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Developing a Counterselection System to Facilitate Genetic Modification of Synechococcus sp. PCC 7002.

Construction of counterselection cassettes comprised of sacB and antibiotic resistance genes.

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Preface

This thesis constitutes the completion of my master's degree (MSc.) at the Department of Biotechnology at the Norwegian University of Science and Technology (NTNU). My master project has been carried out under the guidance of Associate Professor Martin F. Hohmann-Marriott and Adjunct Associate Professor and Researcher Rahmi Lale. I am deeply grateful for all you have taught me this last year, and for all the inspiration, guidance and patience provided throughout the work on my thesis.

I will also like to thank the remaining members of the PhotoSynLab research group. You have all been very helpful and fun to be around. A special thanks goes to Anne Vogel and Gunvor Røkke. Anne, you have always been very helpful in the lab, and have taught me a lot about general lab work and methods on how to work with *Synechococcus* sp. strain PCC 7002. And Gunvor, thank you for all the laughs and the decorations at my lab bench. This year would not have been the same without you.

Finally, I would like to thank all my classmates and friends in Trondheim. The last 5 years has been very joyful and rewarding, and you are all a big part of that. I will always look back at all the good times and memories we have shared. And to my family, I want to thank you for all the patience and continuous support I have received during these five years. You have all been paramount to the completion of my thesis.

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Abstract

Cyanobacteria have recently gained a great deal of attention as hosts for metabolic engineering, due to their innate ability to convert CO_2 and sunlight into a variety of chemical products. Cyanobacteria are of special interest as hosts for production of biofuels, which are important for combating global climate change. Compared to eukaryotic algae and plants, many cyanobacteria are easier to genetically engineer, and can grow faster. Furthermore, they are capable of growing under a wide variety of temperatures, light condition and salinities, and cultivation of the bacteria does not compete with the food industry for arable land. Cyanobacterial model strains have already been engineered to produce a wide variety of products. However, the production rate is slow, and the price of commodity goods generated by cyanobacteria is too high to make the production economically competitive. More research and synthetic biology tools are needed to overcome these limitations (Liu et al., 2011, Berla et al., 2013, Taton et al., 2014).

This thesis represents a small contribution to the goal of giving *Synechococcus* sp. strain PCC 7002 and other cyanobacteria a toolbox of genetic parts, that can be utilized to establish cyanobacteria as successful biotechnological platforms for the production of renewable chemicals and biofuels. The aim of the study was to construct a genetic system that facilitates the genetic modification of wild-type *Synechococcus* by counterselection. The *sacB* gene from *Bacillus subtilis* was chosen as the counterselectable marker utilized for this purpose. The gene confers conditional lethality to Gram-negative bacteria harboring the gene, where growth in the presence of 5% sucrose leads to death of the host cells (Gay et al., 1985, Pelicic et al., 1996, Viola et al., 2014).

Various counterselection plasmids were constructed and cloned into *Escherichia coli* DH5α, each containing a counterselection cassette comprised of *sacB* and one or two antibiotic resistance genes, i.e. kanamycin resistance (*kan*^{r}) and chloramphenicol resistance (*Tn9cat*). The cassettes were surrounded by synthesized sequences identical to the A0160, A0935 or A2843 neutral integration sites within the genome of wild-type *Synechococcus*. The flanking sequences were utilized to facilitate homologous recombination, and thereby incorporation of the cassettes within the neutral sites of the *Synechococcus* genome. One of the constructed plasmids, pA0160-KanR-sacB, was investigated by sequencing. The results verified the presence of *sacB* and the *kan*^{r} gene, and confirmed that the genes were incorporated in the expected place and in the right orientation within the constructed plasmids.

