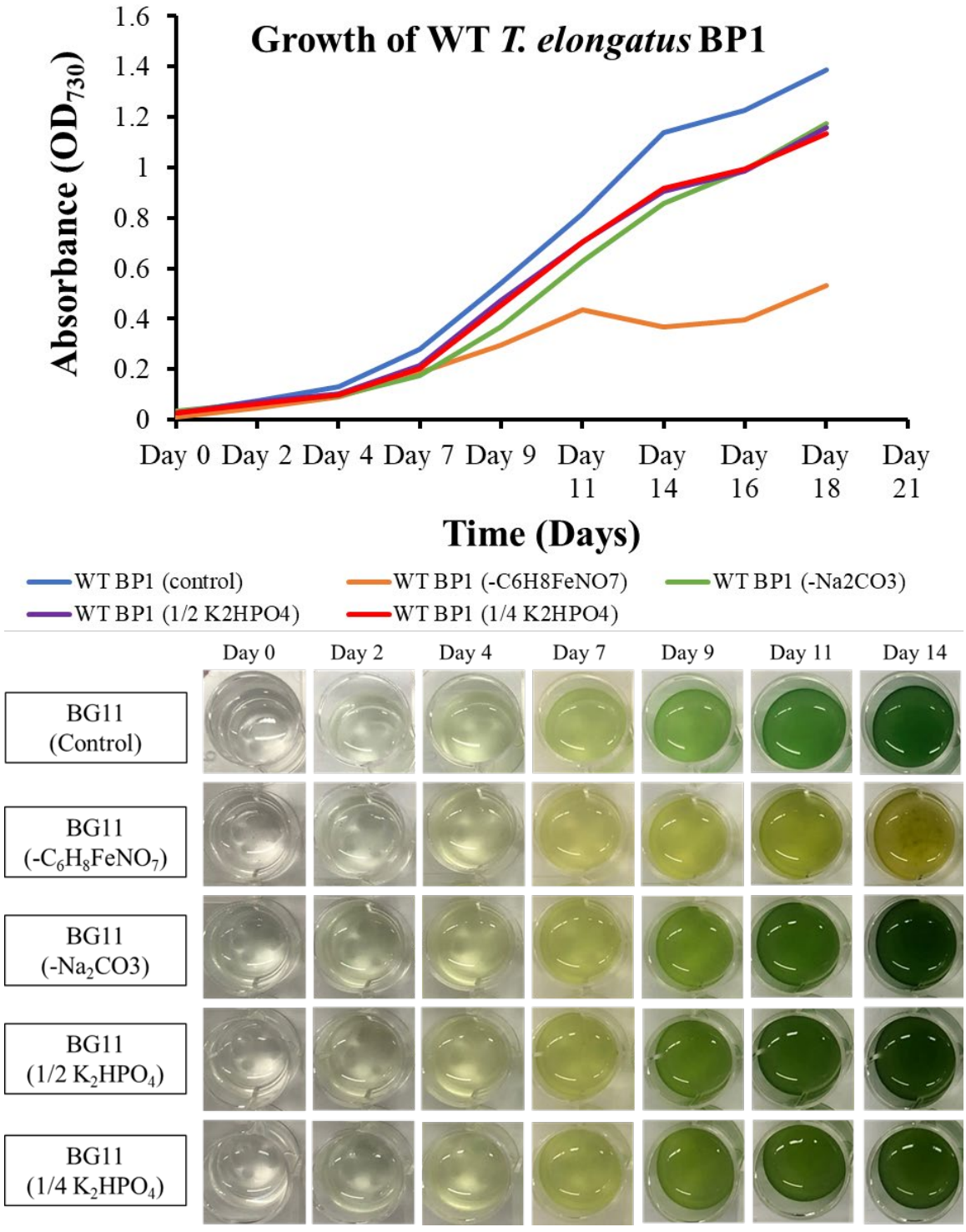
Position	Residue (ref)	Residue 1 (-kan)	Residue 2 (-kan)	Residue 3 (-kan)	Position	Residue (ref)	Residue 1 (-kan)	Residue 2 (-kan)	Residue 3 (-kan)
39	Glutamic Acid	Lysine	Lysine	Lysine	280	Leucine	Gap	Gap	Leucine
44	Isoleucine	Valine	Valine	Valine	281	Glycine	Gap	Gap	Glycine
80	Isoleucine	Valine	Valine	Valine	282	Valine	Gap	Gap	Valine
107	Aspartic Acid	Glutamic Acid	Glutamic Acid	Glutamic Acid	283	Lysine	Asparagine	Asparagine	Lysine
144	Tyrosine	Valine	Valine	Valine	285	Threonine	Tryptophan	Tryptophan	Threonine
152	Glutamate	Alanine	Alanine	Alanine	286	Aspartic Acid	Asparagine	Asparagine	Aspartic Acid
177	Alanine	Serine	Serine	Serine	287	Serine	Glutamine	Glutamine	Serine
179	Serine	Proline	Proline	Proline	288	Serine	Proline	Proline	Serine
181	Glutamic Acid	Lysine	Lysine	Lysine	290	Glycine	Serine	Serine	Glycine
184	Serine	Asparagine	Asparagine	Asparagine	291	Alanine	Serine	Serine	Alanine
185	Serine	Proline	Proline	Proline	292	Phenylalanine	Stop	Stop	Phenylalanine
187	Threonine	Serine	Serine	Serine	293	Threonine	Cysteine	Cysteine	Threonine
190	Threonine	Serine	Serine	Serine	294	Histidine	Leucine	Leucine	Histidine
191	Glutamic Acid	Aspartic Acid	Aspartic Acid	Aspartic Acid	295	Histidine	Glutamic Acid	Glutamic Acid	Histidine
193	Valine	Glutamic Acid	Glutamic Acid	Glutamic Acid	297	Aspartic Acid	Asparagine	Asparagine	Asparagine
196	Serine	Asparagine	Asparagine	Asparagine	298	Glutamic Acid	Gap	Gap	Glutamic Acid
199	Glutamic Acid	Glutamine	Glutamine	Glutamine	299	Asparagine	Gap	Gap	Asparagine
206	Glutamine	Lysine	Lysine	Lysine	300	Lysine	Gap	Gap	Lysine
209	Valine	Isoleucine	Isoleucine	Isoleucine	301	Methionine	Gap	Gap	Methionine
212	Alaine	Threonine	Theronine	Theronine	302	Isoleucine	Gap	Gap	Isoleucine
216	Valine	Isoleucine	Isoleucine	Isoleucine	303	Serine	Gap	Gap	Serine
221	Leucine	Threonine	Leucine	Leucine	304	Leucine	Gap	Gap	Leucine
222	Glutamic Acid	Asparagine	Gap	Glutamic Acid	305	Asparagine	Gap	Gap	Asparagine
