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Construction of a Site-Specific Integrative Vector for Use in Genetic Engineering of Cronobacter sakazakii

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Construction of a Site-Specific Integrative Vector for Use in Genetic Engineering of *Cronobacter sakazakii*

by

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Abstract

Cronobacter sakazakii is a gram-negative bacillus belonging to the family *Enterobacteriaceae* and is classed as an opportunistic pathogen. Capable of mortality rates in excess of 40% in infected infants and neonates, many investigations have sought to elucidate the pathogenesis of this organism. Constructing a site-specific integrative vector that could be utilized to engineer the species and create a fluorescent *C. sakazakii* strain may provide a useful visual aid in tissue invasion studies. The vector pOSIP-KC was utilized as a plasmid backbone into which the φES15 integrase gene, derived from a *C. sakazakii* lysogenic phage, along with the φES15 *attP* attachment sequence were cloned, creating the novel integrative vector pOSIP-ES15. The efficiency of vector integration and targeting at the predicted *attB* location was tested by transforming electrocompetent *C. sakazakii* NCTC 11467 cells with the vector and confirming integration through PCR analysis. pOSIP-ES15 appeared to preferentially integrate into other unknown locations in the chromosome, only integrating into the *attB* site approximately one third of the time. An expression cassette with a gene encoding the Superfolder GFP protein, driven by the strong, constitutive promoter nptII, was cloned into the vector to create pOSIP-ES15_GFP. Through repeated attempts, although integration of the vector into the NCTC 11467 chromosome was verified, no visual fluorescence was detectable. Fluorescence analysis by spectrophotometry showed no significant difference in emission intensity between integrated strains and parental NCTC 11467 cells. Expression of the cassette when harbored in an episomal plasmid within NCTC 11467 led to high intensity fluorescence, indicating the strain could express the cassette, but once integrated into the chromosome unknown factors caused a silencing of expression. This project demonstrated that site-specific integrative vectors can be powerful genetic engineering tools, but that without prior characterization of integration locations, expression of exogenous DNA introduced cannot always be reliably predicted.

Acknowledgements

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Chapter I: Introduction

Listeria innocua Engineering Project

The title of this thesis clearly conveys the primary focus and scope of the project. However, the idea and execution of the primary body of work stemmed from an initial investigation into genetic modification of bacteria. A collaboration with a local biotechnology company led to a proposed venture in genetically engineering the microbe *Listeria innocua* so that a strain could be generated that was constitutively, and stably, fluorescent.

Genetic modification of bacteria is not a new field of study within molecular biology. Indeed, bacteria have been genetically modified for nearly half a century (Cohen et al. 1973). However, in those succeeding decades, an array of techniques has been developed to engineer bacteria for different purposes. One very important use has been the delivery of a gene encoding a protein known as green fluorescent protein (GFP) to act as a visual reporter for several different applications. One of the most basic applications has been the generation of strains of bacteria that have the ability to fluoresce when excited with blue visible light or ultraviolet light. In the simplest experiments this has been accomplished by transforming, or inserting, an extrachromosomal plasmid containing the GFP gene into bacterial cells and inducing the cells to express the protein from the plasmid. For some purposes, this is a sufficient method for creating a fluorescent strain. However, as cells must expend resources on replicating these plasmids, this leads to a loss in energy for metabolism (Pinheiro et al., 2008). Therefore, the cells will only maintain the plasmid if it is essential for survival such as conferring resistance to an antibiotic when the cells are grown on selective media. This observation dictates the applications in which these cells may be used. For some investigations, such as studying *in vivo* pathogenesis

mechanisms involved in bacteria crossing plant or animal tissue barriers and using fluorescence as a means of tracking the bacteria, this method would not be useful as it would not always be possible to perform these studies in the presence of an antibiotic. The bacteria would not maintain the plasmid, leading to a loss of fluorescence and the subsequent ability to track the cells as they invade or infiltrate a tissue. Integrating the GFP gene into the chromosome would circumvent this and allow the fluorescence to be stably maintained in absence of a selective pressure (Pinheiro et al., 2008).

The desire by certain groups to obtain a strain of *Listeria innocua* engineered in such a manner was the prompting that led to this collaborative project. *L. innocua* is a nonpathogenic, saprotrophic, gram-positive facultatively anaerobic rod that is the sister species to the well-known foodborne pathogen *L. monocytogenes* (Velge & Roche, 2010). *L. monocytogenes* is responsible for the majority of listeriosis cases that occur through consumption of contaminated food products, mostly meat and dairy (Farber and Peterkin, 1991). Although a relatively uncommon foodborne infection compared to the more prevalent pathogens *Salmonella* and *E. coli*, *L. monocytogenes* nonetheless presents the highest hospitalization rate among those infected and results in a remarkably high fatality rate, many times exceeding 30% (Schuppler and Loesner, 2010) (Ward et al. 2004). An evolutionary offspring, *L. innocua* lost the prfA virulence gene cluster present in the pathogenic species *L. monocytogenes* while maintianing similar metabolism and growth characteristics (Velge & Roche, 2010). This has made this species a desirable surrogate for use in studies or applications where researchers may wish to avoid working directly with the pathogenic species *L. monocytogenes* and substitution with *L. innocua* is acceptable.

As strains of *L. monocytogenes* modified to be constitutively fluorescent with an expression cassette containing *gfp* engineered into their chromosomes were already in existence, this initial project became somewhat simplified. A cursory literature review into the methods utilized was warranted so that an experiment could be planned to test the techniques within *L. innocua*. Integration of *gfp* into these strains was accomplished utilizing site-specific recombination with a plasmid that had been designed to target a known location within the chromosome through the action of a protein encoded by the plasmid. The phage known as PSA, isolated from *L. monocytogenes* strain Scott A, had been previously demonstrated to integrate into multiple *Listeria* species (Lauer et al. 2002). This action is accomplished through an integrase protein contained within the phage genome that recognizes an attachment site (*attB*) located within the $tRNA^{ARG}$ gene and catalyzes a recombination event between the *attB* site on the bacterial chromosome and the homologous attachment site on the phage chromosome *(attP)*. A plasmid was constructed that contained the integrase gene along with the *attP* site. Lauer and colleagues labeled this plasmid pPL2 and demonstrated its efficiency in integrating into several different species of *Listeria* (2002). This presented an ideal tool for delivering exogenous genes into *Listeria* strains. Another group took advantage of this and designed an expression cassette that they cloned into pPL2, creating pPL3e. This expression cassette contained a strong constitutive promoter to drive expression of a GFP mutant known as GFPmut2 that exhibited increased fluorescence (Shen and Higgens, 2005). This was successfully used to create a fluorescent strain of *L. monocytogenes*. The efficiency of this plasmid in generating fluorescent strains of *L. monocytogenes* was further demonstrated in another study that utilized it to deliver four different expression cassettes designed with GFP derivatives (Balestrino et al. 2010). With

this information, it was decided that the pPL3e plasmid would be tested to determine if it would integrate into the $tRNA^{ARG}$ gene in *L. innocua*, and whether this species would express the expression cassette in an efficient manner that would allow visual detection of fluorescence.

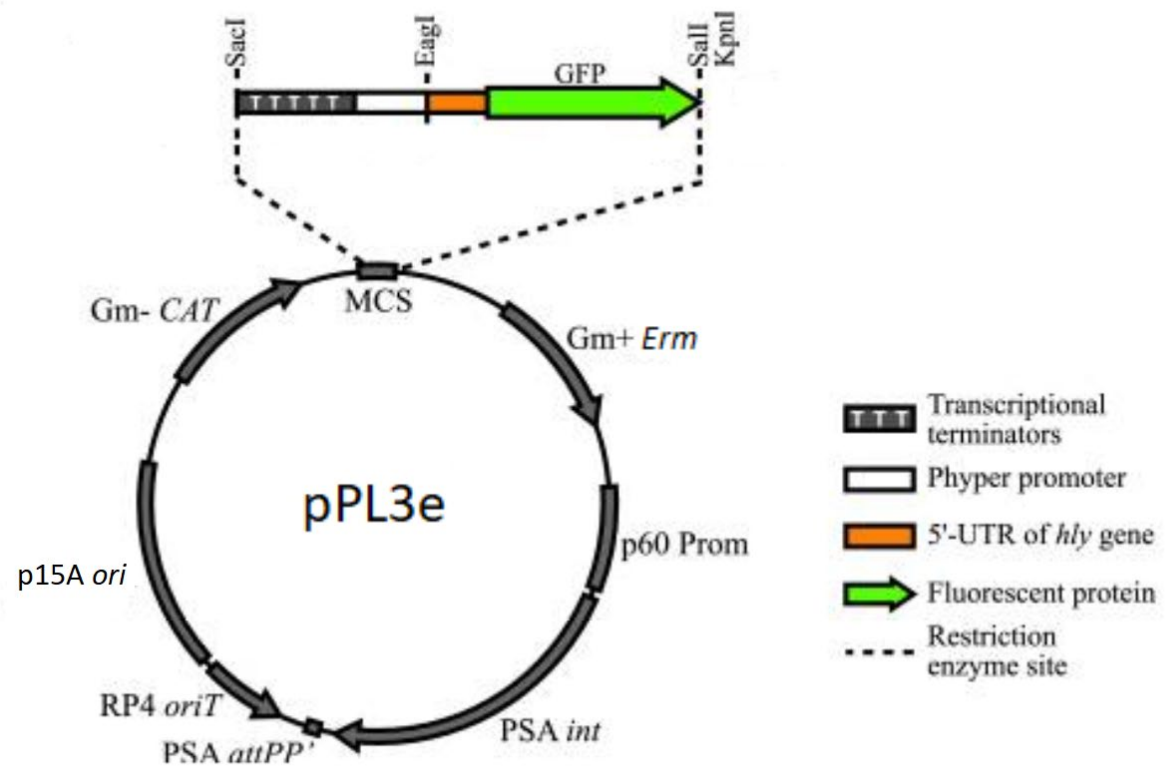

Figure 1. Plasmid map of the integrative plasmid pPL3e designed by Shen & Higgens, which is a derivative of the plasmid pPL2. The plasmid utilizes the integrase gene from the PSA listeriophage for site-directed integration into Listeria species. The fluorescence expression cassette includes the constitutive promoter Hyper-SPO1 fused to the 5' UTR of the Listeria hly gene, which acts post-transcriptionally to increase expression of downstream genes, which is gfp in this instance. An erythromycin resistance gene allows for selection of positive transformants, while a gram-negative chloramphenicol gene and origin of replication allow for propagation in E. coli.

Materials and Methods

With the pPL3e plasmid obtained through a most generous donation from the source laboratory, proceeding to test its ability to integrate into *L. innocua* required a straightforward transformation experiment. The transformation protocol was based upon a well-established protocol, which first involved the preparation of electrocompetent cells (Hupfeld et al., 2018). *L. innocua* NCTC 11288 was inoculated into 5 mL Brain Heart Infusion (BHI) broth media (Hardy Diagnostics) and was grown overnight at 37°C, shaking at 250 rpm. The following morning, 2 mL of this culture was inoculated into two separate volumes of 40 mL BHI broth and incubated at 30°C shaking at 180 rpm until reaching an optical density of OD_{600} of 0.23. The following procedure was conducted for each 40 mL culture. Once this optical density was reached, the 40 mL culture was divided into four separate 10 mL cultures. All four cultures were inoculated with Penicillin G at a concentration of 10 µg/mL. These cultures were then placed back into the 30°C incubator, shaking at 180 rpm, for two hours of growth with the added Penicillin G. The cultures were then removed and had lysozyme (VWR Life Science) added. Two of the cultures had lysozyme added at a concentration of 10 µg/mL, while the other two at a concentration of 5 µg/mL, to empirically determine proper concentration level. The cultures were placed back into the incubator at the same settings for an additional 10 minutes. They were then removed, and the cultures transferred to chilled 50 mL conical tubes (Falcon) and placed onto ice for 5 minutes. Following this, the tubes were centrifuged at 3220 x g at 4°C for 10 minutes. The supernatant was poured off after, and 1.5 mL of ice-cold Sucrose Glycerol Wash Buffer (SGWB; 0.5 M Sucrose, 10% glycerol, pH 7.4) was added to resuspend the pellet by gentle swirling while on ice. The tubes were centrifuged again at the same parameters, supernatant poured off, and pellet

resuspended with 1 mL SGWB. This step was repeated a final time, with a final resuspension in 60 µL SGWB for each pellet. These resuspensions were moved to chilled 1.5 mL microfuge tubes.

1 µg of pPL3e plasmid DNA was added to four cell resuspensions. (two 10 µg/mL lysosome, two 5 µg/mL lysozyme) and incubated on ice for 1 minute. A Harvard Apparatus BTX 830 Electroporator was utilized for the electroporation. Electroporation pulse parameters were set at 2.2 kV, 400 Ω, 25 µF. Each resuspension of electrocompetent NCTC 11288 was added to chilled 2 mm electroporation cuvettes (VWR Life Science), inserted into the electroporation apparatus and delivered an electric pulse. After pulse delivery, each cell suspension was immediately moved from the cuvette to 1 mL warmed BHI + 0.5M Sucrose broth in a microfuge tube. These were then placed into a 30°C static incubator to recover for 90 minutes. After recovery, the cells were plated onto warmed BHI Erythromycin agar plates (5 µg/mL). An electroporation positive control was conducted with a resuspension of cells with no DNA added to verify cells could survive pulse parameters, with the cells being plated onto nonselective BHI agar plates. Another resuspension that was not electroporated was plated directly onto a BHI Erythromycin agar plate to act as a negative control. All growth plates were placed into a 30°C incubator for 48 hours. After this period, they were removed and observed for growth. Transformant colonies were viewed under a light microscope at 10X magnification, illuminated by an integrated LED light at 470 nm wavelength to determine if visual fluorescence was detectable. Transformant colonies were also illuminated with a NightSea BlueStar UV Flashlight at 480 nm wavelength and viewed special filtered glasses by naked eye.

Integration of the plasmid into the chromosome at the predicted location was performed by PCR analysis. Two primers, NC16 and PL95, were utilized that amplify a 499-bp DNA fragment in strains that have the pPL3e vector integrated into the *attB* site (Lauer et al. 2002). The sequences are included in Table 1. Colony PCR was performed on the colonies by first inoculating each colony into separate 100 µL sterile diH_2O aliquots which were then vortexed to ensure a well-mixed suspension. 25 µL PCR reactions were prepared by mixing 2.5 µL 10X PCR Buffer (-Mg), 0.5 µL 10 mM dNTP, 0.75 µL 50 mM $MgCl_2$, 0.1 µL Platinum Taq DNA Polymerase (ThermoFisher Scientific), and 2 µL of the colony suspension. A negative control was prepared utilizing native NCTC 11288 cells. A thermocycler was programmed with an initial 4-minute boil step at 95°C to ensure the cells would be lysed. The rest of the program followed a standard PCR run, with a 30 second 95°C denaturation, followed by 30 second annealing at 48°C, and 30 second elongation at 72°C. 30 total cycles were programmed and completed. Once finished, the PCR reactions were combined with EZ-Vision Two DNA Dye and loaded onto a 1% agarose gel. Gel electrophoresis was conducted at 140V/200mA until the lower band of dye was approximately 5 cm from the bottom of the gel.

Table 1. Primers utilized to confirm integration of pPL3e into the *attB* site within the $tRNA^{ARG}$ gene in *L. innocua* NCTC 12288 chromosome. Primer sequences obtained from Lauer et al. 2002.

Primer Name	**Melting Temp. (°C)**	**Primer Sequence**
NC16	49.1	5’-GTCAAAACATACGCTCTTATC-3’
PL95	52.8	5’-ACATAATCAGTCCAAAGTAGATGC-3’

The fitness of the integrated strain was also tested against the native strain of NCTC 11288 to evaluate whether the integration of the plasmid negatively affected its growth rate. Four colonies of both NCTC 11288 and NCTC 11288-GFP were inoculated into 5 mL BHI broth and grown at 37°C, shaking at 250 rpm, until reaching an optical density of OD_{600} 2.0. The cultures were diluted 10^{-6} and 40 µL inoculated into 360 µL BHI broth in a 36-well plate. A negative control well was prepared with BHI broth with no inoculum. This was then loaded into a Bioscreen C automated growth curve analyzer, set at 37°C, shaking at 250 rpm. The Bioscreen recorded optical density at OD_{600} every 15 minutes for a period of 24 hours and a growth curve was constructed from the data.

Stability of the integrated plasmid was determined through a multigenerational growth experiment. A colony of NCTC 11288-GFP was inoculated into 10 mL BHI broth with no antibiotics added and placed into a 37°C incubator, shaking at 250 rpm. After 12 hours growth, the culture was removed and 50 µL of the culture was transferred to a fresh 10 mL of BHI broth. This was placed back in the incubator at the same parameters, and this process was repeated an additional 6 times, until 96 hours of growth had been achieved. At this point, the culture was diluted 10^{-6} and plated onto a nonselective BHI agar plate. The plate was placed into a 37°C incubator. After 24 hours of growth, 108 colonies were chosen at random and plated onto BHI Erythromycin agar plates (5 µg/mL) which were then placed into the 37°C incubator for 24 hours. The plate was then assessed to determine how many colonies could sustain growth on selective media and fluoresce.

Results

Upon transforming *L. innocua* NCTC11288 with pPL3e, the first experiment appeared successful as colonies were found growing on the selective media after 48 hours of growth. Utilizing a light microscope with an integral LED light emitting at a wavelength of 470 nm at 10X magnification, the colonies were observed to be visibly fluorescent as compared to a negative control of native NCTC 11288 cells viewed under the same conditions. The colonies were also easily determined to be visibly fluorescent with the naked eye when viewed with the BlueStar UV flashlight and filtered glasses. While fluorescence presumptively indicated that integration of the pPL3e plasmid had occurred, it was necessary to verify this with PCR analysis. As indicated, the primer pair utilized for confirmation only amplifies a band when integration of the vector has occurred. This is resultant from NC16 annealing to a location on the NCTC 11288 chromosome upstream of the *attB* site, while the PL95 primer anneals to a location on the pPL3e plasmid. The gel image in Figure 2 clearly demonstrated that this event had occurred. Visible in lanes 2 & 3 are amplified bands at approximately 500 bp with lanes 4 & 5 being negative controls. The band appears in the appropriate location for a strain in which the plasmid at integrated at the predicted site.