Several successful transformations were performed utilizing this system. However, verification of cassette functionality proved to be problematic, as *sacB*-mediated counterselection was observed to be NaCl-sensitive. In *E. coli* host cells this problem could be circumvented by excluding NaCl from the growth plates. However, for *Synechococcus* host cells, the problem was more difficult to circumvent. NaCl was removed from the growth media, but sodium and chloride were still present as part of other media constituents. At this point, *sacB*-mediated counterselection could not be achieved within *Synechococcus* host cells. While the system shows great promise, appropriate selection conditions have to be established before it can be fully implemented in *Synechococcus.*

Sammendrag

Cyanobakterier har nylig fått mye oppmerksomhet som potensielle vertsorganismer for metabolsk modifisering. Dette skyldes deres naturlige evne til å omdanne CO_2 og sollys til en rekke kjemiske produkter. Av spesiell interesse er produksjon av biodrivstoff, som er en viktig del av å bekjempe global klimaendring. I sammenligning med eukaryote alger og planter er cyanobakterier mye lettere å genetisk modifisere, i tillegg til at de vokser raskere. De er i stand til å vokse under et bredt spekter av temperatur, lysforhold og konsentrasjon av salt. Videre er at konkurrerer ikke dyrking av cyanobakteriene med næringsmiddelindustrien for dyrket mark. Flere stammer har allerede blitt utviklet for å fremstille et bredt utvalg av biologiske produkter. Produksjonshastigheten er imidlertid treg, og prisen på forbruksvarer produsert av cyanobakterier for høy til å gjøre produksjonen økonomisk konkurransedyktig. Mer forskning og biologiske verktøy er nødvendig for å overkomme disse begrensningene (Liu et al., 2011, Berla et al., 2013, Taton et al., 2014).

Denne avhandlingen representerer et lite bidrag til målet om å gi *Synechococcus* sp. PCC 7002 og andre cyanobakterier en verktøykasse av genetiske bestanddeler, som kan bidra til å etablere organismene som vellykkede bioteknologiske plattformer for produksjon av fornybare kjemikalier og biodrivstoff. Målet med studien var å utvikle et genetisk system som kan forenkle genmodifisering av villtype Synechococcus gjennom motseleksjon. *sacB* fra *Bacillus subtilis* ble valgt som motseleksjonsmarkøren som skal anvendes til dette formålet. Genet gir betinget dødelighet til Gram-negative bakterier som bærer genet, da vekst i nærvær av 5% sukrose fører til død av vertscellene (Gay et al., 1985, Pelicic et al., 1996, Viola et al., 2014).

Forskjellige motseleksjonsplasmid ble konstruert og klonet inn i *E. coli* DH5α. Hvert plasmid inneholder en motseleksjonskassett bestående av *sacB* og ett eller to antibiotikaresistensgen, dvs. kanamycin-resistens (*kan*^{r}) og kloramfenikol-resistens (*Tn9cat*). Kassettene er omkranset av syntetiserte sekvenser identiske med et av de tre nøytrale integrasjonsstedene (A0160, A0935 eller A2843) i genomet til villtype *Synechococcus*. De flankerende sekvensene ble anvendt til homolog rekombinasjon, og dermed til inkorporering av kassettene i de nøytrale integrasjonsstedene i *Synechococcus*. Ett av de konstruerte plasmidene, kalt pA0160-KanR-sacB, ble undersøkt ved hjelp av sekvensering. Resultatene bekreftet tilstedeværelsen av både *sacB* og *kan*^{r}, samt bekreftet at genene ble inkorporert i forventet posisjon og orientering inne i konstruktene.

Flere vellykkede transformasjoner ble utført ved anvendelse av dette systemet. Verifikasjon av kassettfunksjonalitet viste seg imidlertid å være problematisk, siden *sacB*-mediert motseleksjon ble observert å være NaCl-sensitivt. I *E. coli* vertsceller ble dette problemet unngått ved å utelukke NaCl fra vekstplatene. For *Synechococcus* vertsceller viste dette seg å være mer problematisk. NaCl ble fjernet fra vekstmediet, men natrium og klorid var fremdeles til stede som en del av andre mediebestanddeler. *sacB*-mediert motseleksjon kan dermed ikke bekreftes innen *Synechococcus* vertsceller på det nåværende tidspunktet. *sacB*-mediert motseleksjon viser seg å være svært lovende, med det er nødvendig å undersøke og rette opp i flere problemer før systemet kan implementeres i *Synechococcus*.