223	Lysine	Serine	Gap	Lysine	306	Isoleucine	Gap	Gap	Isoleucine
224	Threonine	Leucine	Gap	Threonine	307	Aspartic Acid	Gap	Gap	Aspartic Acid
225	Valine	Leucine	Gap	Valine	308	Glutamic Acid	Gap	Gap	Glutamic Acid
226	Threonine	Arginine	Gap	Threonine	309	Glycine	Gap	Gap	Glycine
227	Glutamine	Histidine	Gap	Glutamine	310	Isoleucine	Gap	Gap	Lysine
228	Phenylalanine	Stop	Gap	Phenylalanine	311	Isoleucine	Gap	Gap	Isoleucine
229	Gap	Threonine	Gap	Gap	312	Phenylalanine	Gap	Gap	Phenylalanine
230	Valine	Leucine	Gap	Leucine	313	Asparagine	Serine	Serine	Asparagine
231	Serine	Glutamic Acid	Gap	Serine	314	Lysine	Glutamine	Glutamine	Glutamic Acid
232	Glutamic Acid	Lysine	Gap	Lysine	315	Valine	Threonine	Theronine	Serine
233	Threonine	Valine	Gap	Threonine	316	Valine	Leucine	Leucine	Isoleucine
234	Lysine	Glutamine	Gap	Lysine	317	Glutamic Acid	Glutamine	Glutamine	Glutamine
235	Leucine	Leucine	Gap	Histidine	318	Aspartic Acid	Glutamine	Glutamine	Asparagine
236	Proline	Methionine	Gap	Threonine	319	Phenylalanine	Glutamic Acid	Glutamic Acid	Phenylalanine
237	Isoleucine	Lysine	Gap	Isoleucine	320	Aspartic Acid	Histidine	Histidine	Aspartic Acid
238	Threonine	Leucine	Gap	Threonine	321	Phenylalanine	Leucine	Leucine	Phenylalanine
239	Threonine	Serine	Gap	Threonine	322	Arginine	Proline	Proline	Glutamic Acid
240	Leucine	Proline	Gap	Leucine	323	Alanine	Isoleucine	Isoleucine	Serine
241	Aspargine	Histidine	Histidine	Asparagine	324	Valine	Isoleucine	Isoleucine	Leucine
243	Glycine	Stop	Stop	Glycine	325	Valine	Stop	Stop	Isoleucine
244	Lysine	Glutamic Acid	Glutamic Acid	Lysine	326	Serine	Methionine	Methionine	Serine
245	Serine	Serine	Serine	Arginine	327	Serine	Lysine	Lysine	Serine
246	Alanine	Isoleucine	Isoleucine	Serine	328	Leucine	Isoleucine	Isoleucine	Leucine
247	Valine	Methionine	Methionine	Valine	329	Serine	Lysine	Lysine	Leucine
248	Aspartic Acid	Gap	Gap	Aspartic Acid	330	Glutamic Acid	Stop	Stop	Aspartic Acid