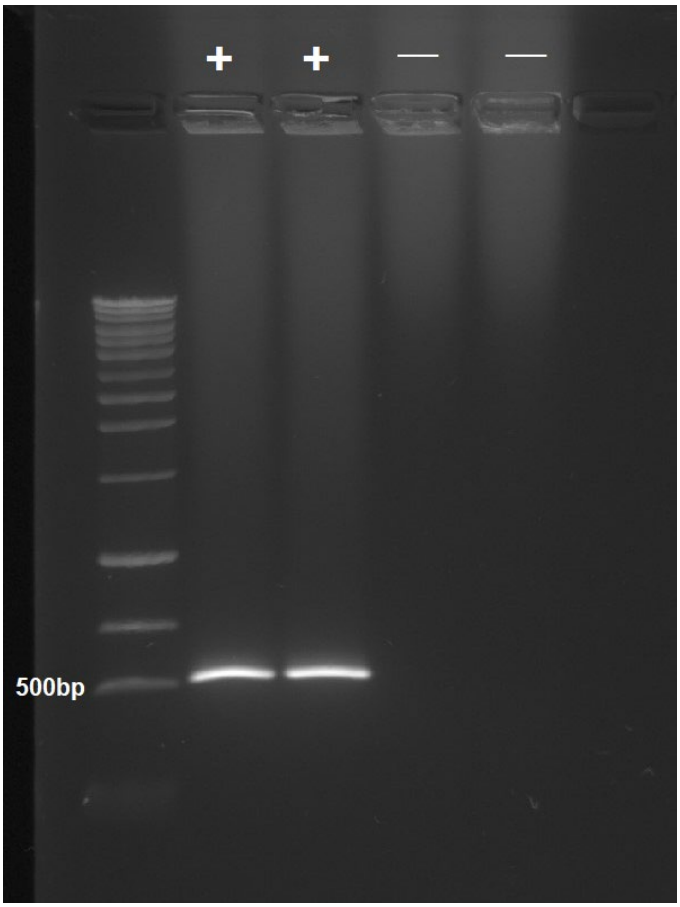

Figure 2. Confirmation of chromosomal integration by colony PCR, utilizing the primers NC16 and PL95. Bands present in Lanes 2 & 3 were amplified from transformant colonies growing on BHI media containing erythromycin. Bands appear at ~500 bp as expected. Lanes 4 & 5 are negative controls from PCR utilizing the same primer pair on parental NCTC 11288 colonies. Ladder is Quanti-Marker 1kb.

Stability of the integrated plasmid was tested in the multigenerational growth experiment. Over 100 generations of cell growth had been achieved by the multiple passaging. As was evident in the final plating onto selective Erythromycin media, all 108 colonies were able to survive and maintain fluorescence. Four of these colonies were chosen to proceed with the fitness assay. After 24 hours of growth in the Bioscreen C, a growth curve was constructed from the collected measurements. This curve is presented in Figure 3. Cultures 1-4 are the integrated NCTC 11266-GFP, while cultures 5-8 are the parental non-engineered strain. The growth curves

from the eight cultures all follow the same trend. A One-Way ANOVA was conducted, comparing each culture against one another as individual groups, resulting in an $F = 0.0576$, $p = 0.996$. This indicated there was no statistical significance in growth between any of the samples.

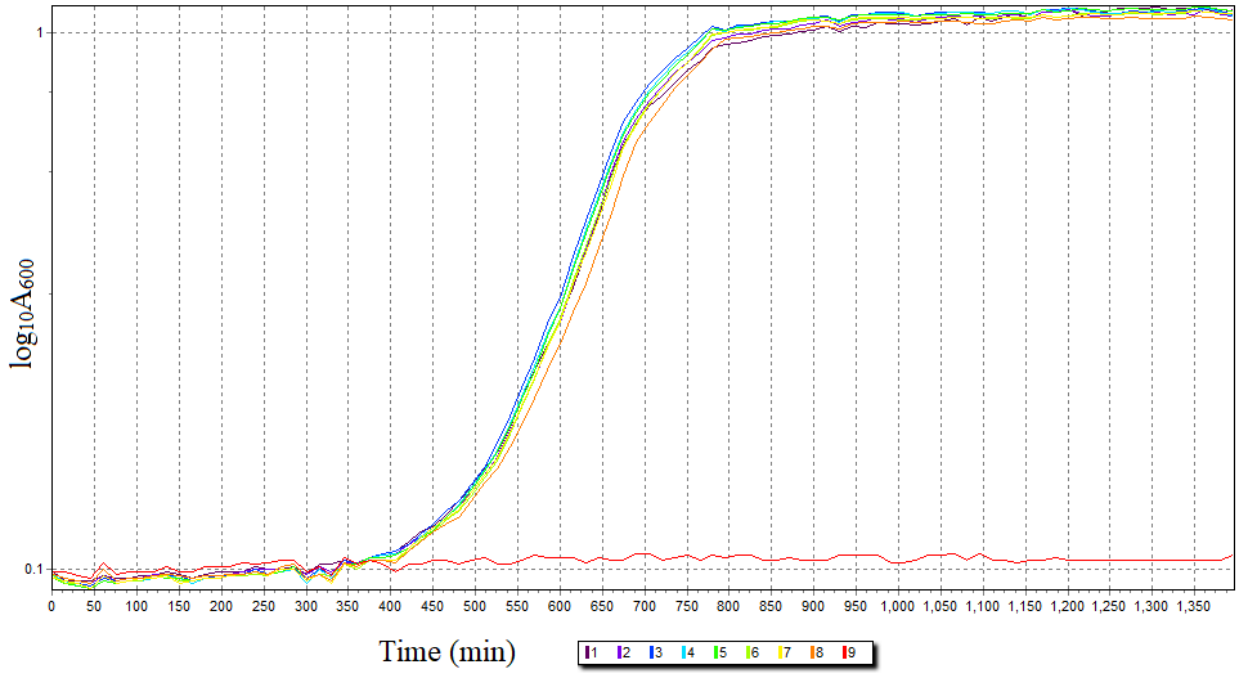

Figure 3. Fitness test comparing growth curves from the engineered strain and the parental strain performed on an automated growth curve Bioscreen C system. Samples 1-4 are cultures of L. innocua-GFP, while samples 5-8 are parental strain NCTC 11288. Sample 9 is a negative control. One-Way ANOVA showed no statistical differences between the gfp-integrated cultures and the parental cultures.

Discussion

In engineering *L. innocua* to generate a stable, constitutively fluorescent strain, there were several lessons and observations that were gleaned. A group looking to engineer a bacterial species of interest must consult the published literature to determine if there is a genetic tool that has already been developed for use in the specific species or family that is of interest. The groups that first developed the pPL2 plasmid, that was further modified to create the pPL3e plasmid

utilized here put forth much time and effort to determine what genetic elements would be necessary in generating these tools that allowed precise integration and efficient expression. This is no small task, and the effort here in engineering *L. innocua* would have been magnified greatly if a cursory literature review had not been first conducted that shed light on the existence of these plasmids and how they were constructed. While this may seem quite obvious, oversight in finding pertinent literature has led to countless hours being squandered in laboratories.

The pPL3e plasmid integrated into the predicted *attB* site on the *L. innocua* chromosome upon the first transformation experiment conducted. The transformant colonies that were obtained were highly fluorescent and easily detectable with the naked eye. PCR analysis confirmed that the integration occurred in the predicted location, as observed in Figure 2. Amplification was only possible if the plasmid was present, as NC16 annealed the vector while PL95 annealed the genome. The basic fitness tests demonstrated that in basic growth conditions the integrated plasmid did not induce major metabolic strain upon the cells and that they grew at the same rate as the parental strain of cells lacking the plasmid.

The most important observation from this project was the sheer proficiency exhibited by the integrative plasmid in generating an engineered bacterial strain. What was expected to be a project that could have been quite extensive ended up an exercise in electrotransformation technique. For a group that had never conducted any similar experiments in genetic modifications of bacteria, the site-specific targeting combined with the efficiency established the notion that for future projects this method may be the most preferable to explore.

Chapter II: *Cronobacter sakazakii*

Introduction

Cronobacter sakazakii is a gram-negative bacillus belonging to the family *Enterobacteriaceae* that is classified as an opportunistic pathogen (Iversen, et al., 2007). Of the members of the genus *Cronobacter*, *C. sakazakii* has been of the most interest to the research community due to its ability to cause sepsis, necrotizing enterocolitis, and meningitis in infected infants and neonates (Yan et al., 2012). Often, the mortality rate in those infected infants is in excess of 40% (Yan et al., 2012). It has been linked to, and identified within, several tragic outbreaks including the 1994 France outbreak where 17 new-born children were infected in a single hospital NICU, resulting in 3 infant deaths (Feeney, Kropp, O'Connor, & Sleator, 2014). The pathogenesis of the organism is still under current investigation, with several virulence factors identified that may aid in its ability to invade and cross tissue barriers (Singh, Goel, & Raghav, 2015).

While it has a variety of natural reservoirs, the primary mode of transmission to humans is through contaminated powdered infant milk formula (Feeney, Kropp, O'Connor, & Sleator, 2014). Unfortunately, this organism has the ability to survive well in desiccated environments and this increases the likelihood of contamination of powdered formula products post-pasteurization (Breeuwer, Lardeau, Peterz, & Joosten, 2003). While not being an extremely thermotolerant organism, the lipid A core endotoxin it possesses has demonstrated the ability to remain biologically active in these products post-pasteurization and heat treatment, which makes detection of *C. sakazakii* a high priority (Townsend, Barron, Loc-Carrillo, & Forsythe, 2007).

These factors have made *C. sakazakii* a primary organism of concern to manufacturers of powdered formula.

Reviewing this information, it was proposed that there could be utility and applications for a strain of *C. sakazakii* engineered to be brightly fluorescent. Such a strain would be useful in studies where tracking cells in tissue invasion or membrane infiltration would be made simpler through visual analysis rather than enumeration assays. Constructing an engineering tool that would allow efficient integration into the *C. sakazakii* chromosome at a defined location would allow for delivery of an expression cassette containing the GFP gene to accomplish this. Additionally, this tool could be useful in other studies where engineering this species with exogenous genetic elements may be useful. As the pPL3e integrative plasmid worked so well in this manner in the *Listeria* project, it was decided that construction of a site-specific integrative vector for use in *C. sakazakii* would be pursued.

Cronobacter **Engineering Vector**

Before this project could begin, it was necessary to determine if there was a characterized, temperate phage isolated from *C. sakazakii* whose full genome sequence had been published. As integrative vectors rely upon the action of a phage integrase protein to perform the integration mechanism, constructing a vector is dependent upon the ability to clone an integrase gene. Furthermore, as phages coevolve with their host bacterial species their molecular machinery evolves to function with host-encoded factors and sequences (Koskella & Meaden, 2013). To construct an integrative vector for a target species of bacteria, it can thus be expected to operate most efficiently when the integrase is derived from a lysogenic phage isolated from the species. A review of the pertinent literature revealed that there was indeed a lysogenic phage

that had been induced from its prophage state from a strain of *C. sakazakii* by UV induction, and the genome sequenced.

φES15 is a phage that was first identified as a prophage existing within the genome of the *C. sakazakii* strain ES15, isolated from ground whole grains (Lee et al., 2012). Once isolated, the genome was sequenced by the source laboratory and published on NCBI. This genome had been automatically annotated by gene prediction programs and ribosomal binding site locators, which gave predictions based upon homology (Lee et al., 2012). Analyzing this genomic data, the gene at locus tag φES15_003 was found to be called as a putative integrase. Additionally, a 26-bp sequence was identified as a potential attachment site *attP*, upon which the putative integrase would integrate into the 26-bp identical *attB* sequence on the host chromosome. It is worthy to note the mechanism that integrases operate by so that it is clear why these sequences were important to find. Integrase proteins operate by recognizing sequence similarity between the *attP* in the phage genome and the homologous *attB* in the host genome (Groth & Calos, 2004). The integrase then binds and cleaves the phosphodiester backbone at these sites through a catalytic amino acid residue present in their active sites; either a serine or tyrosine depending upon which evolutionary class the protein falls in (Groth & Calos, 2004). The nucleotide strands are then swapped between the two chromosomes and ligated to one another, allowing integration of the phage chromosome into the host. Integrative vectors exploit these sequences to target a known location and integrate into that site. With the sequences of these two elements known, an experiment could be planned to build an integrative vector that would be tested in engineering the type strain *C. sakazakii* NCTC 11467.

Using NCBI Blast, this 26-bp *attB* was identified within the genome of NCTC 11467. Located in an intergenic region, it appeared an ideal site for integration of a vector, as it should not be disruptive to genes or other major metabolic elements. The decision was made to move forward with a proposed project, which would seek to collect data to answer these three hypotheses:

Hypothesis 1: An integrative vector constructed utilizing the φES15 integrase gene and *attP* sequence will integrate into *C. sakazakii* NCTC 11467 chromosome at the predicted location

Hypothesis 2: The constructed engineering vector can be utilized to integrate a fluorescent expression cassette into *C. sakazakii* genome, to generate a fluorescent strain

Hypothesis 3: The putative φES15 integrase performs a molecular recombination between the putative viral *attP* sequence and host *attB* sequence

Chapter III: Materials and Methods

Integrative Vector Design

In order to proceed with designing and constructing an integrative engineering vector for use within *C. sakazakii*, choosing a suitable plasmid backbone was necessary. As there have been many excellent choices of previously designed integrative vectors discussed in the scientific literature, it was decided that one of these would afford the best backbone. The plasmid decided upon was a member of a line of plasmids known as the One-Step Integration Plasmids, or pOSIP, originally designed to efficiently integrate into the chromosome of *Escherichia coli* (St-Pierre et al., 2013). The plasmids in this family all shared the same basic design but differed in the specific integrase genes and respective *attP* sites that were designed in each. Similar to all integrative plasmids, they were constructed with an integrase gene, *attP* site, multiple cloning site, antibiotic resistance gene, and a conditional method of replication. The design features that made these plasmids attractive as a donor backbone were the R6Kγ origin and the λ temperature sensitive repressor. The R6Kγ origin would only allow this plasmid to be replicated in the propagation strain of *E. coli* containing the PIR1 gene, thereby forcing recipient cells of C. sakazakii to integrate it into their chromosomes or they would be unable to replicate and grow. The λ temperature sensitive repressor allows for efficient expression of the integrase gene at the permissive temperature of 37°C, but prevents expression at 30°C, thus allowing a temperature-inducible control of plasmid integration.

The specific plasmid member chosen for this project was known as pOSIP-KC and contained the integrase gene φC31 and its respective *attP* site. pOSIP-KC (see Figure 4) contained convenient restriction sites at the sequences that needed to be excised, and the same sites were not present within the sequence designs that were to be cloned into the construct.

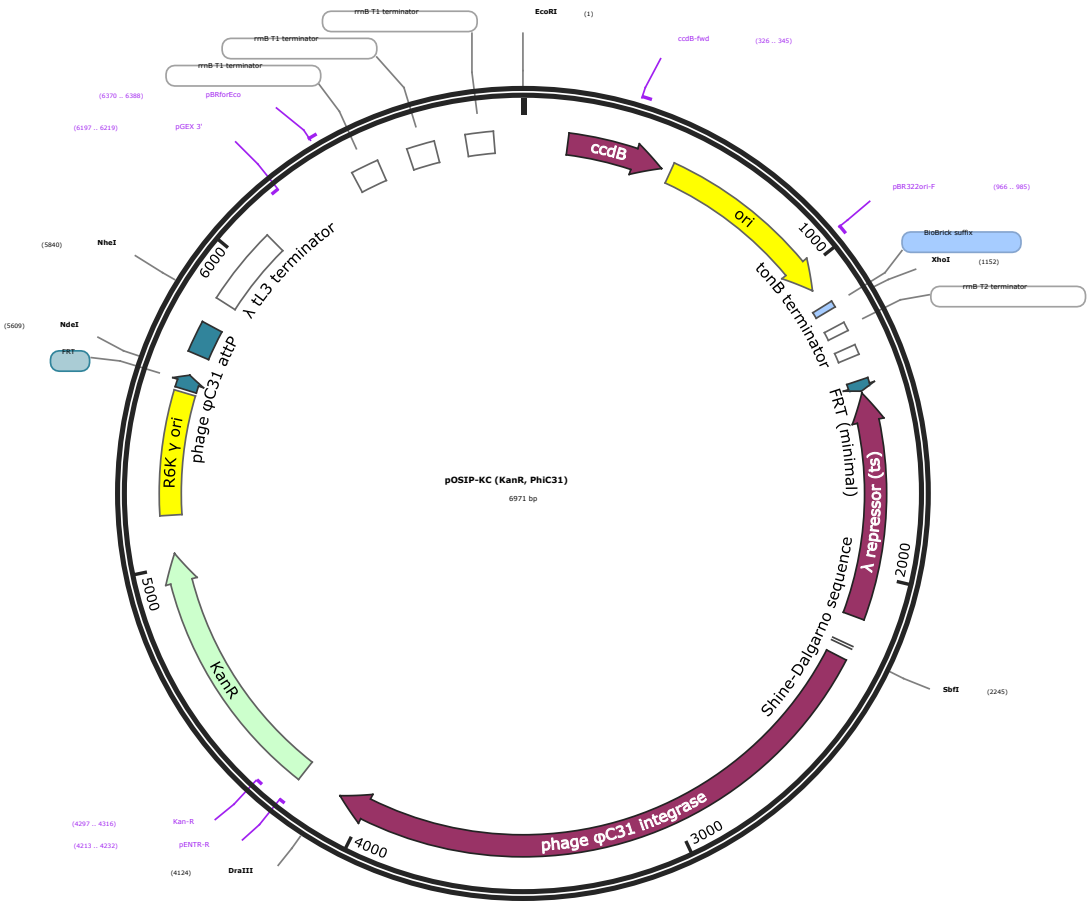

Figure 4. Plasmid map of the integrative vector pOSIP-KC, chosen as the plasmid backbone from which an engineering vector for use in *C. sakazakii* could be constructed. This vector was chosen due to its desirable design attributes, including a convenient multiple cloning site, conditional origin of replication, and a temperature-sensitive repression system that allows selective expression of the integrase gene.

With a backbone vector chosen, the insert sequences could be designed. First, a short noncoding 255-bp sequence was designed for replacement of the original sequence present within the multiple cloning site. This noncoding fragment would act as a placeholder within the multiple cloning site for initial integration testing of the construct before a coding sequence would replace it. It was designed with an EcoRI and XhoI restriction site on either side. The second fragment designed was a 1305-bp fragment containing the coding sequence for the

φES15 integrase gene. A synthetic ribosomal binding site allowing for upregulated translation of the transcribed mRNA was inserted before the start codon and was designed utilizing the online RBS Calculator by the Salis Lab (Espah Borujeni et al., 2017; Espah Borujeni & Salis, 2016; Espah Borujeni, Channarasappa, & Salis, 2013; Salis, Mirsky, & Voigt, 2009). DraIII and SbfI restriction sites flanked the sequence, allowing for directional insertion in the proper orientation for the λ repressor protein to act upon the native φES15 integrase promoter. The third sequence was a 206-bp fragment containing the φES15 *attP* site derived from the phage genome. This fragment was designed with flanking NheI and NdeI restriction sites. These first three fragments were to be cloned into the pOSIP-KC backbone, yielding the base integrative plasmid pOSIP-ES15, at a size of 5452-bp. All three of these fragments were ordered from, and synthesized by, the company Biomatik and arrived cloned within separate pBSK plasmids (Delaware, USA). Fragment sequences are included in Appendix A.