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Abbreviations

Bp	=	Base Pairs
B. subtilis	=	*Bacillus subtilis*
Cfu	=	Colony Forming Units
Chl	=	Chloramphenicol
ChlR	=	Chloramphenicol Resistance
dNTP	=	Deoxynucleoside Triphosphate
dsDNA	=	Double-Stranded DNA
E. coli	=	*Escherichia coli*
Kan	=	Kanamycin
KanR	=	Kanamycin Resistance
LA	=	Lysogeny Agar
LB	=	Lysogeny Broth
NEB	=	New England Biolabs Inc.
OD	=	Optical Density
PCR	=	Polymerase Chain Reaction
S. cerevisiae	=	*Saccharomyces cerevisiae*
SOC	=	Super Optimal Broth with Catabolic Repressor
Ta	=	Annealing Temperature (PCR Primers)
Tm	=	Melting Temperature (PCR Primers)
WT	=	Wild-Type

1 Introduction

This chapter provides the background information needed to understand the experimental work conducted through the work on this thesis. Section 1.1 provides the motivation for the project, and section 1.2 to 1.5 the experimental tools needed to perform the genetic engineering. Section 1.6 is the final part of this chapter, and gives a detailed description of the aim of the project and the methods by which the experimental work will be conducted.

1.1 *Synechococcus* sp. Strain PCC 7002

1.1.1 Cyanobacteria

Synechoccoccus is a genus of cyanobacteria, one of the major phyla of Bacteria. The phylum is comprised of a large group of morphologically, ecologically, and physiologically diverse photosynthetic, Gram-negative bacteria. Both unicellular and filamentous forms are known, with cell sizes ranging from 0.5 to 40 µm in diameter. The bacteria are divided into 5 different morphological groups, as presented in Figure 1-1 (next page). All cells contain chlorophyll a and phycobilins, which are pigments and accessory pigments utilized during photosynthesis. Cells that contain phycocyanins, a class of phycobilins, possess the blue-green color seen in most cyanobacteria (Madigan et al., 2012).

Cyanobacteria were the first oxygen-evolving phototropic organisms on Earth, which converted an originally anoxic atmosphere to the highly oxic atmosphere present today. They are capable of using sunlight as their energy source, CO_2 from the air as their carbon source, H_2O as an electron donor, and some are even able to use atmospheric N_2 as their nitrogen source. The bacteria are widely distributed in nature, in both terrestrial, freshwater and marine habitats. Some are capable of growing under extreme conditions, including environments with high temperatures and/or salinity, i.e. hot springs, desert soils, and highly saline lakes. Small unicellular cyanobacteria such as *Synechococcus* and *Prochlorococcus* are currently the most abundant phototrophs in the oceans, and are responsible for maintaining the biosphere by fixating a significant amount of CO_2 (Madigan et al., 2012, Palenik et al., 2003, Nomura et al., 1995, Huang et al., 2010, Heidorn et al., 2011).