249	Glutamic Acid	Gap	Gap	Glutamic Acid	331	Leucine	Phenylalanine	Phenylalanine	Leucine
250	Serine	Gap	Gap	Asparagine	332	Lysine	Histidine	Histidine	Serine
251	Leucine	Gap	Gap	Leucine	333	Glycine	Stop	Stop	Glycine
252	Proline	Gap	Gap	Proline	334	Isoleucine	Threonine	Threonine	Isoleucine
253	Serine	Gap	Gap	Serine	335	Glutamic Acid	Stop	Stop	Glutamic Acid
254	Phenylalanine	Gap	Gap	Phenylalanine	336	Tyrosine	Threonine	Threonine	Tyrosine
255	Leucine	Gap	Gap	Leucine	337	Glutamic Acid	Lysine	Lysine	Glutamic Acid
256	Glycine	Gap	Gap	Glycine	338	Glycine	Glutamic Acid	Glutamic Acid	Glycine
257	Isoleucine	Gap	Gap	Isoleucine	339	Glutamine	Lysine	Lysine	Lysine
258	Tyrosine	Gap	Gap	Tyrosine	340	Tyrosine	Phenylalanine	Phenylalanine	Tyrosine
259	Asparagine	Gap	Gap	Asparagine	341	Isoleucine	Leucine	Leucine	Isoleucine
260	Glycine	Gap	Gap	Glycine	342	Aspartic Acid	Threonine	Threonine	Aspartic Acid
261	Lysine	Gap	Gap	Lysine	344	Glutamine	Alanine	Alanine	Lysine
262	Leucine	Gap	Gap	Leucine	345	Tyrosine	Serine	Serine	Glutamine
263	Serine	Gap	Gap	Serine	346	Glutamic Acid	Lysine	Lysine	Glutamic Acid
264	Glutamic Acid	Gap	Gap	Glutamic Acid	347	Glutamic Acid	Isoleucine	Isoleucine	Glutamic Acid
265	Isoleucine	Gap	Gap	Proline	348	Phenylalanine	Leucine	Leucine	Phenylalanine
266	Serine	Gap	Gap	Asparagine	349	Isoleucine	Isoleucine	Isoleucine	Valine
267	Leucine	Gap	Gap	Leucine	350	Gap	Leucine	Leucine	Gap
268	Lysine	Gap	Gap	Lysine	351	Gap	Asparagine	Asparagine	Gap
269	Asparagine	Gap	Gap	Glutamic Acid	354	Serine	Serine	Serine	Asparagine
270	Phenylalanine	Gap	Gap	Phenylalanine	355	Alanine	Gap	Gap	Alanine
272	Glutamic Acid	Asparagine	Asparagine	Glutamic Acid	356	Proline	Proline	Proline	Leucine
274	Alanine	Glutamine	Glutamine	Alanine	359	Glutamine	Stop	Stop	Glutamine
275	Aspartic Acid	Serine	Serine	Aspartic Acid	360	Aspartic Acid	Threonine	Threonine	Aspartic Acid
276	Phenylalanine	Leucine	Leucine	Phenylalanine	361	Arginine	Stop	Stop	Arginine
279	Methionine	Lysine	Lysine	Methionine	362	Leucine	Gap	Gap	Leucine