Following the presumptive integration tests with this plasmid, it would then be utilized to deliver an expression cassette contained within the multiple cloning site. This 1408-bp cassette was designed to contain the necessary regulatory elements that would allow constitutive expression of the target gene. As the goal was to create a fluorescent strain of *C. sakazakii*, the target gene would code for a fluorescent protein. The gene that was chosen for this project encoded the green fluorescent protein variant Superfolder GFP, which produces fluorescence that is approximately three times the brightness over that of the native *Aequorea victoria* green fluorescent protein (Pedelacq et al., 2006). Driving expression of the Superfolder GFP gene would be the nptII promoter. This promoter has been demonstrated to be a strong, constitutive promoter within *Enterobacteriaceae*, and was originally derived from the neomycin

phosphotransferase II gene conferring kanamycin resistance to the *E. coli* strain it was first discovered in (Remus-Emsermann, Gisler, & Drissner, 2016). This would allow for constant transcription of the Superfolder GFP gene. Following the promoter, a synthetic ribosomal binding site, once again designed using the online RBS Calculator, was inserted in front of the start codon to allow for upregulated translation. The gene was followed by a rrnB1 transcriptional terminator and λ t0 transcriptional terminator. This 1.4-kb fragment was designed with an EcoRI restriction site and an XhoI site for directional insertion. Following insertion into the multiple cloning site, this would yield a final 6380-bp integrative engineering plasmid that would deliver an expression cassette into the *C. sakazakii* chromosome to allow fluorescence.

Integrative Vector Construction

Construction of the vector was accomplished through a series of successive restriction enzyme cloning steps. All restriction enzymes and associated buffers utilized were obtained from New England Biolabs (Ipswich, MA). The pOSIP-KC vector was ordered from Addgene (Watertown, MA) and arrived as an episomal plasmid contained within *E. coli*. The *E. coli* was inoculated into 5 ml of liquid LB broth and incubated overnight at 37°C shaking at 250 rpm. The following morning, the culture was harvested at 7000 x G for 5 minutes and plasmid DNA was extracted utilizing the Hi-Speed Mini Plasmid Kit from IBI Scientific according to the kit protocol (IBI Scientific, Dubuque Iowa). The first round of restriction enzyme cloning involved a removal of the expression cassette contained within the multiple cloning site of pOSIP-KC to be replaced with the MCS noncoding fragment. Two micrograms of pOSIP-KC plasmid DNA were digested with 1 µl EcoRI, 1 µl XhoI, 5 µl CutSmart buffer, and a volume of diH20 to bring the total volume to 50 µl. At the same time, 2 micrograms of pBSK plasmid DNA containing the

noncoding fragment were digested with the same reagents and amounts. Both reactions were incubated at 37°C for two hours to ensure complete digestion. 1 µl of shrimp alkaline phosphatase (rSAP, New England Biolabs) was added to each reaction and incubated for a further 30 minutes at the same temperature, followed by an incubation at 65°C for five minutes to inactivate the rSAP enzyme. 10 µl of EZ-Vision Two DNA dye (VWR Life Science) was added to each reaction, once cooled to room temperature. Both 60 µl volumes were loaded into separate wells of a 1% agarose gel, alongside a 1-kb DNA ladder (GeneMate Quanti-Marker) and electrophoresis was conducted at settings of 140V/200A for 90 minutes, until the lower dye band was approximately 2 centimeters from the bottom of the gel. The gel was visualized with an Omega Lum gel imaging system (Aplegen) and the respective bands of DNA excised for extraction from the gel (5820-bp pOSIP-KC, 255-bp noncoding Linker). DNA extraction was performed using a Gel Extraction Kit (IBI Scientific) following the protocol included. The extracted linear fragments were then used in a ligation reaction. Ligation reaction calculations were performed using the NEB Ligation Calculator. The reaction was setup using a 3:1 insert : vector ratio, utilizing 80 ng of vector to 11.6 ng of insert in a 20 µl ligation reaction consisting of 2 ul T4 ligase buffer, 1 µl T4 ligase and diH20. The ligation reaction was incubated at 10°C for a period of approximately 16 hours, which was followed with a 10 minute heat inactivation at 65°C. 2.5 µl of this ligation reaction was then transformed into One Shot™ PIR1 Chemically Competent *E. coli* (ThermoFisher Scientific). The transformation was conducted according to the protocol supplied with the cells. Following the recovery step in the protocol, the cells were diluted 100-fold and plated onto LB-Kanamycin selective agar plates and incubated for 24 hours at 30°C, as this temperature would ensure suppression of the encoded integrase gene and prevent

the propagation cells from integrating the plasmid. Transformants were successfully recovered from the plates after the incubation period. Verification of the ligation reaction was conducted by selecting five colonies at random from the plates. These colonies were inoculated into LB-Kanamycin liquid broth for overnight growth at 30°C shaking at 250 rpm. Plasmid DNA was extracted from these colonies following the same procedures detailed above. Insert ligation was confirmed by digesting one microgram of extracted plasmid DNA from each of the five colonies with both EcoRI and XhoI in reactions prepared in the same manner as described previously. Once EZ-Vision Dye was added, the digestion reactions were loaded onto a 1% agarose gel and ran according to the same parameters. The gel was visualized using the Omega Lum imaging system and confirmed that the ligation worked as expected and that this first subcloning step was successful.

The following two subcloning steps were conducted in the same manner as described for the cloning of the first insert. The second subclone involved the removal of the φC31 integrase gene from pOSIP-KC using DraIII and SbfI and the insertion of the φES15 integrase gene. The third subclone removed the φC31 *attP* site using NheI and NdeI, to allow for the insertion of the φES15 *attP* site. Following this last subcloning step and isolation of the plasmid DNA from the propagation PIR1 E. coli, this final vector construct was confirmed by performing four separate restriction enzyme digestions of the plasmid. The first reaction was a single digestion with ApaLI, which confirmed both the expected final size and the φES15 *attP* site insertion as this site was only present within this sequence. The next three digestions were with the same enzyme pairs utilized for the subcloning steps.

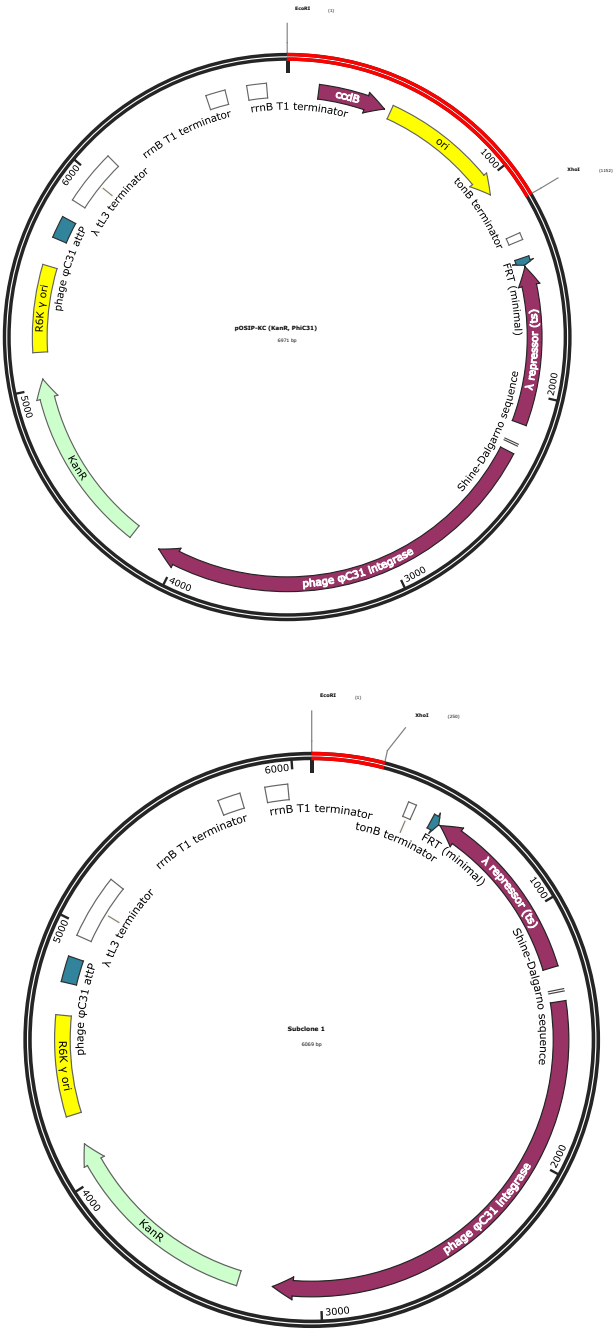

Figure 5. First subcloning step in construction of pOSIP-ES15. The cassette contained within the multiple cloning site of pOSIP-KC was removed with the restriction enzymes EcoRI and XhoI. In its place, a 255-bp noncoding fragment was ligated. The fragments are highlighted in red. This resulted in the plasmid labeled Sublcone 1.

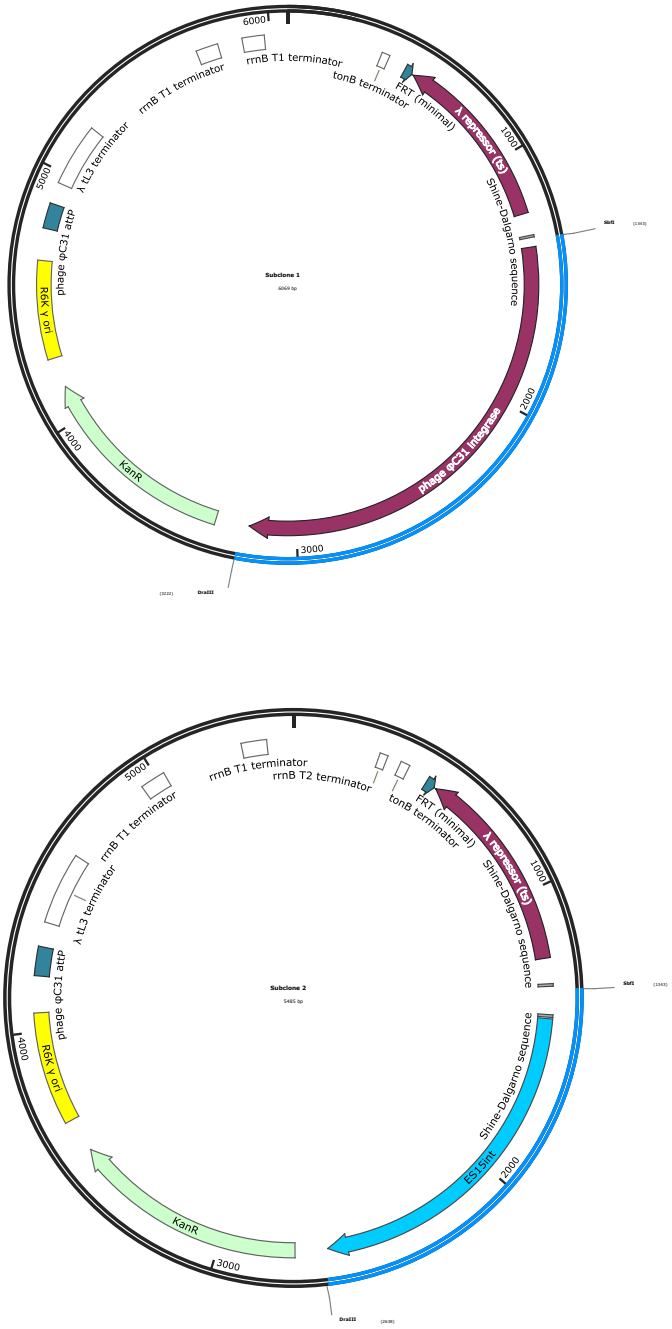

Figure 6. Second subcloning step in construction of pOSIP-ES15. The φC31 integrase gene present in Subclone 1 was removed using DraIII and SbfI restriction enzymes. The 1405-bp φES15 integrase gene was ligated into its place. The fragments involved in this step are highlighted in blue. This resulted in the plasmid labeled Subclone 2.

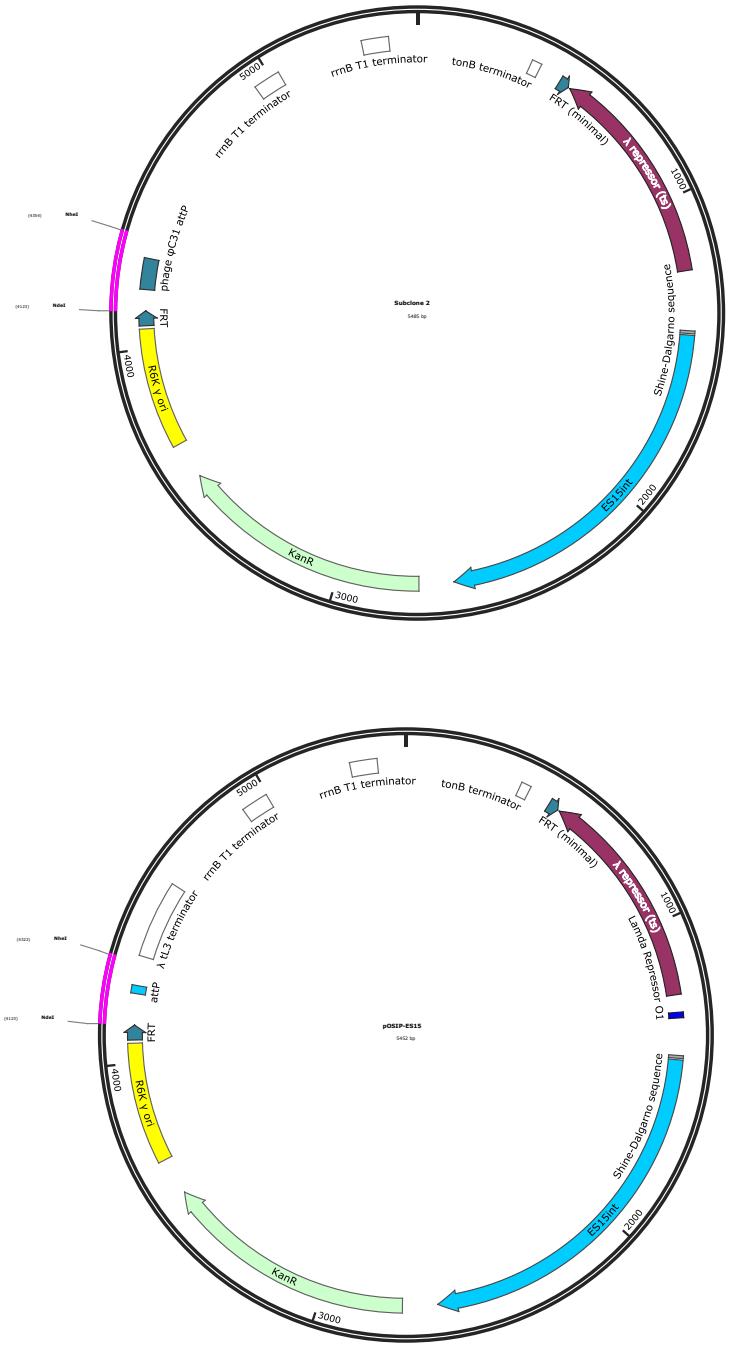

Figure 7. Third subcloning step in construction of pOSIP-ES15. The φC31 *attP* site was removed using the restriction enzymes NheI and NdeI. The 206-bp φES15 *attP* fragment was ligated in its place. The fragments involved in this step are highlighted in magenta. This resulted in the 5452-bp integrative vector labeled pOSIP-ES15.

Transformation by Electroporation

Once the pOSIP-ES15 integrative vector construction had been confirmed, it was necessary to test its ability to integrate before attempting to utilize it to deliver an expression cassette into the chromosome. This test simply involved a transformation of competent *C. sakazakii* cells with the vector and observing if transformant colonies could be obtainable on kanamycin selective media. Confirmation of integration would be confirmed by PCR analysis. The first step involved a preparation of *C. sakazakii* electrocompetent cells. 50 ml of LB broth was inoculated with 500 µL of an overnight, turbid culture of *C. sakazakii* NCTC 11467 and placed into a 37°C incubator, shaking at 250 rpm. Optical density of the culture was measured at a wavelength of 600 nm at regular intervals of 30 minutes until a OD_{600} measurement of 0.6 was reached. The cells were transferred to a chilled 50 mL conical centrifuge tube and chilled on ice for 30 minutes. All following steps were performed to ensure that the cells remained cold throughout the entire process. The culture was then centrifuged at 1000 x g, 4°C for 15 minutes, and the supernatant decanted. The cell pellet was resuspended in 50 mL ice-cold 10% glycerol while being kept on ice and was centrifuged again at 1000 x g, 4°C for 10 minutes. The supernatant was once again decanted, and the cell resuspended in 25 mL ice-cold 10% glycerol. Centrifugation was repeated, and two more successive wash steps, first in 10 mL then in 5 mL 10% glycerol were performed in the same manner as above. After the final centrifugation following the 5 mL wash, the supernatant once again was decanted, and the pellet resuspended in a final volume of 500 µL 10% glycerol. From this, 60 µL aliquots of the now-electrocompetent cells were prepared in chilled microfuge tubes and kept on ice. The cells were used immediately in electroporation in order to ensure that transformation efficiency would remain the highest. A

Harvard Apparatus BTX 830 Electroporator was utilized for the electroporations. 100 ng of pOSIP-ES15 plasmid DNA was added to a 60 µL aliquot of cells. The aliquot was mixed gently by flicking and incubated on ice for 1 minute. The cells were then transferred to a frozen (-20°C) 2 mm electroporation cuvette (VWR Signature) and immediately placed into the electroporation apparatus to ensure the cuvette remained cold. The cells were electroporated at settings of V = 2.5 kV, 200 Ω, 25 µF. They were then transferred from the cuvette to a microfuge tube containing 950 µL SOC media, and incubated at 37°C for one hour, shaking at 250 rpm. A positive control was performed using 100 ng pBSK Simple-Kan vector, while a negative control was performed with 5 µL diH_2O. After incubation, cells were plated onto LB Kanamycin agar plates (25 µg/mL) and placed into a 30°C incubator for 24 hours. The negative control was plated onto both LB Kanamycin plates as well as nonselective LB plates to act as a positive control of cell survival through electroporation process.

Confirmation of Integration

After 24 hours of growth, the plates were removed from the incubator and colonies were observed to be growing on the media. This was a presumptive indication that the vector had integrated but a series of confirmation experiments were necessary to validate this observation. 64 colonies were selected and re-streaked onto LB kanamycin plates at double the kanamycin concentration (50 µg/mL) to rule out possible false positives. The colonies that were able to grow on the higher concentration antibiotic media were then prepared for PCR analysis. A primer pair was designed that selectively annealed at positions flanking the hypothesized integration location in the chromosome. Primers were synthesized and obtained from IDT DNA (Coralville, IA). These primers were labeled Flank Forward and Flank Reverse, with sequences

contained in Table 2. Colony PCR was performed on the colonies by first inoculating each colony into separate 100 µL sterile diH_2O aliquots which were then vortexed to ensure a well-mixed suspension. 25 µL PCR reactions were prepared by mixing 2.5 µL 10X PCR Buffer (-Mg), 0.5 µL 10 mM dNTP, 0.75 µL 50 mM $MgCl_2$, 0.1 µL Platinum Taq DNA Polymerase (ThermoFisher Scientific), and 2 µL of the colony suspension. A positive control was prepared with 10 ng linearized pOSIP-ES15 plasmid, and a negative control with native *C. sakazakii* cells. A thermocycler was programmed with an initial 4-minute boil step at 95°C to ensure the cells would be lysed. The rest of the program followed a standard PCR run, with a 30 second 95°C denaturation, followed by 30 second annealing at 53°C, and 6-minute elongation at 72°C. 30 total cycles were programmed and completed. Once finished, the PCR reactions were combined with EZ-Vision Two DNA Dye and loaded onto a 1% agarose gel. Gel electrophoresis was conducted at the same parameters described previously.