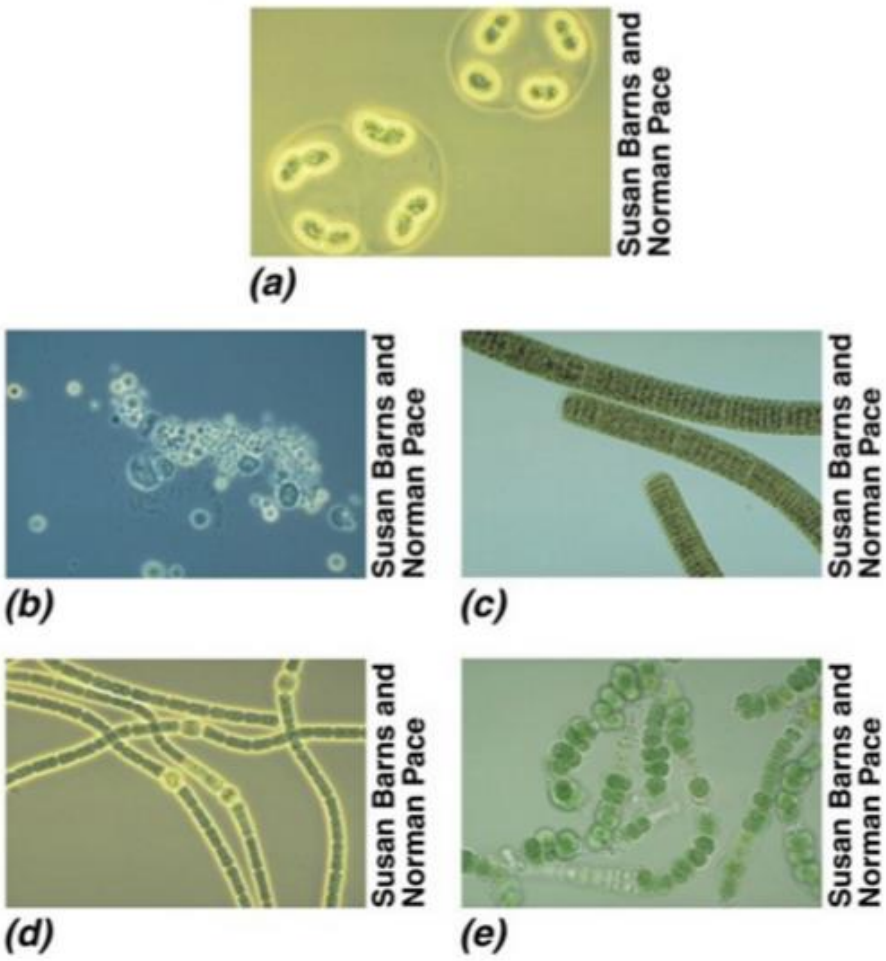

Figure 1-1. Cyanobacteria: the five major morphological types of cyanobacteria. *(a)* Unicellular, *Gloeothece*; a single cell measures 5-6 µm in diameter; *(b)* colonial, *Dermocarpa*; a large cell is about 8 µm in diameter; *(c)* filamentous, *Oscillatoria*; a single cell measures about 15 µm wide; *(d)* filamentous heterocystous, *Anabaena*; a single cell measures about 5 µm wide; *(e)* filamentous branching, *Fischerella*; a cell is about 10 µm wide. Micrographs in parts a, b, and d are photographed with phase contrast, and parts c and e with bright field. The figure corresponds to figure 18.24 in Brocks Biology of Microorganisms (Madigan et al., 2012).

According to the endosymbiotic hypothesis, chloroplasts of modern-day eukaryotes arose from the engulfment of a cyanobacterial ancestor cell that carried out oxygenic photosynthesis (Madigan et al., 2012). The photosynthetic machinery of cyanobacteria is therefor similar to the machinery of eukaryotic plants and algae. In addition, cyanobacteria are much easier to genetically engineer and grows faster than eukaryotic algae and plants, and are therefore utilized as model organisms to study photosynthesis (Liu et al., 2011).

Recently, model strains of cyanobacteria have been engineered to produce a wide variety of products, including sucrose, fatty acids, long-chain alcohols, alkanes, polyhydroxybutyrate, ethylene, 2,3-butanediol, ethanol, and hydrogen. Precursors for biofuel production are of special interest, due to the observed global climate change and the limiting energy resources. Cyanobacteria are capable of growing under a wide variety of temperatures, light condition and salinities, and cultivation of the bacteria do not compete with the food industry for arable land. However, the production rate is slow, and the price of commodity goods generated by cyanobacteria is too high to make the production economically competitive. Attempts to maximize the productivity have previously been obstructed by a limited set of genetic tools available for metabolic engineering of cyanobacteria. The goal remains to increase product yield at the expense of biomass accumulation, i.e. cell growth and division, without negatively influencing metabolic flux associated with the assimilation and direction of carbon to the metabolic pathway of interest. Due to the recent interest in utilizing cyanobacteria as cell factories for production of biomolecules, several advances have been made within this field. Synthetic biology tools have been developed for a select group of cyanobacterial strains, including *Synechococcus* sp. PCC 7002 (Nomura et al., 1995, Berla et al., 2013, Jacobsen et al., 2011, Markley et al., 2014, Taton et al., 2014, Liu et al., 2011, Davies et al., 2014).