Position	Residue (ref)	Residue 1 (-kan)	Residue 2 (-kan)	Residue 3 (-kan)	Position	Residue (ref)	Residue 1 (-kan)	Residue 2 (-kan)	Residue 3 (-kan)
363	Tryptophan	Gap	Gap	Tryptophan	453	Serine	Histidine	Histidine	Alanine
364	Glutamine	Gap	Gap	Glutamine	454	Isoleucine	Serine	Serine	Isoleucine
365	Alanine	Gap	Gap	Alanine	455	Arginine	Glutamine	Glutamine	Arginine
366	Valine	Alanine	Alanine	Valine	456	Glutamic Acid	Glutamine	Glutamine	Glutamic Acid
368	Serine	Stop	Stop	Asparagine	457	Lysine	Histidine	Histidine	Lysine
369	Leucine	Asparagine	Asparagine	Leucine	458	Leucine	Stop	Stop	Isoleucine
371	Glutamine	Lysine	Lysine	Glutamine	459	Asparagine	Glutamic Acid	Glutamic Acid	Asparagine
372	Serine	Glutamic Acid	Glutamic Acid	Serine	460	Proline	Alanine	Alanine	Proline
374	Glutamic Acid	Isoleucine	Isoleucine	Glutamic Acid	461	Isoleucine	Lysine	Lysine	Isoleucine
375	Threonine	Serine	Serine	Threonine	462	Cysteine	Leucine	Leucine	Cysteine
377	Valine	Lysine	Lysine	Valine	463	Phenylalanine	Glutamine	Glutamine	Phenylalanine
378	Alanine	Serine	Serine	Alanine	465	Isoleucine	Lysine	Lysine	Isoleucine
379	Glutamic Acid	Lysine	Lysine	Glutamic Acid	466	Asparagine	Lysine	Lysine	Asparagine
380	Glutamine	Lysine	Lysine	Glutamine	467	Asparagine	Alanine	Alanine	Asparagine
381	Glycine	Gap	Gap	Glycine	469	Glycine	Threonine	Threonine	Glycine
382	Threonine	Gap	Gap	Threonine	470	Tyrosine	Phenylalanine	Phenylalanine	Tyrosine
383	Serine	Gap	Gap	Serine	471	Threonine	Tyrosine	Tyrosine	Threonine
384	Phenylalanine	Gap	Gap	Phenylalanine	472	Valine	Leucine	Leucine	Valine
385	Phenylalanine	Gap	Gap	Phenylalanine	473	Glutamic Acid	Leucine	Leucine	Glutamic Acid
386	Glycine	Gap	Gap	Glycine	474	Arginine	Valine	Valine	Arginine
387	Alanine	Gap	Gap	Alanine	475	Glutamic Acid	Methionine	Methionine	Glutamic Acid
388	Serine	Gap	Gap	Serine	476	Isoleucine	Valine	Valine	Isoleucine
389	Threonine	Threonine	Threonine	Serine	478	Glycine	Phenylalanine	Phenylalanine	Glycine
390	Isoleucine	Leucine	Leucine	Isoleucine	479	Proline	Asparagine	Asparagine	Proline
392	Leucine	Gap	Gap	Leucine	480	Threonine	Leucine	Leucine	Asparagine
393	Lysine	Gap	Gap	Lysine	482	Serine	Cysteine	Cysteine	Serine
394	Serine	Histidine	Histidine	Proline	483	Tyrosine	Lysine	Lysine	Tyrosine
395	Asparagine	Glutamine	Glutamine	Lysine	485	Aspartic Acid	Stop	Stop	Aspartic Acid
396	Serine	Methionine	Methionine	Serine	486	Isoleucine	Aspartic Acid	Aspartic Acid	Isoleucine
397	Arginine	Arginine	Arginine	Histidine	487	Proline	Stop	Stop	Proline
399	Isoleucine	Tyrosine	Tyrosine	Isoleucine	488	Methionine	Glutamine	Glutamine	Methionine
400	Glycine	Histidine	Histidine	Glycine	489	Tryptophan	Serine	Serine	Tryptophan
401	Glutamine	Lysine	Lysine	Glutamine	490	Asparagine	Glutamic Acid	Glutamic Acid	Asparagine
402	Proline	Threonine	Threonine	Proline	491	Tyrosine	Lysine	Lysine	Tyrosine
403	Leucine	Alanine	Alanine	Leucine	492	Serine	Lysine	Lysine	Serine
404	Tryptophan	Tyrosine	Tyrosine	Tryptophan	493	Lysine	Serine	Serine	Lysine
406	Serine	Lysine	Lysine	Serine	494	Leucine	Isoleucine	Isoleucine	Leucine