Table 2. Primer pairs used in confirmation of integration at predicted *attB* location and presence of pOSIP-ES15 vector.

Primer Name	**Melting Temp. (°C)**	**Primer Sequence**
Flank Forward	55.9	5'-GCAGCTTAATAACCTGCTTAGAGC-3'
Flank Reverse	57.3	5'-GTAGGTCCCAAGGCTCATCTTG-3'
KanR Reverse	54.9	5'-ATCGCGAGCCCATTTATACC-3'
ES15 Forward	55.0	5'-ATCGACTTTATCGGAAACACCG-3'

Another secondary PCR analysis was conducted utilizing the primers KanR Reverse and ES15 Forward, that selectively annealed within the integrative vector only. While the prior primer pair would confirm integration into the hypothesized location, this primer pair would only

confirm presence of the vector, in the possible instance of non-specific integration into off-target sites. PCR reactions were prepared in the same manner as just described, with the exception of an annealing temperature of 50°C and a shortened 1-minute elongation. These samples were subjected to gel electrophoresis analysis by the same method as described.

Expression Cassette Subcloning

The expression cassette containing the gene for Superfolder GFP was the last insert to be subcloned into the pOSIP-ES15 vector. As mentioned previously, all DNA fragments were synthesized by Biomatik, including this one, and arrived in the cloning plasmid. Subcloning of this cassette followed the same procedures as for the previous fragments, using the restriction enzyme pair EcoRI and AatII, which removed the noncoding fragment from the multiple cloning site of the pOSIP-ES15 vector to be replaced with this cassette. Confirmation of successful subcloning was performed with a restriction digest and gel electrophoresis to confirm insert size and location.

pOSIP-ES15_sGFP Integration

Following successful insertion of the expression cassette into the integrative vector, it was necessary to transform the vector into the *C. sakazakii* NCTC 11467 to allow chromosomal integration and test for expression of fluorescence. Transformation was conducted by electroporation in the same manner as described with the pOSIP-ES15 vector lacking the expression cassette, and electroporated cells were plated onto LB Kanamycin agar plates (25 µg/mL). Plates were incubated for 24 hours at 30°C. Following successful obtainment of transformants growing on the media plates, confirmation of chromosomal integration was conducted by PCR analysis with the same primer pairs as utilized previously according to the

same protocols. Detection of fluorescence was performed visually by observing transformant colonies underneath a light microscope at 10X magnification with light supplied by an integrated LED light at 470 nm wavelength.

Integration of the pOSIP-ES15_sGFP vector was also conducted within *E. coli* as a secondary control. *E. coli* K12 cells were made electrocompetent according to the same protocol as NCTC 11467 and transformed under the same parameters and conditions. Cells were plated onto LB Kanamycin agar plates (25 μg/mL), incubated for 24 hours at 30°C, and transformant colonies obtained were subject to the same PCR analysis to confirm integration utilizing the KanR Reverse and ES15 Forward primer pair.

Expression Cassette Control

A secondary control was conducted to confirm that the expression cassette could be efficiently transcribed and translated by *C. sakazakii*. This was performed by transforming a vector containing the expression cassette along with an origin of replication functionally recognized into competent *C. sakazakii* cells. The plasmid utilized was pOK12, with the expression cassette cloned into the multiple cloning site. The plasmid was transformed into competent cells according to the same protocols as previously described. The cells were plated onto LB Kanamycin agar plates (50 μg/mL), incubated overnight at 37°C and transformant colonies observed under the LED microscope.

Fluorescence Assay

An assay was performed to measure the levels of fluorescence emitted by the strains of *C. sakazakii* and E. *coli* that were generated through chromosomal integration of the pOSIP-ES15_sGFP vector. This was conducted using a Varian Cary Eclipse fluorescence

spectrophotometer. Three strains of both *C. sakazakii* and *E. coli* were measured. These included the strains containing a chromosomal integration of the vector, the strains harboring the episomal pOK12-sGFP plasmid, and the native strains to act as the baseline measurements. Three colonies of each were inoculated into LB broth so that measurements could be taken in triplicate. The cultures were incubated at 37°C shaking at 250 rpm until an optical density of 0.5 at OD_{600} was reached. Each culture was then measured, undiluted, in the fluorescence spectrophotometer in a clear quartz cuvette. Excitation was set at 485 nm, and the spectrophotometer recorded emission spectra in the range of 490–550 nm.

Active Site Mutation Assay

A set of experiments was conducted to empirically determine if integration of the pOSIP-ES15 vector into the chromosome of *C. sakazakii* was reliant upon the action of the ES15 integrase protein and that integration was not occurring through homologous recombination events. To determine this, the gene encoding the integrase was mutated to generate an inactive form of the protein. Using NCBI Conserved Domain Database, the sequence of the protein was compared against other integrase proteins to determine phylogeny (Lu et al., 2020). The tyrosine in the active site responsible for performing nucleophilic attack on the phosphodiester backbone was identified by comparison against other members in the protein family (see Figure 19). With a single base substitution, this tyrosine could be changed to a phenylalanine by switching the codon TAC to TTC. This was accomplished by having the gene synthesized with this single base pair change. This ES15-mutant gene was then subcloned from the vector it arrived in, into pOSIP-ES15 in place of the native gene using the same subcloning methods and protocol described previously. Once proper insert subcloning was confirmed by digestion and gel

electrophoresis, the vector was transformed into *C. sakazakii* by electroporation. A series of concurrent transformations were conducted, with NCTC 11467 transformed with pOSIP-ES15, pOSIP-ES15_GFP, and pOSIP-ES15_MUTANT in triplicate. The cells were then plated onto LB Kanamycin agar plates (25 µg/mL) and placed in a 30°C incubator for 24 hours, after which they were observed for growth.

Intermolecular Plasmid Integration Assay

An intermolecular plasmid integration assay was performed to collect additional supporting evidence that the ES15 integrase protein performs an integration event between the *attP* site and the *attB* site. If pOSIP-ES15 were harbored in a cell along with another plasmid that contained the *C. sakazakii* NCTC 11467 *attB* site sequence, a recombination could be expected with the two plasmids becoming a single larger plasmid as the pOSIP-ES15 integrates into the other. To test this hypothesis, two primers, named *attB* EcoRI Forward and *attB* BamHI Reverse, were designed that would amplify a 639-bp fragment from the *C. sakazakii* NCTC 11467 genome containing the 26-bp *attB* site along with flanking elements. Primer sequences are contained in Table 4. As their names implied, the primers were utilized to add restriction sites to either end of the amplicon to allow for cloning of the fragment into a vector. Annealing temperatures in the PCR amplification were programmed for 53°C for 10 cycles, followed by an increase to 60°C for 20 cycles to compensate for the overhang. Otherwise, the PCR reaction was performed as previously described, utilizing a colony of NCTC 11467 cells as the genomic template. The PCR reaction was subjected to restriction enzyme digestion with EcoRI and BamHI before fragment isolation utilizing gel electrophoresis. The amplicon was extracted from the gel utilizing the Gel Extraction Kit (IBI Scientific) according to the protocol provided. This

extracted fragment was then ligated into pUC19 plasmid that had been linearized with EcoRI and BamHI according to ligation procedures described earlier.

Table 3. Primer pairs used in cloning of 639-bp *attB* site fragment from NCTC 11467 genome.

Primer Name	**Melting Temp. (°C)**	**Primer Sequence**
attB EcoRI Forward	62.7	5'-GTCAAGGAATTCGCAGCTTAATA ACCTGCTTAGAGC–3'
attB BamHI Reverse	63.2	5'–GTATAAGGATCCGTAGGTCCCAA GGCTCATCTTG–3'

Commercial NEB 5 Alpha chemically competent *E. coli* cells were concurrently transformed with 100 ng pOSIP-ES15_sGFP and 100 ng pUC19-NCTC11467_*attB* according to the protocol provided. Following recovery, the cells were plated onto LB Kanamycin/Ampicillin agar plates (50 µg/mL / 100 µg/mL). These plates were then placed into a 30°C incubator for 24 hours. Transformant colonies that were obtained were subjected to plasmid DNA extraction. The plasmid DNA was then digested with the restriction enzyme pair NheI and XhoI and the digestion subjected to gel electrophoresis. Following this, the same transformation procedure was repeated with the pOSIP-ES15_mutant plasmid with the codon-substituted ES15 gene and attempts to obtain transformant colonies were conducted.

Chapter IV: Results

Integrative Vector Construction

The three subcloning steps that were described in the construction of the vector pOSIP-ES15 are visually represented in Figures 5-7. The plasmid map of the final integrative vector pOSIP-ES15 is included in Figure 8. Restriction enzymes utilized for cloning steps, as well as the various features are labeled.

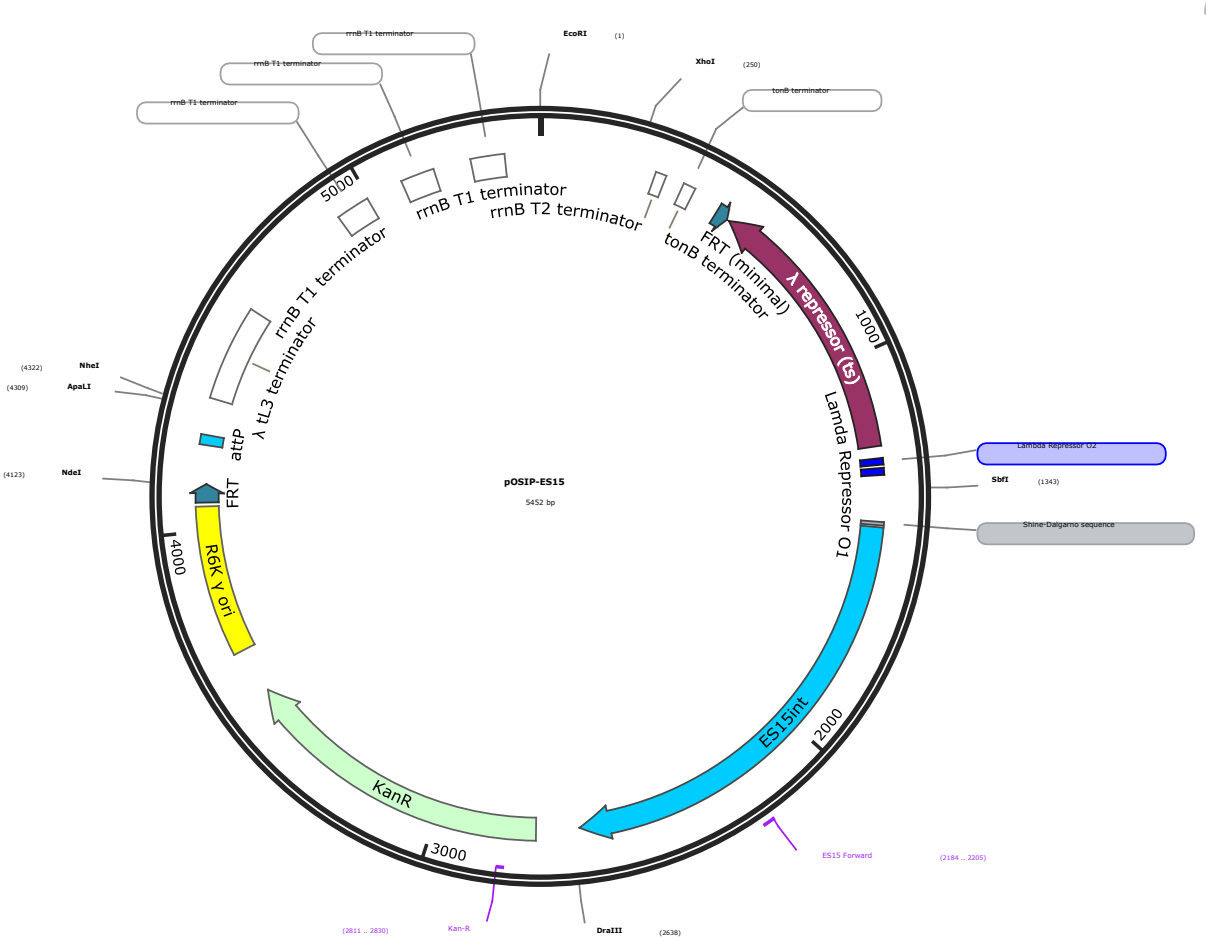

Figure 8. Plasmid map of pOSIP-ES15. Enzymes utilized in restriction cloning are labeled, along with the primer pair used to verify presence of the vector within transformant cells. Individual features and orientations are indicated.

Once the final cloning step was completed and the vector obtained by plasmid DNA extraction, confirmation of insert sizes and proper locations was conducted by restriction enzyme digest. Banding patterns are presented in the gel image in Figure 9. ApaLI confirmed the expected size of the vector, while also confirming presence of the proper third insert. The following three lanes confirmed proper inserts by digestion with the enzyme pairs used in cloning.

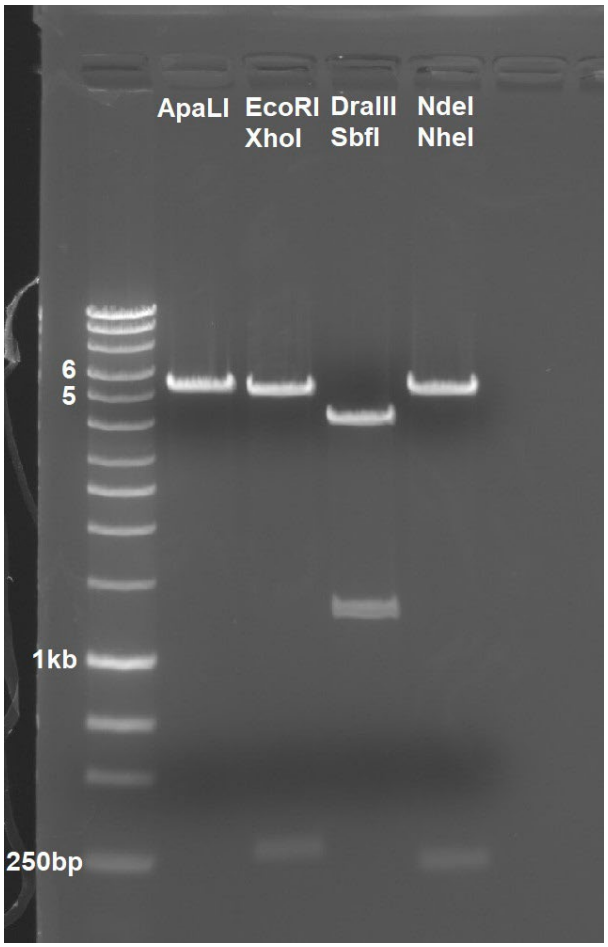

Figure 9. Gel image of final confirmation of pOSIP-ES15 construction. Expected size is confirmed with ApaLI, and proper size inserts at expected locations are confirmed with the restriction enzyme pairs used in cloning. Insert one (EcoRI/XhoI) present at 255-bp. Insert two (DraIII/SbfI) present at 1305-bp. Insert three (NdeI/NheI) present at 206-bp. Ladder is Quanti-Marker 1 kb.

pOSIP-ES15 Integration Confirmation

After transforming NCTC 11467 cells by electroporation with pOSIP-ES15, colonies were observed growing on the selective kanamycin media 24 hours after being placed in the incubator. A total of 30 colonies were obtained in the first transformation experiment. This presumptively indicated that the cells were positive integrants, and that the plasmid had inserted into the chromosome as predicted. As it was possible that these colonies were not true integrants or that integration occurred in locations other than the predicted *attB* site, PCR analysis was performed to confirm. The results from ten integrant clones are presented in Figure 10. Lane 1 is the DNA ladder, followed by clones in lanes 2-11 and the positive control in lane 12.

The banding patterns in Figure 10 resulted from amplification with Flank Forward and Flank Reverse. These primers anneal into the chromosome upstream and downstream of the *attB* site. If integration of the vector occurred at the predicted *attB* site, the primers would amplify the entire 5.4-kb plasmid. This was observed in lanes 7-9, where a faint band is visible. If integration did not occur at the *attB* site, the primer pair would amplify a genomic fragment 639-bp in size, as is clearly visible in the positive control in the final lane. The ~640-bp band is visible in six other lanes, indicating one of two possibilities. The first being that these clones were mutants not carrying the plasmid that had gained resistance to kanamycin. The second possibility was that the plasmid had integrated into the chromosome, but at a location other than the predicted site. To rule out the former and verify the latter, another PCR analysis was conducted using a separate primer pair. This primer pair, KanR Reverse and ES15 Forward, anneal at two locations in pOSIP-ES15 (indicated in Figure 8) and amplify a fragment 647-bp in size when the vector is present within a cell. PCR was conducted utilizing this primer pair and the same ten clones

presented in Figure 10. The results of the PCR analysis are presented in Figure 11. A band is present at approximately 650-bp in every lane. This demonstrated the vector's presence in all ten clones and ruled out the possibility that these were false positive mutants that had gained kanamycin resistance. PCR analysis on 20 more clones revealed similar results as the ten presented here. Approximately 25% of all integrant colonies presented pOSIP-ES15 integration into the expected location. The remaining colonies had vector presence verified, but integration location was indeterminable with PCR analysis.