1.1.2 *Synechococcus* sp. strain PCC 7002

Synechococcus sp. strain PCC 7002 (hereafter referred to as *Synechococcus*) is a unicellular, euryhaline cyanobacterium, part of morphological group 1 (*(a)* in Figure 1-1). The strain was isolated from a mud sample on Magueyes Island in Puerto Rico. The natural habitat consists of marine estuarine systems and tidal zones, where salinity, irradiance, moisture and nutrients can fluctuate dramatically. As a result, the bacteria are capable of growing under a wide range of NaCl concentrations, and are capable of growing under high light and variable temperatures. Under optimal conditions, i.e. 38ºC, 1% (v/v) CO_2, and 250 µmol m^{-2} s^{-1} photons, the bacteria have a doubling time of about 2.6 hours. This is one of the fastest recorded for any cyanobacteria (Chen and Widger, 1993, Ludwig and Bryant, 2012).

The strain naturally harbors one chromosome and 6 endogenous plasmids, as demonstrated in Table 1-1. The plasmid copy numbers are in the range of 1 to 8 per chromosome, with an approximate

chromosome copy number of 6 per cells. The exact copy numbers per cell varies somewhat with the growth conditions utilized. Genetic modification can be conducted for both the plasmids and the chromosome, as explained in section 1.2 (KDRI, Xu et al., 2011, Berla et al., 2013).

Table 1-1. Genome size of *Synechococcus* sp. PCC 7002.
The information is collected from CyanoBase (KDRI).

Name:	Size (bp):	Number of genes:
Chromosome	3,008,047	2,872
pAQ1	4,809	3
pAQ3	16,103	17
pAQ4	31,972	30
pAQ5	38,515	39
pAQ6	124,030	109
pAQ7	186,459	165
Total:	3,409,935	3,235

Synechococcus possesses several characteristics that make it an excellent platform for biotechnological applications, including in biofuel production. These properties include, but are not limited to, the fast growth rate, tolerance of high light intensities, tolerance of salt and temperature variations, its natural transformability, and a completely sequenced genome (available from http://www.ncbi.nlm.nih.gov/assembly/GCF_000019485.1/). Furthermore, the cells are capable of growing both as photoautotrophs and heterotrophs. In its natural habitat, *Synechococcus* use CO_2 as the sole carbon source. However, the strain is also capable of utilizing glycerol as the sole carbon source. Moreover, *Synechococcus* has additional properties which make is amenable for biofuel production, including a naturally high tolerance for potential biofuel products and a previously demonstrated capability of producing high quantities of free fatty acids – the key precursors for many advanced biofuels (Ruffing et al., 2015, Ludwig and Bryant, 2012, Xu et al., 2011).

Several synthetic biology tools exist for genetic engineering of *Synechococcus* and other cyanobacteria. The tools and methods available are presented in the following section.

1.2 Genetic Modification of Cyanobacteria

Synechococcus and various other cyanobacteria shows great promise as biotechnological platforms, using photosynthesis to convert CO_2 into higher-value products such as biofuels, food

related molecules, and various chemical precursors. Synthetic biology tools can help unlock the potential of cyanobacteria as a producer of these products, but unfortunately the tool development has not come as far as for other model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae*. Compared to other systems, there are few examples of simple and effective tools, as the tools developed for the *E. coli* and *S. cerevisiae* often do not function as designed when utilized in cyanobacteria. Furthermore, genetic engineering of cyanobacterial strains requires special considerations due to several features related to the phylum. These features include, but are not limited to, the polyploidy nature of the bacteria, and the existence of a circadian rhythm. Moreover, the genome size, content and codon usage, as well as ability to undergo natural transformation, can be strikingly different between cyanobacterial species. These variations may affect the strains ability to express and introduce genes of interest, resulting in strain-specific tools. Some broad host range tools have been developed, but these are likely to vary in efficiency between strains (Heidorn et al., 2011, Berla et al., 2013, Ruffing et al., 2015, Taton et al., 2014). Despite of the challenges that remains to be solved, several cyanobacterial strains are known that are readily amenable to genetic modification.