407	Isoleucine	Glutamine	Glutamine	Isoleucine	495	Proline	Gap	Gap	Proline
408	Glycine	Leucine	Leucine	Glycine	496	Glutamic Acid	Gap	Gap	Glutamic Acid
409	Tyrosine	Lysine	Lysine	Tyrosine	497	Threonine	Glutamine	Glutamine	Threonine
411	Phenylalanine	Stop	Stop	Phenylalanine	499	Glycine	Alanine	Alanine	Glycine
412	Proline	Leucine	Leucine	Proline	500	Alanine	Leucine	Leucine	Alanine
413	Alanine	Lysine	Lysine	Alanine	501	Threonine	Leucine	Leucine	Threonine
415	Leucine	Methionine	Methionine	Leucine	502	Glutamic Acid	Serine	Serine	Glutamic Acid
416	Glycine	Lysine	Lysine	Glycine	503	Aspartic Acid	Gap	Gap	Glutamic Acid
417	Serine	Glutamine	Glutamine	Serine	504	Arginine	Gap	Gap	Arginine
418	Glutamine	Serine	Serine	Glutamine	505	Valine	Gap	Gap	Valine
419	Isoleucine	Leucine	Leucine	Isoleucine	506	Valine	Isoleucine	Isoleucine	Valine
420	Alanine	Leucine	Leucine	Alanine	507	Serine	Methionine	Methionine	Serine
421	Aspartic Acid	Asparagine	Asparagine	Aspartic Acid	508	Lysine	Methionine	Methionine	Lysine
423	Glutamic Acid	Gap	Gap	Glutamic Acid	509	Isoleucine	Valine	Valine	Isoleucine
424	Serine	Glycine	Glycine	Serine	510	Valine	Isoleucine	Isoleucine	Valine
425	Arginine	Histidine	Histidine	Arginine	511	Arginine	Glutamine	Glutamine	Arginine
427	Leucine	Serine	Serine	Leucine	512	Threonine	Serine	Serine	Threonine
429	Phenylalanine	Alanine	Alanine	Phenylalanine	513	Glutamic Acid	Lysine	Lysine	Glutamic Acid
430	Isoleucine	Leucine	Leucine	Isoleucine	514	Asparagine	Glutamic Acid	Glutamic Acid	Asparagine
431	Glycine	Histidine	Histidine	Glycine	515	Glutamic Acid	Lysine	Lysine	Glutamic Acid
432	Aspartic Acid	Glutamine	Glutamine	Aspartic Acid	517	Valine	Methionine	Methionine	Valine
433	Glycine	Phenylalanine	Phenylalanine	Glycine	518	Serine	Alanine	Alanine	Serine
435	Leucine	Stop	Stop	Leucine	519	Valine	Glutamine	Glutamine	Valine
436	Glutamine	Asparagine	Asparagine	Glutamine	520	Methionine	Isoleucine	Isoleucine	Methionine
437	Leucine	Glutamine	Glutamine	Leucine	522	Glutamic Acid	Alanine	Alanine	Glutamic Acid
438	Threonine	Arginine	Arginine	Threonine	523	Alanine	Threonine	Threonine	Alanine
440	Gap	Isoleucine	Isoleucine	Gap	524	Gap	Methionine	Methionine	Gap
441	Gap	Leucine	Leucine	Gap	525	Gap	Isoleucine	Isoleucine	Gap
442	Gap	Leucine	Leucine	Gap	526	Gap	Phenylalanine	Phenylalanine	Gap
443	Gap	Valine	Valine	Gap	528	Alanine	Cysteine	Cysteine	Alanine
444	Gap	Asparagine	Asparagine	Gap	529	Aspartic Acid	Glycine	Glycine	Aspartic Acid
445	Gap	Proline	Proline	Gap	530	Valine	Isoleucine	Isoleucine	Proline
446	Gap	Tyrosine	Tyrosine	Gap	531	Asparagine	Threonine	Threonine	Asparagine
447	Gap	Glycine	Glycine	Gap	532	Arginine	Glutamine	Glutamine	Arginine
448	Glutamine	Aspartic Acid	Aspartic Acid	Glutamine	533	Methionine	Asparagine	Asparagine	Methionine
449	Glutamic Acid	Glutamine	Glutamine	Glutamic Acid	535	Tryptophan	Glutamine	Glutamine	Tryptophan
451	Glycine	Aspartic Acid	Aspartic Acid	Glycine	536	Isoleucine	Asparagine	Asparagine	Isoleucine
452	Leucine	Isoleucine	Isoleucine	Leucine	537	Glutamic Acid	Histidine	Histidine	Glutamic Acid