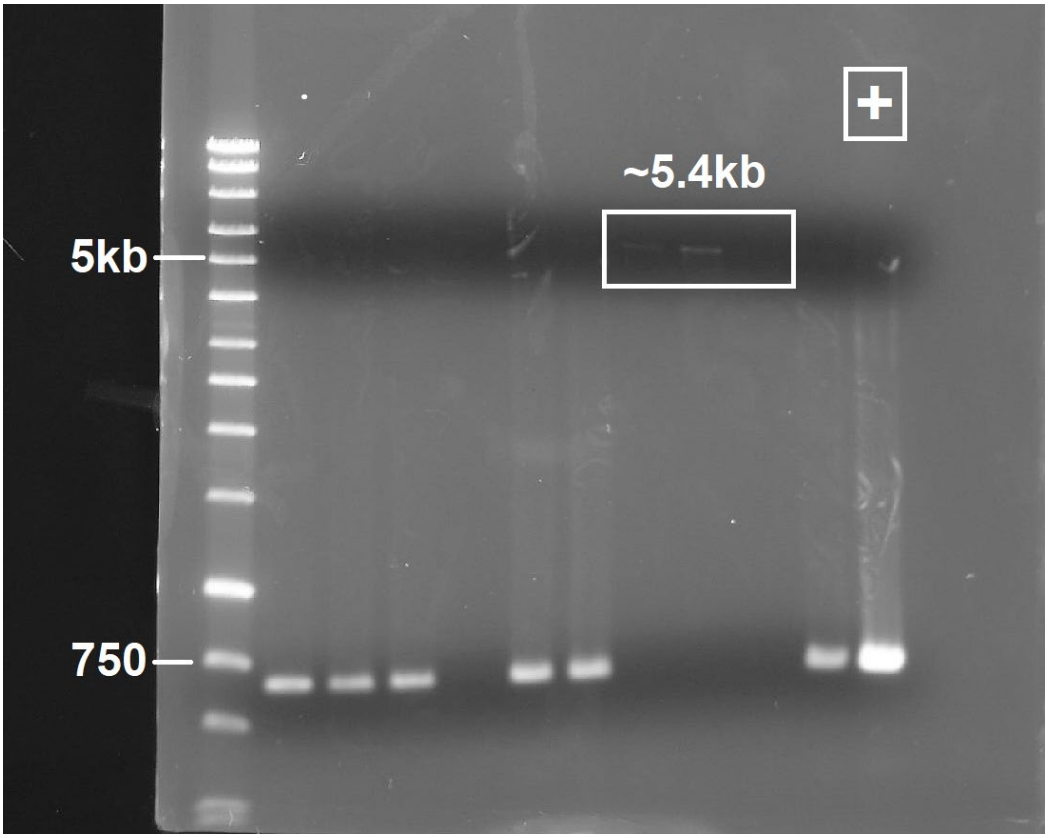

Figure 10. Amplification of bands from ten integrant colonies using Flank Forward and Flank Reverse. Integration into the predicted *attB* site would result in amplification of the entire 5.4-kb integrated vector. This is observed in lanes 7-9. Six other lanes amplified the ~640-bp band if vector was not present at *attB*.

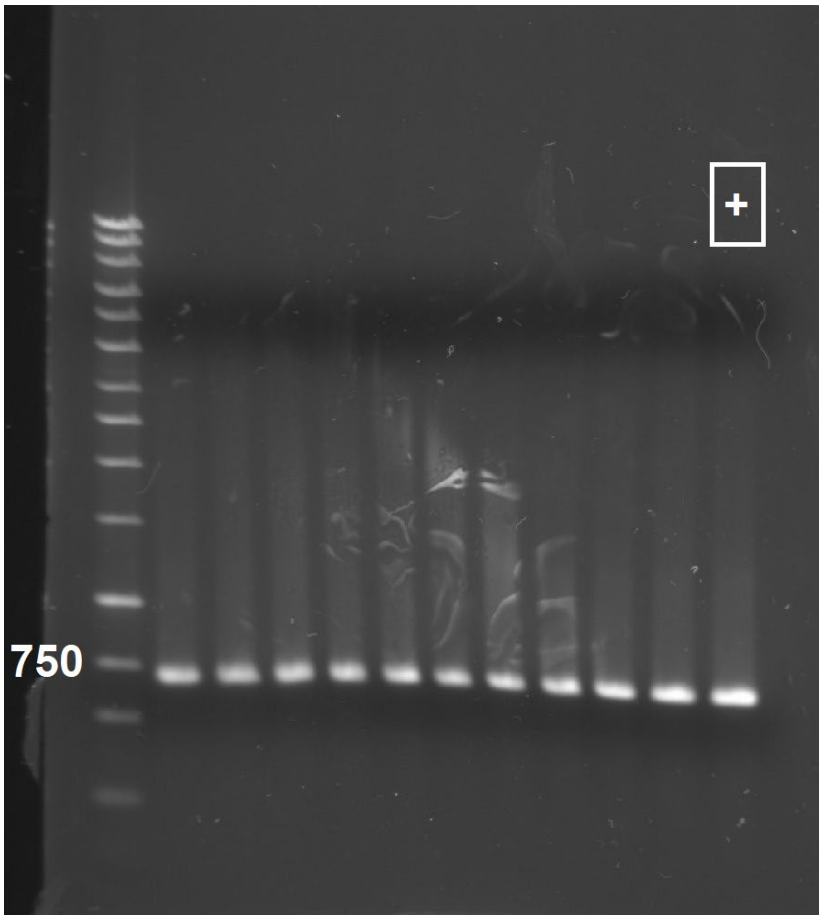

Figure 11. Gel image of PCR analysis on same ten clones as in Figure 10, conducted with primer pair KanR Reverse and ES15 Forward which anneal within pOSIP-ES15. Primer pair amplifies a ~650-bp band when vector is present within the integrant. All ten clones amplified the band at the expected location. Positive control utilized linearized pOSIP-ES15 as template.

pOSIP-ES15_sGFP Construction

After confirming that the pOSIP-ES15 vector does integrate into the expected *attB* location on the NCTC 11467 chromosome, albeit at a low frequency, it was decided that an attempt would be made to utilize the vector to deliver the expression cassette that would allow for constitutive fluorescence. The plasmid map of this vector pOSIP-ES15_GFP is presented in Figure 12. The 1408-bp cassette was ligated into pOSIP-ES15 after digestion with EcoRI and AatII. Confirmation of final vector size and insert location is presented in Figure 13.

Cloning of the expression cassette into pOSIP-ES15 went smoothly, with transformant PIR1 colonies all housing the recombined plasmid. It is worthy of note that the *E. coli* cells propagating this vector were quite visibly fluorescent upon a cursory examination using a Blue LED flashlight. This gave initial confidence that the construct could allow for visible fluorescence once integrated into *C. sakazakii* NCTC 11467.

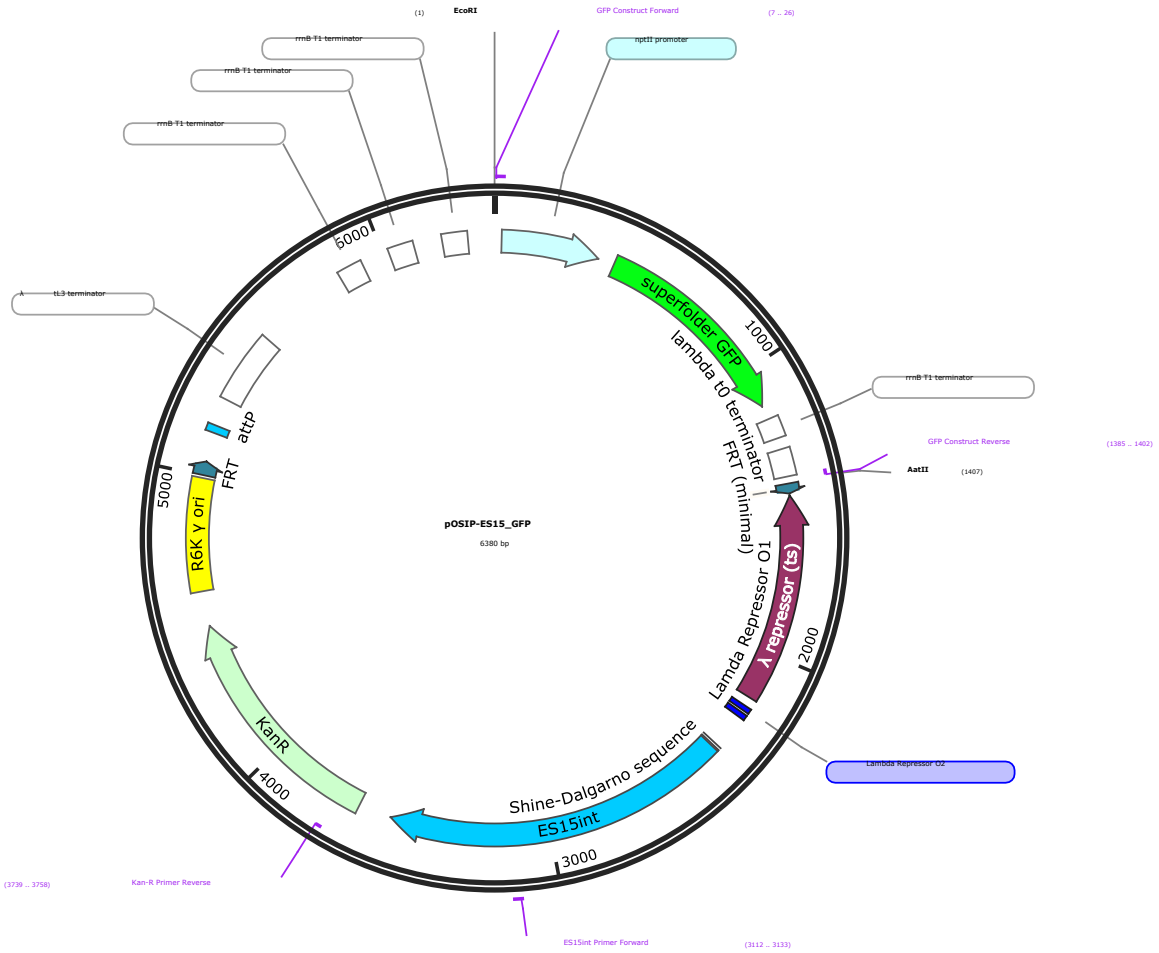

Figure 12. Plasmid map of pOSIP-ES15_GFP. Final vector size was 6380-bp with the 1408-bp expression cassette cloned into the multiple cloning site. The orientation and design of the expression cassette are visible, with the constitutive nptII promoter situated upstream of the Superfolder GFP gene, followed by the rrnB T1 and lambda T0 transcriptional terminators.

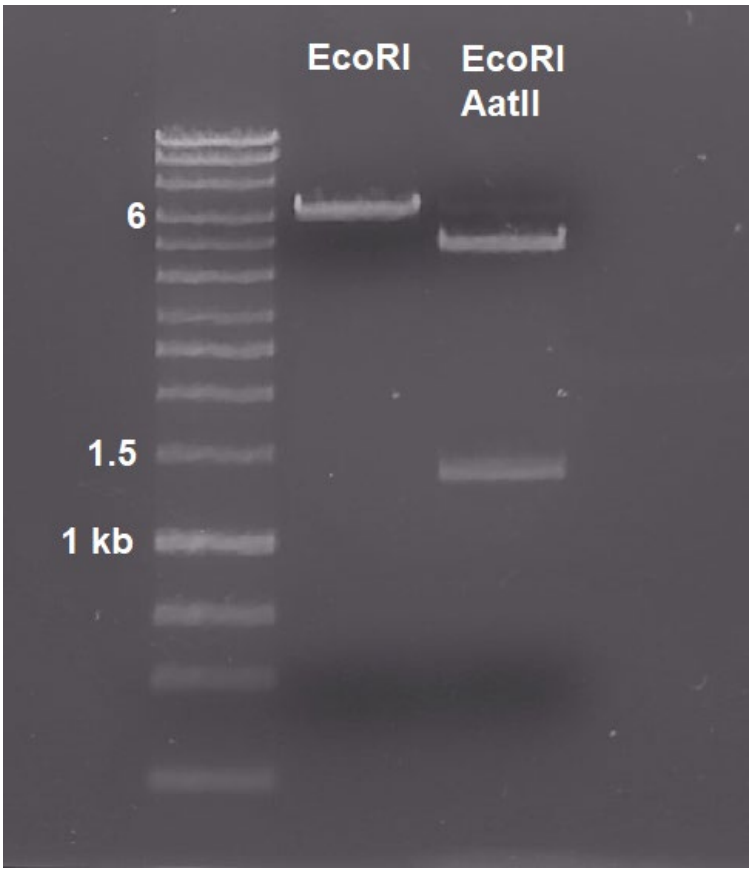

Figure 13. Confirmation of pOSIP-ES15_GFP construction by restriction enzyme digest. Digestion with EcoRI confirmed 6380-bp size and digestion with EcoRI/AatII confirmed proper insert location and size.

pOSIP-ES15_sGFP Integration

As with the empty pOSIP-ES15 vector, transformant colonies were observed growing on the kanamycin selective media after the first attempt at electroporation, albeit at a lower number. Integration of the vector into the chromosome at the predicted *attB* site needed to be verified once again. Using the primer pair Flank 1 and Flank 2, attempts were made to locate a transformant that amplified a 6.4-kb fragment from the *attB*. However, as is illustrated in the gel image example in Figure 14 created from a sample of six random transformant colonies, a positive transformant with integration at the *attB* site was not located. Of the 36 colonies

obtained in the initial round of transformation, none amplified a 6.4-kb band, and all amplified the ~640-bp band present when the native *attB* site is intact with no vector integration.

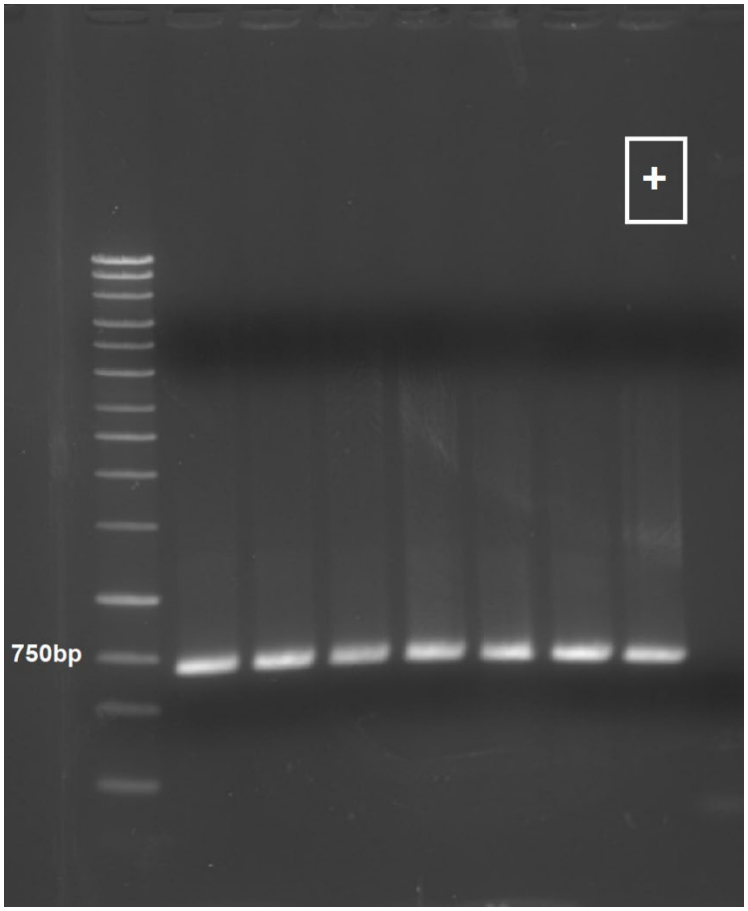

Figure 14. Confirmation of pOSIP-ES15_GFP integration by PCR analysis with primer pair Flank Forward and Flank Reverse. In the six clones presented here, all six amplified at ~640-bp band, indicating vector integration at a location on the chromosome other than the *attB*. In none of the integrant colonies obtained was integration at the *attB* site verified by amplification of a 6.4-kb band.

Integrant colonies were analyzed with the KanR Reverse and ES15 primers as performed with the pOSIP-ES15 integrants to confirm that the vector was present somewhere within the chromosome. The gel image in Figure 15 illustrate the results from the same six clones presented in Figure 14. All six of these clones amplified the band at ~640-bp that would be expected if the

vector is present in the chromosome. The results do not indicate any location, merely that the vector did indeed integrate somewhere.

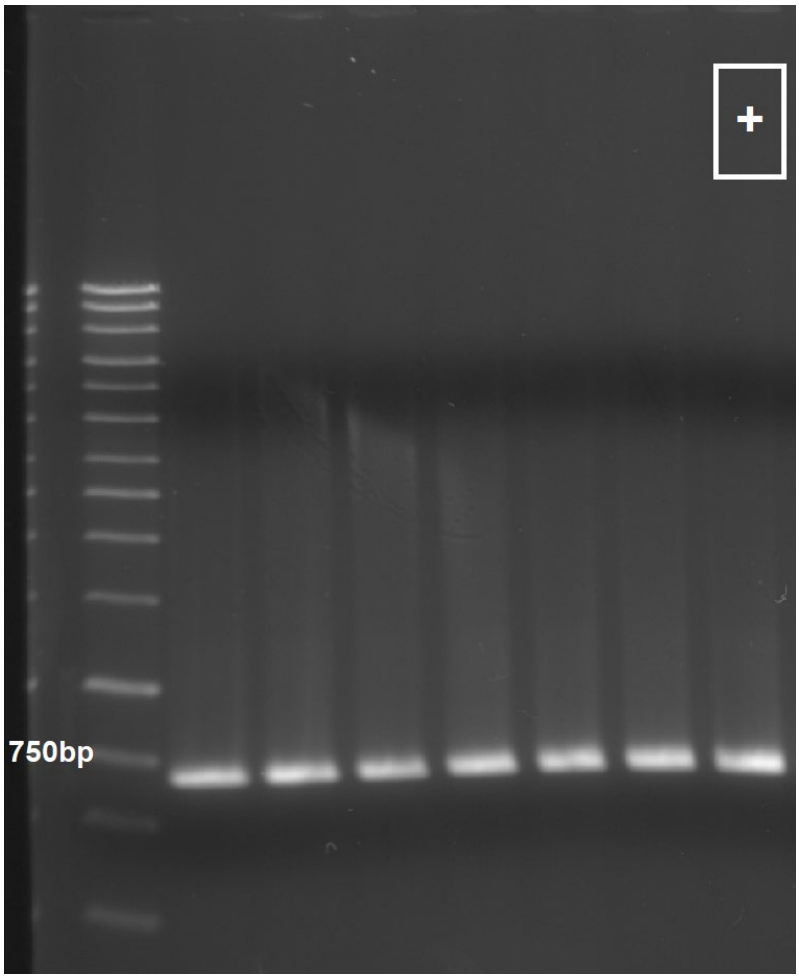

Figure 15. Confirmation of pOSIP-ES15_GFP integration by PCR analysis using primer pair KanR Reverse and ES15 Forward. The six clones presented here, which are also included in Figure 14, all amplified a ~650-bp band, indicating that the integrative vector was present within the chromosome, but at a location other than the predicted *attB* site. Unfortunately, none of the colonies were visibly fluorescent.

Although the PCR analyses dd present evidence that integration of the vector had occurred, none of the integrant colonies appeared fluorescent when viewed at 10X magnification under a microscope illuminated with 470 nm wavelength LED light. From the attempts with excitation with wide-wavelength UV lights, none of the colonies were discernable from non-

transformant NCTC 11467 cells. As this indicated a number of possible explanations, the first control that was conducted was integration of the vector into *E. coli* K12 cells to determine if expression of the cassette was detectable, as these cells were visibly fluorescent when they housed the episomal pOK12-GFP plasmid the synthesized cassette arrived in. Verification of integration was confirmed with KanR Reverse and ES15 Forward, as indicated in Figure 16, but as with NCTC 11467 no fluorescence was visually detectable. This gave support to the notion that there may be issues with expression of the vector once integrated.