Genetic modification of cyanobacteria can be performed either in *cis* (chromosome editing) or in *trans* (plasmid addition). The transformation efficiency via homologous recombination is affected by the amount of transforming DNA, whether the DNA to be incorporated is transformed as a circular or linear molecule, the growth phase of the host cells, the incubation time and temperature, agitation, and the light conditions utilized. Experiments have been performed successfully for both *cis* and *trans* approaches (Berla et al., 2013, Ruffing et al., 2015). However, as the experimental work in this thesis is conducted in *cis*, this will be the sole focus of the remainder of this thesis.

The most common approach in cyanobacterial synthetic biology is genetic modification performed in *cis*, i.e. chromosome editing. The approach takes advantage of the natural transformation and homologous recombination that occurs in many cyanobacterial strains, including *Synechococcus*. The goal is to create insertions, deletions or replacements within the chromosome of the cyanobacterial cells. The approach facilitates knockout or knock-in mutations, which can create a strain that is optimized for the production of a specific compound. Knockout mutations cause inactivation or deletion of the target gene, and can be utilized to investigate gene function.

Alternatively, gene inactivation can be used to create a desired phenotype. Furthermore, knock-in mutations can substitute a wild-type gene for another, or can insert a new gene or sequence of interest into the genome under study. This method can replace non-functioning genes, or can create a desired phenotype (Berla et al., 2013, Jacobsen et al., 2011, Snustad and Simmons, 2012, Reece, 2004).

Originally, genetic engineering by homologous recombination occurred by transforming cells with a suicide vector that was not able to replicate within the host cells. This method is not very efficient on its own, as the frequency of double crossover events may be quite low. Furthermore, single crossover might occur, leading to incorporation of the whole plasmid. As a result, the number of true transformants might represent only a small fraction of the colonies appearing, and might be difficult to isolate. By linking the gene or DNA sequence of interest to an antibiotic resistance gene and growing the cells in the presence of the corresponding antibiotic, the selection pressure favors growth of cells that have incorporated the cassette. This creates mutants that have gained antibiotic resistance in addition to the insertion, deletion or replacement performed. Furthermore, by including a counterselectable marker with conditional lethality within the backbone of the suicide plasmid, mutants that have incorporated the whole plasmid by single crossover can be eliminated in the presence of the counterselectable compound (Reyrat et al., 1998, Berla et al., 2013).

The method has later on been refined to facilitate markerless mutations. A counterselectable marker is then included in the cassette utilized for transformation, usually linked to an antibiotic resistance gene and the gene or DNA sequence of interest. Two separate transformations are conducted; one to incorporate the whole cassette, and another to excise the incorporated counterselectable marker and antibiotic resistance gene and replace it with another exogenous DNA sequence (Berla et al., 2013). This method is based on counterselection cassettes, and is emphasized in the following section.

1.3 Counterselection and the Counterselectable Marker *sacB*

1.3.1 Counterselection

Counterselection is an important tool for genetic manipulation of microorganisms, and is of particular importance in cyanobacterial engineering. This is due to the presence of multiple copies of the chromosome, which makes it difficult to achieve homozygous strains using antibiotic resistance genes alone (Begemann et al., 2013).

A counterselectable marker is a gene that confers conditional lethality in microorganism harboring the gene. Under specific growth conditions, it promotes the death of the organism. The markers are usually utilized in combination with a positive selectable marker, such as an antibiotic resistance gene (Reyrat et al., 1998).