Position	Residue (ref)	Residue 1 (-kan)	Residue 2 (-kan)	Residue 3 (-kan)	Position	Residue (ref)	Residue 1 (-kan)	Residue 2 (-kan)	Residue 3 (-kan)
539	Valine	Glutamic Acid	Glutamic Acid	Valine	569	Gap	Glutamic Acid	Glutamic Acid	Gap
540	Leucine	Glutamine	Glutamine	Leucine	570	Gap	Cysteine	Cysteine	Gap
541	Glutamic Acid	Glutamine	Glutamine	Alanine	571	Gap	Threonine	Threonine	Gap
543	Gap	Asparagine	Asparagine	Gap	572	Gap	Glycine	Glycine	Gap
545	Aspartic Acid	Stop	Stop	Aspartic Acid	573	Gap	Leucine	Leucine	Gap
546	Alanine	Serine	Serine	Alanine	574	Gap	Serine	Serine	Gap
547	Proline	Arginine	Arginine	Proline	575	Gap	Stop	Stop	Gap
549	Leucine	Serine	Serine	Leucine	576	Gap	Phenylalanine	Phenylalanine	Gap
551	Gap	Glutamic Acid	Glutamic Acid	Gap	577	Gap	Trytophan	Tryptophan	Gap
552	Gap	Leucine	Leucine	Gap	578	Gap	Glutamine	Glutamine	Gap
553	Gap	Lysine	Lysine	Gap	582	Glycine	Histidine	Histidine	Glycine
554	Gap	Methionine	Methionine	Gap	583	Lysine	Glutamine	Glutamine	Lysine
555	Gap	Asparagine	Asparagine	Gap	584	Leucine	Lysine	Lysine	Leucine
556	Gap	Leucine	Leucine	Gap	586	Alanine	Stop	Stop	Alanine
557	Gap	Cysteine	Cysteine	Gap	587	Glutamic Acid	Lysine	Lysine	Glutamic Acid
558	Gap	Leucine	Leucine	Gap	588	Glutamine	Lysine	Lysine	Glutamine
559	Gap	Leucine	Leucine	Gap	589	Gap	Tryptophan	Tryptophan	Gap
560	Gap	Stop	Stop	Gap	590	Gap	Valine	Valine	Gap
561	Gap	Lysine	Lysine	Gap	591	Gap	Asparagine	Asparagine	Gap
562	Gap	Lysine	Lysine	Gap	592	Gap	Tyrosine	Tyrosine	Gap
563	Gap	Leucine	Leucine	Gap	593	Gap	Leucine	Leucine	Gap
564	Gap	Lysine	Lysine	Gap	594	Gap	Leucine	Leucine	Gap
565	Gap	Glutamine	Glutamine	Gap	597	Gap	Isoleucine	Isoleucine	Gap
566	Gap	Isoleucine	Isoleucine	Gap	598	Stop	Asparagine	Asparagine	Serine
567	Gap	Glutamine	Glutamine	Gap	599	Gap	Histidine	Histidine	Stop
568	Gap	Isoleucine	Isoleucine	Gap	600	Gap	Asparagine	Asparagine	Stop