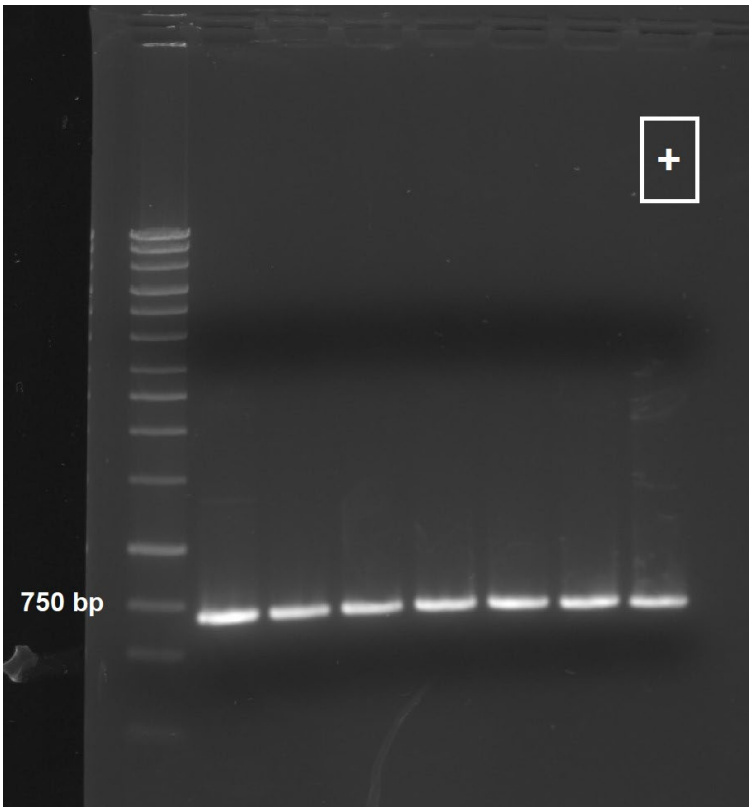

Figure 16. Confirmation of pOSIP-ES15_GFP integration into *E. coli* cells by PCR analysis using primer pair KanR reverse and ES15 forward. As the *E. coli* cells utilized in this control experiment lacked the PIR1 gene necessary for replication of the vector, they acted as a suitable control to determine if integration would occur. All transformant colonies analyzed contained the vector, but as with *C. sakazakii*, no fluorescence was detectable.

Expression Cassette Control

After confirming that the pOSIP-ES15_GFP vector had the ability to integrate into the chromosome of NCTC 11467 but that no fluorescence could be detected, a secondary expression control was conducted. As the expression cassette arrived in the vector pOK12, which *C. sakazakii* could replicate, this vector was transformed into the strain. The panels in Figure 17 show the results. *C. sakazakii* cells harboring the pOK12-GFP vector were brightly fluorescent as compared to cells with integrated pOSIP-ES15-GFP. This confirmed the strain could indeed express the cassette, and that single-copy integration could present fluorescence levels too low to be detected visually.

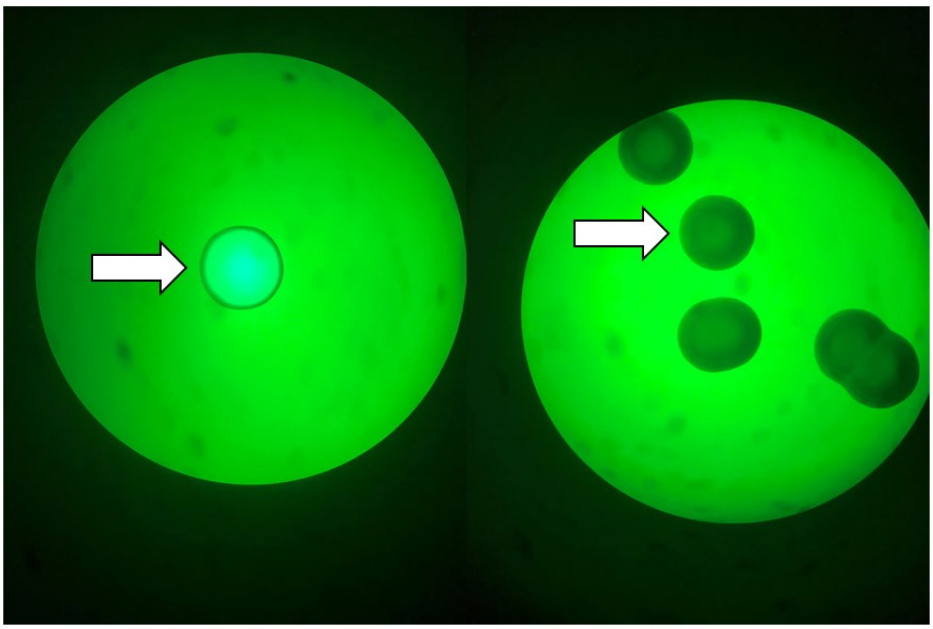

Figure 17. Images obtained at 10X magnification, with cells excited by LED light at wavelength 470 nm. The left panel demonstrates the easily detectable fluorescence of *C. sakazakii* NCTC 11467 cells harboring the replicative pOK12-sGFP vector that contained the same expression cassette as was cloned into pOSIP-ES15_GFP. Panel on right are NCTC 11467 cells with integrated pOSIP-ES15_GFP for comparison in fluorescence emitted. White arrows indicate colonies.

Fluorescence Assay

After confirming that *C. sakazakii* NCTC 11467 could express the cassette when housed in an episomal vector and emit brightly visible fluorescence, and earlier evidence revealed that *E. coli* had the ability as well, experimentation with more highly sensitive techniques than the human eye was necessary. Fluorescence levels generated by *C. sakazakii* pOK12-GFP, *C. sakazakii* pOSIP-ES15_GFP, *C. sakazakii* NCTC 11467, *E. coli* pOK12-GFP, *E. coli* pOSIP-ES15_GFP, and *E. coli* NEB 5 Alpha were recorded in the range of wavelengths 490 nm – 550 nm. With each culture tested in triplicate with three separate samples, the average intensity was recorded at each wavelength, as well as standard deviation. This data is presented in the table in Appendix B. A line graph was constructed from the data and compiled in Figure 18. The *C. sakazakii* and *E. coli* strains carrying the episomal pOK12-GFP vector emitted fluorescence levels at a much greater intensity than any of the other four bacterial strains. When all six samples were compared with One-Way ANOVA analysis, this revealed a F-value = 304.601, $p < 0.05$. This indicated a statistical significance in intensity values. A Tukey's Post-Hoc test revealed that intensity values from both *C. sakazakii* pOK12-GFP and *E. coli* pOK12-GFP were statistically different than the other four samples. When the remaining four samples were compared against one another in One-Way ANOVA with Tukey's Post-Hoc, the only sample that showed a statistically significant difference was NCTC 11467, which had the lowest fluorescence readings of any of the samples and was acting as the baseline control for no fluorescence expression.

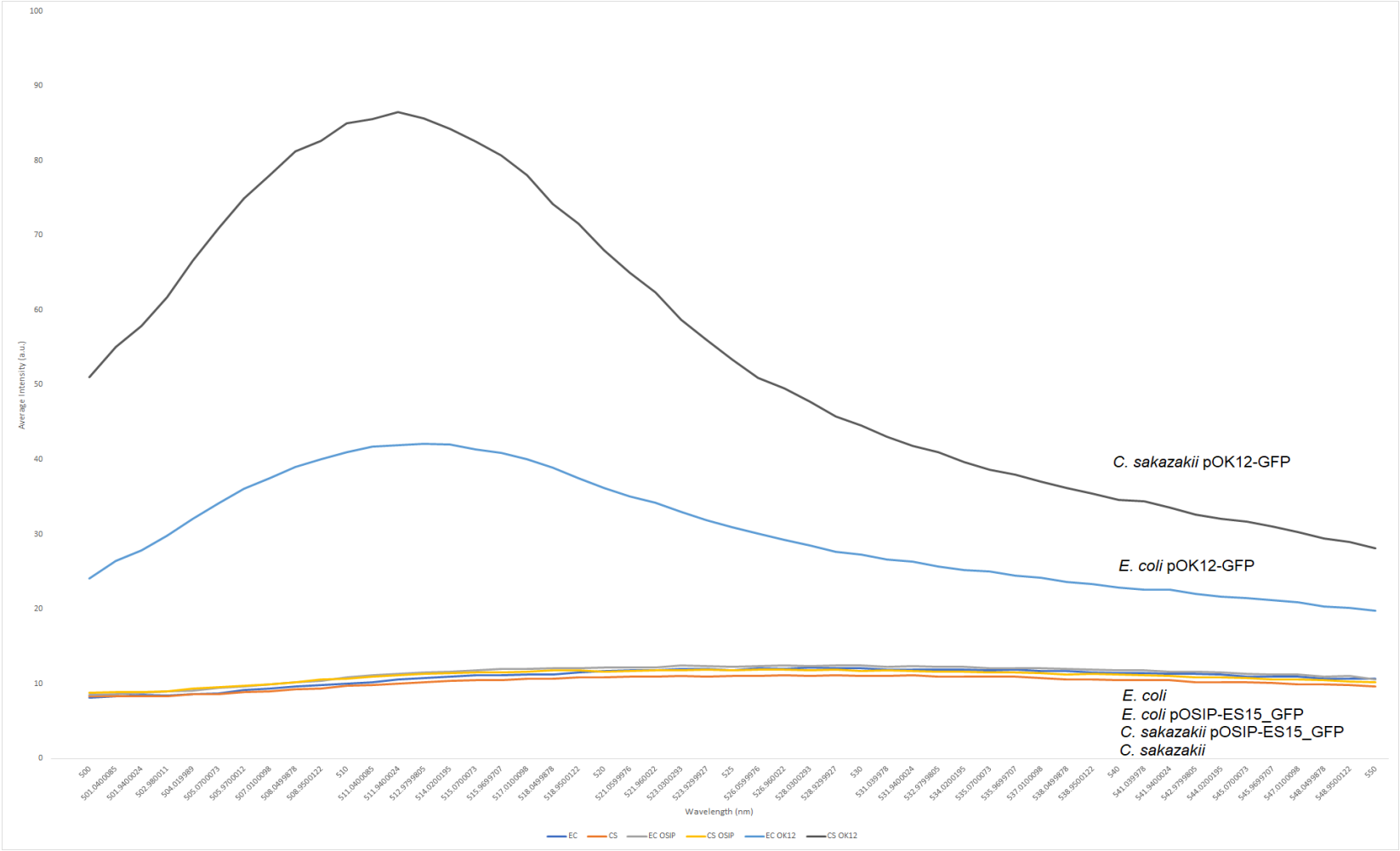

Figure 18. Line graph constructed from fluorescence data collected by spectrophotometry. As is evident in the graph, the *C. sakazakii* NCTC 11467 and *E. coli* NEB 5 Alpha cultures harboring an episomal pOK12-GFP plasmid had much higher emission values than the remaining four. There was no statistical significance in emission intensity values between the native *C. sakazakii* and *E. coli* cultures and the *C. sakazakii* and *E. coli* cultures with integrated pOSIP-ES15_GFP, indicating that expression of the single-copy cassette was undetectable.

Active Site Mutation Assay

The active site mutation assay was conducted to gather evidence to support the third hypothesis. The analysis using the NCBI Conserved Domain Database revealed that the φES15 integrase contained a conserved domain that placed it into the INT_ICEBs1_C_like Tyrosine subfamily. Members of this integrase family contain a conserved tetrad of amino acid residues in their active site, as illustrated in Figure 19. The active tyrosine residue responsible for performing the nucleophilic attack in the mechanism of action for the φES15 integrase was identified at position Y373 based upon this analysis. The codon for the tyrosine was changed to the codon for phenylalanine by single base pair substitution (TAC → TTC) in synthesis of a new gene sequence, which would eliminate the active hydroxyl group while retaining the same aromatic structure to allow for proper protein folding conformation, as shown in Figure 20.

Figure 19. Conserved amino acid residues within the active site of members of the INT_ICEBs1_C_like Tyrosine subfamily highlighted. The top panel shows other members of the subfamily with the active tyrosine residue responsible for nucleophilic attack located 33 residues after the conserved arginine. The φES15 integrase gene was analyzed and the active tyrosine located at Y373. Conserved domains obtained from NCBI Conserved Domain Database.

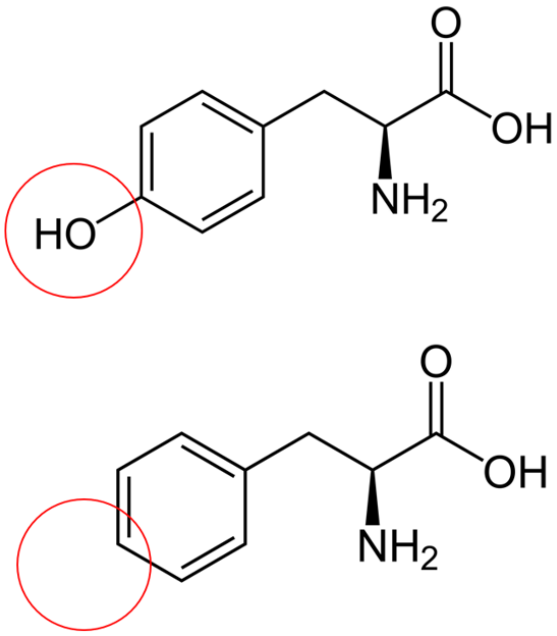

Figure 20. By making a single base pair substitution in the nucleotide sequence for the φES15 integrase gene, the active tyrosine could be mutated to a phenylalanine (TAC → TTC). This would maintain the same aromatic amino acid structure to allow proper protein folding conformation while eliminating the active hydroxyl.

Once the newly synthesized mutant φES15 integrase gene was cloned into pOSIP to make pOSIP-ES15_MUTANT, a series of concurrent transformation experiments were prepared. The results of the experiments are illustrated in Figure 21. Transformations were performed concurrently to act as an internal control that the procedure had worked properly. All three transformations with pOSIP-ES15 and all three with pOSIP-ES15_GFP resulted in transformant colonies growing on the selective kanamycin media. There were no colonies obtained from any of the plates that the cells transformed with pOSIP-ES15_MUTANT. This was the expected negative result that would be obtained if the integrase protein was necessary for integration of the vector and that integration was not resultant from homologous recombination between DNA fragments that shared homology with regions in the chromosome.

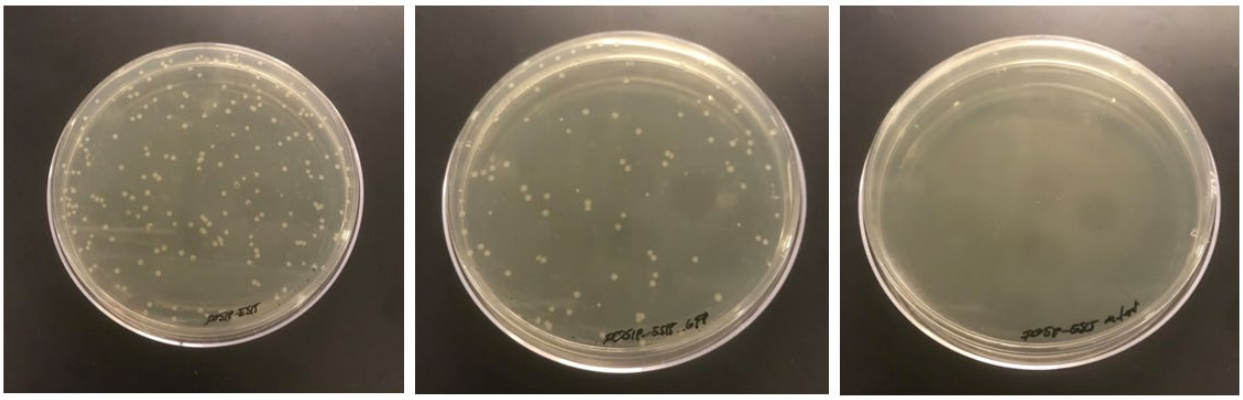

Figure 21. The active site mutation assay was conducted by concurrent transformations of *C. sakazakii* NCTC 11467 cells performed in triplicate with pOSIP-ES15, pOSIP-ES15_GFP, and pOSIP-ES15_MUTANT. As visible in the above panels, transformant colonies were obtained on every plate with the pOSIP-ES15 and pOSIP-ES15_GFP vectors, but no colonies were obtained with pOSIP-ES15_MUTANT. This was the negative result expected if the tyrosine at Y373 was responsible for integration, and plasmid did not rely upon homologous recombination for insertion.

Intermolecular Plasmid Integration Assay

The intermolecular plasmid integration assay provided further support for the third hypothesis. After pUC-NCTC11467_*attB* was constructed and confirmed through restriction enzyme digest, it was co-transformed along with pOSIP-ES15_GFP. The plasmid map for pUC19-NCTC11467_*attB* is shown in Figure 22, with the 639-bp *attB* sequence highlighted in blue. As discussed prior, the *E. coli* NEB 5 Alpha cells would only survive on the kanamycin/ampicillin selective media if the plasmids had recombined into a plasmid 9666-bp in size. The plasmid map of this recombined pOSIP-ES15_GFP-pUC19-NCTC11467_*attB* plasmid can be seen in Figure 23. After the predetermined incubation time, the growth plates were removed from the incubator and transformant colonies were observed to be growing. Digestion of the extracted plasmid DNA from two of these colonies with NheI and XhoI resulted in the banding pattern presented in the gel image in Figure 24.

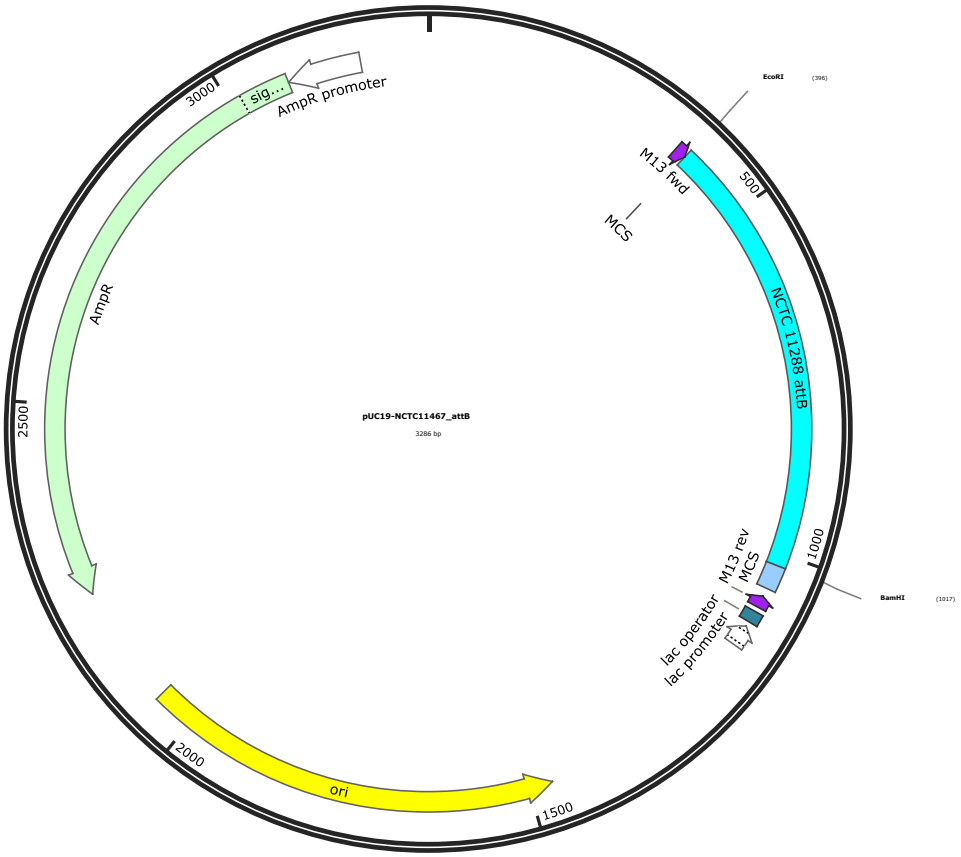

Figure 22. Plasmid map of pUC19-NCTC11467_*attB*. This plasmid was constructed by cloning the *attB* fragment out of NCTC 11467 chromosome using the primers *attB* EcoRI and *attB* BamHI and utilizing restriction enzyme cloning to insert into pUC19.