As previously stated, transformation of cyanobacteria is usually conducted in a two-step process: First, a counterselection cassette containing an antibiotic resistance gene and a conditionally toxic gene is transformed into cyanobacterial host cells. Flanking regions of the cassette, corresponding to a neutral integration site within the cyanobacterial genome, are utilized to facilitate homologous recombination, i.e. double crossover. Transformants are selected based on the capability to grow in the presence of the antibiotic by which the cells have gained resistance. After complete segregation[1] of the cassette, a second transformation is conducted to excise the incorporated counterselection cassette. This is facilitated by another double crossover event, with a markerless DNA sequence. The sequence can, for example, comprise a gene of interest that is desired to be incorporated into the cyanobacterial genome. Transformants are selected by inducing the toxic gene. Cells that have not deleted the cassette are exposed to the toxic effect of the gene, leading to cell death. As a result, only transformants containing the gene or DNA sequence of interest are capable of growing under the specified conditions (Berla et al., 2013, Lagarde et al., 2000).

The DNA to be incorporated may be a transformed into the host cells as a double stranded fragment amplified by PCR, or as a part of a suicide vector. The crossover events are demonstrated in Figure 1-2, utilizing *sacB* as a counterselectable marker.

[1]The cassette is incorporated into all copies of the cyanobacterial genome.

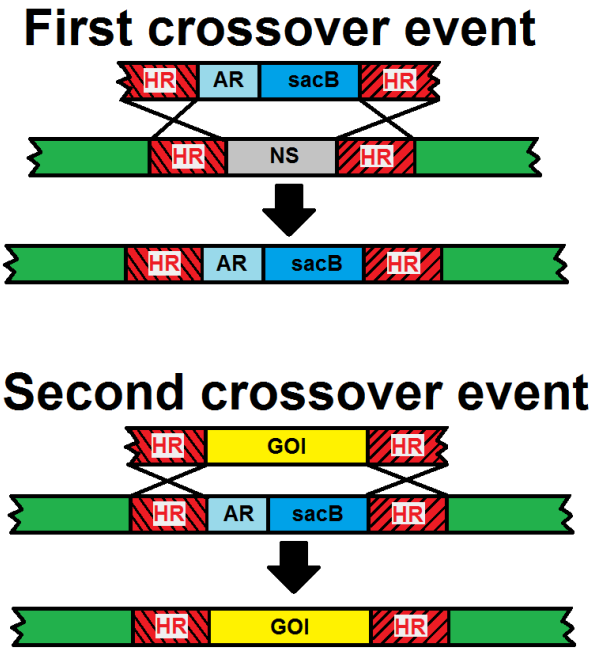

Figure 1-2. Crossover events leading to incorporation of counterselection cassettes, and subsequently, a gene of interest. Integration is facilitated by homologous recombination, utilizing homologous regions (HR). The DNA to be incorporated may be a transformed into the host cells as a double stranded fragment amplified by PCR, or as a part of a suicide vector. In the first crossover event, a cassette comprised of an antibiotic resistance gene (AR) and a counterselectable marker (*sacB*) is incorporated into a neutral integration site (NS) within the genome of a bacteria. In the second crossover event, the counterselection cassette is excised and replaced by a gene of interest (GOI).

This method has several advantages over methods that leaves a scar sequence. Foremost, the system facilitates several transformation events within the same strain. In contrast, if several transformations were carried out with the same selectable marker within the same strain, this might possibly lead to undesirable crossover events between the resulting homologous scar sequences. To circumvent this problem, different selectable markers can be utilized, such as various antibiotic resistance genes. However, a limited number of resistance cassettes are available, and the use of many different antibiotics to select for various inserts could have a cumulative, detrimental effect on cell culture viability. Furthermore, if the gene of interest is to be incorporated downstream of a cyanobacterial promoter, a scar sequence might lead to a frameshift mutation, which can affect downstream gene expression, as well as expression the target gene. Finally, as the antibiotic resistance marker is removed together with the counterselectable marker during markerless genetic

engineering, this limits the opportunity of transferring resistance to other bacteria (Reyrat et al., 1998, Berla et al., 2013, Clerico et al., 2007, Begemann et al., 2013).