APPENDIX G

GROWTH OF WILD-TYPE T. ELONGATUS BP1 IN NUTRIENT-DEFICIENT BG-11 MEDIA



APPENDIX H

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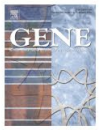

Demonstration of horizontal gene transfer from genetically engineered Thermosynechococcus elongatus BP1 to wild-type E. coli DH5α

Author: Thu H. Nguyen,Cherrelle L. Barnes,Jason P. Agola,Sana Sherazi,Lesley H. Greene,James W. Lee
Publication: Gene
Publisher: Elsevier
Date: 1 July 2019

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Survivability of Wild-Type and Genetically Engineered Thermosynechococcus elongatus BP1 with Different Temperature Conditions

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Publication: Applied Biosafety
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Author: Joachim Kopka et al
Publication: Biotechnology for Biofuels & Bioproducts
Publisher: Springer Nature
Date: Mar 6, 2017

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Photosynthetic activities of a thermophilic blue-green alga

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Yale J Biol Med. 2016 Sep; 89(3): 353–362.
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Metagenomic Assembly: Overview, Challenges and Applications

Jay S. Ghurye, Victoria Cepeda-Espinoza, and Mihai Pop*

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PRESENTATIONS

C. Barnes, J. Lee and L. Greene, "Monitoring the Stability of Transgenes in *Thermosynechococcus elongatus* BP1" Oral presentation delivered at the Virginia Academy of Science Annual Meeting, Norfolk, VA. May 2019.
C. Barnes, J. Lee, and L. Greene, "Assessing the Stability and Expression of Transgenes in Genetically Engineered Cyanobacteria for Biofuel Production" Poster presentation delivered at the American Chemical Society National Conference, Orlando, FL. April 2019.
C. Barnes, O. Sacko, L. Greene, and J. Lee, "Survivability of Genetically Engineered *Thermosynechococcus elongatus* BP1 in Different Temperature Conditions" Poster presentation delivered at the American Chemical Society National Conference, Orlando, FL. April 2019.
C. Barnes, J. Fountain, and R. Haaland, "The Influence of the Gut Microbiome on Tissue Pharmacokinetics of Pre-Exposure of Prophylaxis Regimens for HIV" Oral presentation delivered at the James A. Ferguson Research Symposium, Atlanta, GA. August 2018.
C. Barnes, T. Nguyen, J. Agola, S. Sherazi, L. Greene, and J. Lee, "Demonstration of Horizontal Gene Transfer from Genetically Engineered Cyanobacteria to Wild-type *E. coli*" Poster presentation delivered at the American Chemical Society National Conference, New Orleans, LA. March 2018.
C. Barnes, T. Nguyen, J. Agola, S. Sherazi, L. Greene, and J. Lee, "Demonstration of Horizontal Gene Transfer from Genetically Engineered Cyanobacteria to Wild-type *E. coli*" Poster presentation delivered at the Graduate Research Achievement Day, Norfolk, VA. March 2018.

PUBLICATIONS

Barnes, C.L., Lee, J.W. and Greene, L.H. (2022). Fate and Stability of Transgenes Within the Genome of Genetically Engineered *Thermosynechococcus elongatus* BP1. (*Manuscript in progress*)
Sacko, O.*, **Barnes, C.L**.*, Greene, L.H. and Lee, J.W. (2020). Growth of Genetically Engineered Cyanobacteria *Thermosynechococcus elongatus* BP1 Cyanobacteria at Ambient Temperatures. *Applied Biosafety*, 25(2), 104-117. (*) Co-first authorship
Nguyen, T.H., **Barnes, C.L**., Agola, J.P., Sherazi, S., Greene, L.H. and Lee, J.W. (2019). Demonstration of horizontal gene transfer from genetically engineered *Thermosynechococcus elongatus* BP1 to wild-type *E. coli* DH5α. *Gene*, 704, 49-58.