Visible in lanes 2 & 3 of the gel image in Figure 24 are two bands, the larger at a position approximately 7.7-kb in size and the smaller approximately 1.9-kb in size. Digestion of the 9666-bp plasmid with NheI and XhoI would result in a 7698-bp fragment and a 1968-bp fragment. Thus, this gel provided evidence that the two plasmids had integrated together at the expected attachment sites. The assay was repeated with the pOSIP-ES15_MUTANT form of the integrative plasmid. In no transformations did the use of this plasmid result in transformant colonies growing on the selective media.

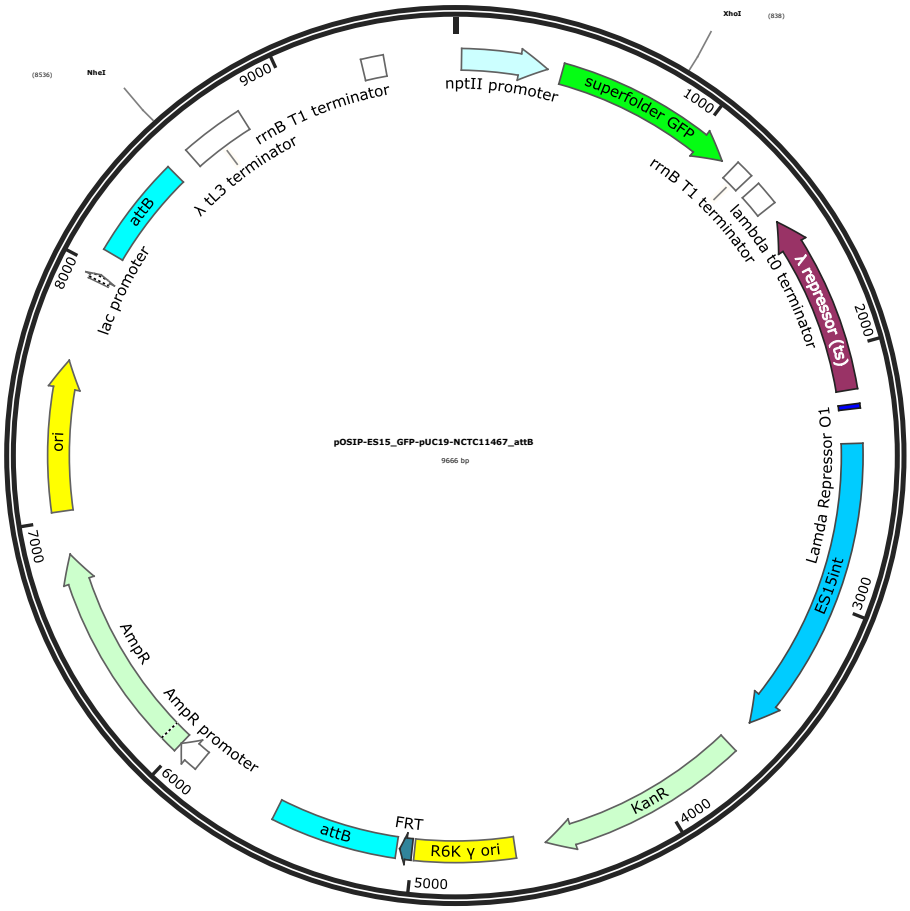

Figure 23. Plasmid map of the combined pOSIP-ES15_GFP-pUC19-NCTC11467_*attB* plasmid that would be obtained when the φES15 integrase protein performs a recombination event between the *attB* site on pUC19-NCTC11467_*attB* and the *attP* site on pOSIP-ES15_GFP. Final vector would be 9666-bp in size. Restriction enzyme sites NheI and XhoI are labeled.

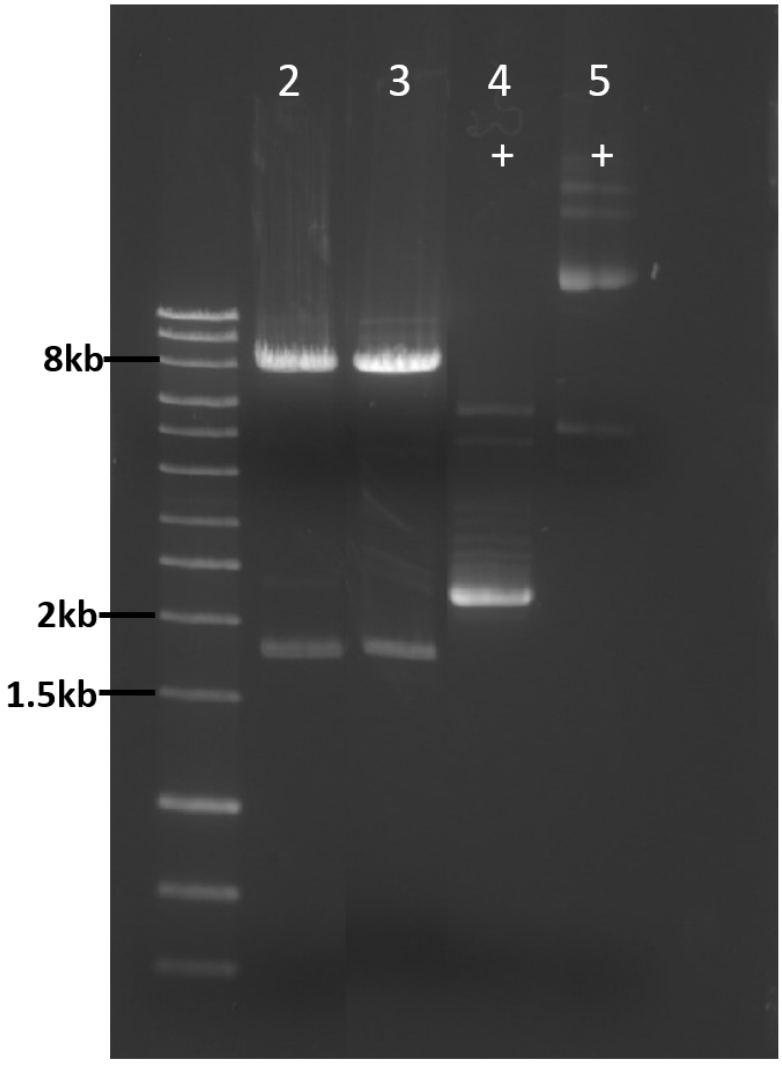

Figure 24. Confirmation of intermolecular plasmid recombination by restriction enzyme digest. Digestion with NheI and XhoI of the recombined 9666-bp plasmid would result in a band at 7698-bp and a band at 1968-bp. These bands are visible in lanes 2 & 3, which are plasmid DNA obtained from two transformant colonies. Lanes 4 & 5 are undigested pUC19-NCTC11467_*attB* and pOSIP-ES15_GFP, respectively.

Chapter V: Discussion

The results from the experiments conducted here to generate a tool that would be useful in engineering *Cronobacter sakazakii* presented some interesting observations and left several unanswered questions. A systematic approach of discussing each hypothesis and the relevant experimental results will aid in elucidation. The first hypothesis stated: An integrative vector constructed utilizing the φES15 integrase gene and *attP* sequence will integrate into *C. sakazakii* NCTC 11467 chromosome at the predicted location. This hypothesis was only partially supported. The results from the initial integration experiments with pOSIP-ES15 showed that the vector was functional as a genetic engineering tool in integrating into the chromosome. However, it did not perform as hypothesized in efficiently integrating into the predicted 26-bp *attB* site on the NCTC 11467 chromosome. The gel image in Figure 10 was typical of the results of the integrant colonies obtained in transformation with this vector. In each round of transformation, approximately 25-30% of the positive integrants obtained amplified a fragment at the expected 5.4-kb size. The other three-quarters of integrant colonies did not, although it was demonstrated that the plasmid did integrate into their chromosomes at some unknown location. What this resulted in was a tool that could still be utilized to deliver a gene of interest or expression cassette into *C. sakazakii*, but there would only be an approximately 25% chance that the colonies obtained from doing so would have the vector at this known location.

Without further experimentation to determine if the vector had a single site that it targeted at a higher rate, or whether there were multiple pseudo-attachment sites, confidence in use of this tool could not be expected. Due to budgetary and time constraints, these necessary experiments were not conducted in the course of this project but will be discussed further below.

Even though it had demonstrated lower efficiency in on-target integration than was desirable, the pOSIP-ES15 vector still demonstrated enough utility to allow for testing in delivery of an expression cassette.

The second hypothesis stated: The constructed engineering vector can be utilized to integrate a fluorescent cassette into *C. sakazakii* genome, to generate a fluorescent strain. This hypothesis was not supported in its entirety, as the data only demonstrated support for the first half. The constructed pOSIP-ES15_GFP vector did integrate into the chromosome as demonstrated by PCR analysis with the primer pair KanR Reverse and ES15 Forward, and the vector was indeed carrying the expression cassette. However, the cells did not express the cassette containing the GFP gene at an efficient enough manner to be detectable by either visual means or instrumentational analysis. The results from the fluorescence experiments presented the most curious results and results that required the most speculation in postulating explanations.

In the first project involving the pPL3e vector integration into *L. innocua*, single-copy cassette integration allowed for brightly visible fluorescence when paired with a strong, constitutive promoter and a GFP protein variant that emitted high fluorescence intensity. The cassette inserted into pOSIP-ES15 and subsequently integrated into the chromosome was designed with analogous features. The nptII promoter has demonstrated to be a strong, constitutive promoter within the *Enterobacteriaceae*, and the synthetic ribosomal binding site was calculated to increase translation rates by nearly three orders of magnitude. In addition, the Superfolder GFP has an even higher fluorescence intensity than the Enhanced GFP cloned into pPL3e. Transformation of NCTC 11467 cells with the episomal plasmid pOK12-GFP that housed this expression cassette resulted in high intensity fluorescence, demonstrating that the

transcription and translational machinery in *C. sakazakii* was able to recognize the regulatory elements in the cassette design and produce functional protein. Therefore, the measuring of fluorescence by spectrophotometry was necessary to determine if expression of the single-copy insert was at a level that simply could not be detected through visual means. As the data from this experiment showed, however, this was not the case. Fluorescence levels were the same between the NCTC 11467 cells integrated with pOSIP-ES15_GFP as with the non-transformed NCTC 11467 cells. Highly sensitive spectrophotometry could not even detect any fluorescence emitted from the cells.

At this point it was necessary to speculate upon possible explanations and further experiments that could be conducted to gather evidence that could further support or refute or refute the hypothesis. There is a well-documented occurrence known as position effect that influences expression of genes that have been integrated into a bacterial chromosome (Bryant, Sellars, Busby, & Lee, 2014). Several groups have shown that expression can vary quite drastically depending upon where the integration occurs. This effect can depend on local regulatory elements, such as transcriptional terminators, or even proximity to a chromosomal origin of replication (Bryant, Sellars, Busby, & Lee, 2014). Integration into highly conserved regions generally presented with higher rates of expression versus areas that were not as highly conserved (Juhas & Ajioka, 2015). This suggests that characterization of proposed integration sites is necessary when engineering a bacterial strain utilizing targeted techniques. As discussed previously, the 26-bp *attB* site present in the NCTC 11467 genome was located in an intergenic region, several hundred nucleotides upstream of an uncharacterized hypothetical protein predicted to be a possible integrase. Further upstream of the *attB*, in the same reading frame was

a sequence identified by NCBI annotation as originating from a transposon. Early in this project, as the integrative vector was still being designed for use, this information appeared promising as it was presumed integration into an intergenic region would be beneficial to prevent disruption to genes or areas metabolically vital. However, this may have actually contributed to decreased expression, as the nearby presence of these sequences could indicate that the targeted site is in an area of low conservation.

This could be a reasonable speculative observation that could explain some of the data, if evidence had been collected that the pOSIP-ES15_GFP was integrating into the predicted *attB* in the first place. Recall, however, that in no instance was a positive integrant located in which a 6.4-kb band was amplified. The vector was targeting one, or multiple, other sites and without knowing where these sites were located, position effects on expression variance can only be speculated highly upon. Other data collected in the course of this experiment could alternatively refute the positional effect speculation. The pOSIP-ES15_GFP vector was also integrated into the chromosome of *E. coli* K12 to verify whether expression of the cassette was possible once integrated into a related member of *Enterobacteriaceae*. As lab strain K12 lacked the PIR1 gene necessary to replicate the vector, confirmation of integration by PCR amplification of vector DNA by primer pair KanR Reverse and ES15 Forward presented in Figure 16 could be considered valid. Fluorescence analysis by spectrophotometry confirmed once again, however, that there was no difference in fluorescence intensity between the integrated strain and parent strain.

An analysis with NCBI Blast in K12 revealed that the 26-bp predicted *attB* sequence constituted the 3’ end of a *ssrA* transfer-messenger RNA gene. This gene, which encodes the

tmRNA, is considered to be well-conserved as the function of tmRNA is quite versatile and plays an important role in *trans*-translational processes, such as recycling of stalled ribosomes and induction of proteolysis for unfinished peptides (Keiler, 2008). Thus, if pOSIP-ES15_GFP was integrating into this 26-bp fragment at this site in the K12 chromosome, integration would presumptively be occurring at a site with a high degree of conservation and yet expression of the cassette was still so low as to be undetectable. This would help demonstrate that conservation of integration location is not necessarily the only factor at play in the apparent repression of expression. Certain members of *Enterobacteriaceae*, including *E. coli*, have genetic regulatory systems known as Nucleoid-Associated Proteins (NAPs) that aid in regulation of horizontally acquired genetic elements (Corcoran, Cameron, and Dorman, 2010). They function by recognizing topology changes when foreign DNA integrates into the host chromosome, as well as regions of higher A-T nucleotide content, and binding to the site to create a repression complex (Corcoran, Cameron, and Dorman, 2010). As an analysis of the UniProt protein sequence database revealed that *C. sakazakii* does produce a NAP homologous to the characterized protein produced by *E. coli*, it is possible that NAPs could be genetically silencing the cassette once integrated into both species.

Interestingly, a re-analysis of the location of the 26-bp *attB* sequence in the *C. sakazakii* genome revealed it did indeed constitute the 3' end of a homolog of the *ssrA* gene identified in *E. coli* K12, but NCBI did not annotate it as such. Even more interesting, was that this homolog was present in every type-species of the *Cronobacter* genus, but always in a location that was surrounded by genetic elements, such as hypothetical integrase or transposase sequences, that indicated it was obtained by lateral gene transfer. While this is information outside the scope of

this project, it nonetheless presents an interesting future project for someone wishing to study the evolution of *ssrA* acquisition in members of *Enterobacteriaceae*.

The third hypothesis stated: The putative φES15 integrase called by gene prediction performs a recombination between the putative *attP* sequence and host *attB* sequence. This hypothesis was supported by the data collected from the last experiments detailed. When a plasmid contains a sequence of DNA that is homologous to a region on the chromosome, integration of the plasmid can occur through the action of native DNA repair enzymes such as RecA that will facilitate recombination with sequences as short as 8 nucleotides in length. (Greene, 2016). Furthermore, the φES15 integrase gene was predicted to encode a functional integrase by machine annotation but the protein had not been characterized and demonstrated to be functional. Therefore, it was necessary to determine that integration of pOSIP-ES15 was in fact occurring due to the action of the encoded integrase and that the annotation of the gene as a functional protein was accurate. By eliminating the single hydroxyl group in the protein responsible for cleaving the nucleotide phosphodiester backbone, it was proposed that integration of the vector would not occur. The concurrent transformation experiments with three pOSIP-ES15 variants presented the evidence that supported this. From each single transformation of electrocompetent NCTC 11467 with both pOSIP-ES15 and pOSIP-ES15_GFP, between 50-200 integrant colonies could be obtained growing on the selective media. In none of the three transformations with pOSIP-ES15_MUTANT was an integrant colony observed.

This was fairly strong evidence for support but being that the result was considered a negative result due to the nature of the test, further evidence was warranted to strengthen the support for the hypothesis. The intermolecular plasmid integration assay added this additional

support. The restriction enzyme digest confirmation in Figure 24 validated that pOSIP-ES15 had been integrated into the pUC19 vector at the expected *attB* location based upon the banding pattern. Attempting to repeat this intermolecular integration between the pUC19-NCTC11467_*attB* and the pOSIP-ES15_MUTANT vector could not produce a single transformant colony on the kanamycin/ampicillin media. This further illustrated that the φES15 integrase protein was indeed functional and responsible for the integration event.

As mentioned, the results of these experiments did leave several unanswered questions. The two primary unknowns were the location(s) that the pOSIP-ES15 vector preferentially targeted, and whether integration into this site was a factor in the lack of fluorescence generated. A series of experiments would need to be performed to verify this hypothesis. With the integrated strains already generated, the next immediate experiment would be expression profiling to verify that the engineered strain was transcribing the expression cassette. A common method that could be utilized for such an analysis would be the use of quantitative real-time PCR (RT-qPCR). This is a powerful technique that is capable of detecting expression of genes that is occurring even at very low levels (Wagner, 2013). To carry out this experiment, a culture of the NCTC 11467-GFP cells would be grown and then lysed, extracting the RNA from the lysate. The mRNA is isolated from the total RNA through commercial kits or established protocols, and cDNA generated through reverse transcription of the mRNA. Primers would be designed that amplify a section of the expression cassette, and this amplification can be quantified in real-time as the reaction occurs. The quantitative data from the amplification can then be utilized to determine the initial levels of transcript and determine if expression of the cassette was low or high initially. If no amplification was possible, it would indicate that transcription was not

occurring. This would give credence to the hypothesis that possible position effect expression variance, or NAP repression, may be responsible.

While a positive result from RT-qPCR would reveal if transcription of the GFP gene was occurring, it would not reveal whether functional GFP was being actively translated from the mRNA transcripts. This would require an additional experiment involving protein isolation and identification using western blot analysis. Superfolder GFP specific antibodies would need to be ordered, and then verification of protein translation and identification through established western blot protocols (Mahmood and Yang, 2012). Detection of a 26.8 kDa protein would confirm that Superfolder GFP was being produced. Since spectrophotometry is an extremely sensitive analytical technique and did not detect any fluorescence from the engineered cell cultures, it is unlikely that if GFP was being produced in the current NCTC 111467-GFP strains, that western blot analysis of would produce detectable results. However, it is important to note that spectrophotometry was performed with intact cells. Before a western blot experiment could be performed, it would be prudent to conduct the spectrophotometry analysis of fluorescence using a cell lysate with isolated proteins from the current strains to determine if intact cells shielded low intensity fluorescence from being detected from a small amount of protein.

Lastly, the most important set of experiments that could be conducted would be a characterization of integration locations in the NCTC 11467 chromosome. As the entirety of this thesis work has illustrated, site-specific integrative vectors can be a very useful tool in efficiently engineering a bacterial species of interest. The *L. innocua* project worked so well because the group that had designed the pPL3e plasmid derived the sequences from a phage integrase that targeted a sequence on a highly conserved genetic element and consistently integrated there. The

predicted *attB* site appeared to be the target for φES15 some of the time, but not all. The fastest, and most comprehensive, method of determining the preferential integration location would be by whole genome sequencing of the NCTC 11467-GFP strains. This would indicate where the vector is currently present. A series of experimental integrations at targeted locations flanking this site could then be prepared by homologous recombination. Using overlap PCR to add short sequences to either end of the GFP expression cassette, these sequences could be designed to allow for homologous recombination at precise locations in intervals of 1000 nucleotides from the current integration site. Fluorescence levels could then be measured by spectrophotometry from a panel of these integrant strains and analyzed to determine if position effect was playing a role in decreased expression, or if endogenous NAPs could still be responsible.

For applications where directed and efficient engineering in bacteria is desirable, and integration locations have been characterized beforehand, integrative vectors present a powerful tool. In projects or situations where characterization is not possible beforehand, or when working with a bacterial species that has not had a fully characterized temperate phage identified, other techniques such as random integration via transposons may present a better option. As we head into an era of molecular biology where tools like CRISPR/Cas9 are being refined to allow for even more efficient engineering approaches, genetic modification of bacteria and other organisms will become an increasingly prevalent technique in research and industry.

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Appendix A

DNA sequences of the designed fragments inserted into the pOSIP backbone to create the vector pOSIP-ES15 and pOSIP-ES15_GFP

Insert Fragments	**DNA Sequence**
φES15 Integrase Gene	cctgcaggaggtttcaaagctgcaggacacctaactgcgcccgatcaagcagcgcataaacctccgtttcacagcggttga ctatcacaagtaaggaggttttacatggtgggcaagaaagaacaaggatcattatctctcccaagaggggtaactattcgcc atcacaaaacaggcgataccctggtaatcacttttacgtataaaggggttctgtgccgcgagccccttccaaaatggaggc aaacgcgcggggtgttaagtatgctgcgcggctgcttggggaaatacaaaatcagatcgcccatgaaacttttgagtatgc gaaatacttccctaattccaaaaaactggagttatttggggttacgaagaaaacaaaaaatattaagtcttacctggacgagta tctcaaaatctgccagaaccgtaacttgtctccgtcgactattaacggctatgaaaagtgcctgtcggcgctgtcagctttgca taaacttcacgtgtcggaactgacacctgcggttcttaaaaactggatagccagccggaaaacaaaactgaaaacgaccag gaataacctttcgtttctgcgcagcgccatcgatgaagctgttacggatggcctgctgaccattaacccggtaaccctcgtca gcgccagccggtaccacgtgatcgacagcaaaccgagccccgacgattacgaggttgacccgttcacgccagcggaaa ccctcgcaatttaccagagctgcaggtacccggaatgggaaaacctgttccggttcgcattcaacaccggactacgtagct ctgaattgtgcgctatacgctggaaggatatcgactttatcggaaacaccgcccacgtgcagatggccagcgtcgtagggg tacttaaatgcactaaaacaaaagccggtacccgtaaggttgagctaaacagtgaggcgctggcagccctgctggcgcag aaacaatacacctttatgaaaagcgagttcatattcagcgacccgaaaacgggagagccttgggcgaacgcagacgctat ccgaaaaaaagcatgggtgccaaccctgaaaaaagctggcgttcggtaccgtaatccctaccagacccgccatacattcg ccaccaaacacatcagccagggagtaaacctttctggcttgcaggtcagatggggcataagggtccagaaatgctgttcc gcaattacggatcatatctggcagtgtacgacggtcgtacatcgataaattctgctatatcgtcatgacctacttacacgttgtg t
φES15 *attP*	catatgatatttttgtcttttctcggtctgcatactgtctggtcagtgtccgtaagtgactgtatttattgcctctgtccggttgcagt cctatcaaaagtggtggagctggcgggagttgaacccatgtctgaggtgtgattaagtggttgaataatagtttttttatttaattc ataatgattttggtgcactttgcgtgctagc
MCS noncoding fragment (Linker)	gaattcatcgacatccaggaccttcaactagcatcgtaaaatcccgtcttcgaacttccttaacctacctacggcatattttcca aggacgttgaccacgtacgacgacttactgactacccaattgttactaatgttaccgtactagccaaatccttttaacgaccga ctaaaacggtacgtacgttgggttttaaaaccaggtaccttagccaattaagggaattaaccgatcagcgtcagtgatcactc gag
GFP Expression Cassette	gaattctttattacacgttcatacgcacgctgccgcaagcactcagggcgcaagggctgctaaaggaagcggaacacgta gaaagccagtccgcagaaacggtgctgaccccggatgaatgtcagctactgggctatctggacaagggaaaacgcaagc gcaaagagaaagcaggtagcttgcagtgggcttacatggcgatagctagactgggcggttttatggacagcaagcgaacc ggaattgccagctgggccgccctctggtaaggttgggaagccctgcaaagtaaactggatggctttcttgccgccaaggat ctgatggcgcaggggatcaagatctgatcaagagacaggatactagtggaggaagaaaaattaaaatttggtttcgaagga ggtaaaggaggttactatgagcaaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaat gggcacaaattttctgtccgtggagagggtgaaggtgatgctacaaacggaaaactcacccttaaatttatttgcactactgg aaaactacctgttccgtggccaacacttgtcactactctgacctatggtgttcaatgcttttcccgttatccggatcacatgaaa cggcatgacttttcaagagtgccatgcccgaaggttatgtacaggaacgcactatatctttcaaagatgacgggacctacaa gacgcgtgctgaagtcaagtttgaaggtgataccttgttaatcgtatcgagttaaagggtattgattttaaagaagatggaaa cattcttggacacaaactcgagtacaactttaactcacacaatgtatacatcacggcagacaaacaaaagaatggaatcaaa gctaacttcaaaattcgccacaacgttgaagatggttccgttcaactagcagaccattatcaacaaaatactccaattggcgat ggccctgtccttttaccagacaaccattacctgtcgacacaatctgtcctttcgaaagatcccaacgaaaagcgtgaccacat ggtccttcttgagtttgtaactgctgctgggattacacatggcatggatgagctctacaaataactgaatgagaggatcggttc cttgtcgagaggatcttccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtc ggtgaacgctctctactagagtcacgtgtaaggaacatctggccaggcattaattctcaccaataaaaaacgcccggcggc

	aaccgagcgttctgaacaaatccagatggagttctgaggtcattactggatctatcaacaggagtcgttgtcgagtaaggatcgacgtc

Appendix B

Table of fluorescence intensity reading averages and accompanying standard deviations collected from the fluorescence assay.

	EC		CS		EC OSIP		CS OSIP		EC OK		CS OK	
Wavelength (nm)	Intensity Average	St Dev	Intensity	St. Dev	Intensity	St. Dev	Intensity	St. Dev	Intensity	St. Dev	Intensity	St. Dev
490	701.918	28.07104	867.9162	66.27459	648.4907	44.00149	761.4711	11.12293	249.6228	17.96633	998.667	2.308908
491.06	420.873	16.32305	516.6308	42.05523	389.3661	27.16003	459.0269	5.168748	151.0511	11.16322	636.7182	28.58232
491.96	154.846	5.743829	192.6098	14.96271	144.0183	9.882383	169.9456	2.896279	60.94273	4.509108	248.429	11.11008
493.03	45.0557	1.663334	55.95692	3.597703	41.94378	2.720037	49.74988	0.479006	24.76579	1.295368	85.96113	2.420926
493.93	14.5136	0.342287	17.20808	1.010735	13.80255	0.731408	15.92987	0.246848	17.26548	1.027492	43.05724	0.658904
495	9.8263	0.139542	10.76072	0.487186	9.624599	0.454035	10.41674	0.142704	17.65468	1.083513	39.01558	0.553504
496.06	8.96966	0.092717	9.696361	0.375733	8.916693	0.217466	9.656207	0.156864	18.37825	1.001108	39.66344	0.885374
496.96	8.29659	0.052251	8.925004	0.276769	8.409593	0.105001	9.068237	0.314183	19.93068	0.952787	42.73587	1.206732
498.03	8.31946	0.047832	8.501827	0.204067	8.430044	0.214636	8.763702	0.137588	22.04371	1.078361	46.25689	1.027951
498.93	8.11784	0.117725	8.351356	0.167303	8.516941	0.079937	8.773641	0.079416	24.11777	1.391377	51.06201	1.222913
500	8.30776	0.095944	8.292118	0.188892	8.601096	0.225527	8.883638	0.147043	26.45257	1.714757	55.02898	1.262859
501.04	8.50113	0.102876	8.286754	0.104775	8.796515	0.133701	8.913266	0.135462	27.88042	1.347842	57.89976	0.992284
501.94	8.4358	0.054492	8.299074	0.185268	8.932456	0.174254	8.975804	0.122872	29.84034	1.563312	61.71942	1.699185
502.98												

		0.2700	8.5710	0.1115	9.0716	0.1509	9.3125	0.0723	32.106	1.8449	66.587	1.3938
504.02	8.60105	98	27	07	19	85	88	75	96	01	99	47
		0.2510	8.6450	0.2159	9.4033	0.2329	9.5210	0.2224	34.105	1.7946	70.922	1.5254
505.07	8.73197	6	42	27	41	44	5	75	82	8	85	61
		0.1618	8.8593	0.1154	9.5939	0.1607	9.7131	0.1701	36.134	1.9488	74.955	1.8728
505.97	9.13306	07	1	15	47	67	59	74	27	7	06	17
		0.0846	8.9328	0.1458	9.9450	0.1865	9.9498	0.0794	37.520	2.0986	78.045	1.9333
507.01	9.35289	42	75	36	59	43	89	11	15	61	72	54
		0.1177	9.2710	0.0821	10.208	0.0939	10.232	0.0371	39.015	2.5675	81.267	2.0580
508.05	9.62205	68	51	42	98	77	78	56	29	35	72	07
		0.1639	9.3930	0.0499	10.398	0.1876	10.533	0.2054	40.076	2.1361	82.667	1.9941
508.95	9.81365	89	85	06	08	63	61	29	61	7	93	89
		0.1336	9.7624	0.0801	10.836	0.2087	10.665	0.1198	41.021	2.2519	85.030	1.9888
510	10.0498	61	13	12	49	29	23	62	68	56	48	53
		0.1160	9.8327	0.0798	11.097	0.1500	10.926	0.1597	41.742	2.3115	85.548	2.0219
511.04	10.2416	27	99	99	4	6	7	58	64	74	03	79
		0.0151	9.9907	0.0913	11.355	0.2903	11.150	0.1802	41.913	2.2839	86.528	2.3118
511.94	10.573	5	65	86	05	33	61	05	42	13	15	69
		0.0926	10.207	0.1340	11.478	0.2897	11.343	0.0663	42.076	2.3366	85.638	2.2806
512.98	10.7362	09	54	63	2	8	25	41	06	41	99	71
		0.2146	10.380	0.1362	11.558		11.384	0.0917	42.008	2.2135	84.291	2.1933
514.02	10.925	25	04	2	88	0.238	31	24	96	14	13	07
		0.1029	10.500	0.1038	11.789	0.1898	11.513	0.2304	41.339	2.2493	82.549	1.9241
515.07	11.1457	71	01	55	61	48	83	42	73	77	21	57
		0.0640	10.486	0.0689	11.939	0.2993	11.527	0.2106	40.917	2.0480	80.717	2.1909
515.97	11.1749	04	26	33	42	14	16	15	11	91	61	5
		0.1254	10.675	0.0884	11.964	0.2755	11.633	0.2594	40.019	2.2200	78.059	1.9831
517.01	11.227	6	49	99	54	85	53	69	59	91	36	31
		0.0944	10.627	0.2421	12.116	0.1531	11.783	0.0938		2.3186	74.181	2.0384
518.05	11.2611	98	82	58	15	41	99	43	38.925	83	98	04
		0.0796	10.881	0.0717	12.029	0.1747	11.758	0.0886	37.461	2.1784	71.613	1.8360
518.95	11.5296	3	93	91	14	52	12	31	18	71	6	81
		0.1490	10.814	0.1359	12.205	0.1758	11.629	0.1049	36.191	2.0116	68.012	1.7827
520	11.6677	94	3	05	92	63	04	66	46	33	45	69

		0.3016	10.905	0.0900	12.134	0.2797	11.702	0.0139	35.109	1.5902	64.984	1.8422
521.06	11.8099	28	16	4	57	76	63	26	74	7	38	96
		0.3277	10.980	0.1733	12.151	0.2038	11.773	0.1412	34.250	1.7871	62.354	0.9263
521.96	11.8305	61	21	61	78	96	91	09	05	22	44	05
		0.2316	11.039	0.1557	12.429	0.3376	11.806	0.2354	32.956	1.8026	58.765	1.7145
523.03	11.9594	49	57	72	55	02	41	51	3	78	51	17
		0.0750	10.938	0.0654	12.324	0.2642	11.881	0.0939	31.896	1.4352	55.993	1.1325
523.93	11.9772	34	61	02	42	02	73	63	04	13	61	79
		0.2350	11.024		12.247	0.2987	11.760	0.2521	30.928	1.3467	53.322	1.4873
525	11.7482	17	2	0.1823	97	01	35	1	48	89	61	22
		0.2439	11.072	0.0632	12.330	0.2631	11.886	0.2581	30.071	1.2149	50.897	1.2806
526.06	12.0911	23	55	22	64	69	66	64	02	9	41	49
		0.2094	11.147	0.0491	12.405	0.2483	11.889	0.2134	29.261	1.1637	49.557	1.3835
526.96	11.9928	01	18	79	27	97	14	11	7	84	3	7
		0.2427	11.068	0.0592	12.309	0.1852	11.771	0.1411	28.489	1.0534	47.708	0.8828
528.03	12.1341	57	26	18	45	89	17	89	04	18	91	83
		0.2014	11.169	0.1240	12.439	0.1166	11.841	0.0950	27.676	0.9991	45.746	1.1684
528.93	12.0436	94	35	31	22	78	35	36	72	7	02	92
		0.0289	11.060	0.1274	12.473	0.1841	11.718	0.0600	27.287	0.9262	44.569	0.9961
530	12.0613	68	22	22	91	6	78	96	3	02	26	48
		0.0619	11.003	0.1397	12.302	0.3372	11.757	0.3659	26.601	1.2480	43.057	1.0404
531.04	11.8949	08	02	9	86	85	4	89	86	88	75	32
		0.2083	11.145	0.0237	12.375	0.2482	11.722	0.1436	26.318	1.1722	41.828	0.8877
531.94	11.8745	26	73	92	73	59	2	84	33	94	31	31
		0.0307	10.978	0.0567	12.229	0.2541	11.646	0.1075	25.640	0.8320	40.942	0.7509
532.98	11.8918	82	42	59	2	35	02	52	74	76	17	44
		0.1333	10.902	0.0901	12.230	0.2324		0.0043	25.209	1.0309	39.651	0.8134
534.02	11.9091	26	87	74	78	25	11.597	23	89	89	31	24
		0.0631	10.920	0.1945	12.028	0.1522	11.542	0.0929	24.983	0.8639	38.676	1.1644
535.07	11.8208	79	67	55	82	02	06	87	49	77	74	66
		0.2218	10.932	0.0590	12.067	0.2183	11.462	0.1746	24.465	0.8476	37.938	1.0033
535.97	11.8849	24	29	96	83	28	11	07	41	16	44	29
		0.3652	10.763	0.0833	12.030	0.1888	11.442	0.1493	24.147	0.8254	36.998	0.6244
537.01	11.6934	3	77	91	99	51	53	15	6	47	88	89

		0.4249	10.526	0.1747	11.959	0.4146	11.209	0.0350	23.588	0.7348	36.219	0.5923
538.05	11.6508	36	69	12	9	74	11	33	87	96	76	05
		0.1488	10.566	0.0610	11.918	0.1337	11.284	0.0924	23.345	0.6177	35.445	0.7815
538.95	11.4899	63	42	86	24	46	54	62	35	94	75	6
		0.1216	10.490	0.1769	11.759	0.2427	11.186	0.0894	22.861	0.7411	34.596	0.7905
540	11.4557	3	41	12	67	31	28	49	57	52	66	05
		0.1125	10.492	0.0924	11.783	0.3720	11.171	0.2262		0.7497	34.372	0.5186
541.04	11.4413	67	98	7	75	42	87	18	22.626	65	02	76
		0.0523	10.445	0.1350	11.592	0.2332	11.053	0.1289	22.553	0.7062	33.570	0.7836
541.94	11.2779	37	62	55	38	92	62	75	29	36	75	78
		0.0396	10.234	0.1248	11.582	0.1710	10.806	0.0436	21.999	0.9487	32.637	0.7220
542.98	11.2777	84	63	11	42	97	88	34	82	92	38	27
		0.0531	10.170	0.1244	11.470	0.3381	10.878	0.0238	21.633	0.6854	32.085	0.5366
544.02	11.2426	75	91	79	07	95	59	06	49	29	65	91
		0.2105	10.219	0.0149	11.359	0.3246	10.791	0.0222	21.470	0.8018	31.675	0.8620
545.07	10.9452	56	39	9	47	87	74	15	22	59	81	24
		0.1802	10.092	0.1289	11.266	0.1685	10.534	0.1034	21.149	0.5439	31.014	0.5183
545.97	10.9664	33	49	48	94	62	19	1	78	98	12	35
		0.0635	9.9527	0.1286	11.215	0.0833	10.578	0.1729	20.912	0.7121	30.237	0.3495
547.01	10.9341	38	39	09	3	57	55	18	51	17	29	12
		0.2085	9.8681	0.2038	10.992	0.2842	10.459	0.0848	20.344	0.6668	29.419	0.4842
548.05	10.6827	61	38	71	09	69	66	08	9	64	43	83
		0.2278	9.8546	0.1186	11.016	0.2613	10.262	0.1680	20.143	0.5672	28.924	0.8361
548.95	10.6937	65	11	26	69	65	33	36	74	91	62	25
		0.1175	9.6131	0.1664	10.611	0.1764	10.186	0.1340	19.799	0.7621	28.129	0.6353
550	10.6218	75	33	73	9	26	8	6	44	28	47	86