A selection of counterselectable markers that have been proven useful in genetic engineering is presented in Table 1-2. The most popular ones are genes that confer sucrose, streptomycin, or fusaric acid sensitivity. The mechanisms of toxicity are described in the table (Reyrat et al., 1998, Berla et al., 2013). Perhaps the most common counterselectable marker is *sacB*, which will be utilized used in this project. The remainder of this section will therefore focus on the function and selection conditions of this marker.

Table 1-2. A selection of counterselectable markers and a description of their mechanism of toxicity. The table is modified from Table 1 in an article published by Reyrat et al. (1998).

Marker:	Mechanism of toxicity:
sacB	*B. subtilis* gene encoding levansucrase. The enzyme converts sucrose to levans, which is harmful to the bacteria.
rpsL (strA)	Encodes the ribosomal subunit protein S12, which is a target of streptomycin.
tetAR	Confers resistance to tetracycline but sensitivity to lipophilic compounds, such as fusaric and quinalic acids.
pheS	Encodes the α subunits of Phe-tRNA synthetase, which renders the bacteria sensitive to *p*-chlorophenylalanine (a phenylalanine analog).
thyA	Encodes thymidylate synthetase, which confers sensitivity to trimethoprim and related compounds.
lacY	Encodes lactose permease, which renders bacteria sensitive to t-*o*-nitrophenyl-β-D-galactopyranoside.
gata-1	Encodes a zink finger DNA-binding protein which inhibits the initiation of bacterial replication.
ccdB	Encodes a cell-killing protein which is a potent poison of bacterial gyrase.

1.3.2 sacB Gene Obtained from Bacillus subtilis

Levansucrase (sucrose:2,6-β-D-fructan 6-β-D-fructosyltransferase; EC 2.4.1.10) is a secreted enzyme (i.e. exoenzyme) encoded by the *sacB* gene in *Bacillus subtilis*. The enzyme is synthesized after induction by sucrose, and catalyzes the transfer of β(2→1)-D-fructosyl residues from sucrose,

to high-molecular-weight branched fructose polymers called levans (Steinmetz et al., 1985, Shimotsu and Henner, 1986, Gay et al., 1983, Gonçalves et al., 2015):

General reaction: Sucrose + Acceptor → Glucose + Acceptor-fructose

Levan synthesis: Sucrose + $Levan_n$ → Glucose + $Levan_{n+1}$

Levansucrase has a molecular weight of 50 kDa, and consists of a single polypeptide chain. The peptide sequence of the secreted enzyme contains 444 amino acids, preceded by a 29 amino acid long extracellular signal peptide (Gay et al., 1985, Gay et al., 1983, Steinmetz et al., 1985). The DNA sequence of the *sacB* gene and its genetic control sites have been determined. The amino acid sequence of the exoenzyme produced by *B. subtilis* strain BS5 was elucidated by A. Delfour (1981), and the DNA sequence of the *sacB* fragment of the *B. subtilis* Marburg genome determined by M. Steinmetz et al. (1985). Several genetic control sites within the DNA sequence have been proposed: a promoter 200 base pairs (bp) or more upstream of the coding sequence containing -35 and -10 regions, a possible attenuator sequence likely to control gene expression, and a possible transcriptional terminator sequence downstream of the coding sequence (Steinmetz et al., 1985, Shimotsu and Henner, 1986, Wrobel et al.). Furthermore, the gene has successfully been cloned and expressed in other organisms, including the popular model organism *E. coli* (Gay et al., 1983).

In *B. subtilis*, the enzyme is secreted after induction by sucrose. In its natural environment, the expression of the gene is harmless to the bacterium. According to Dogsa et al. (2013), the synthesized levans play a structural, albeit not essential, part in biofilm formation. Furthermore, levans are possibly stabilizing components of the biofilm, and might serve as a nutritional reserve. In contrast, in Gram-negative bacteria, *sacB* expression is lethal for cells harboring the gene when grown in media with a sucrose concentration of 5% or higher. The molecular basis for this toxicity is still not fully understood, but it has been proposed that the toxicity involves accumulation of levans in the periplasm, causing cell lysis (Recorbet et al., 1993, Wrobel et al., Gay et al., 1985, Viola et al., 2014, Pelicic et al., 1996). The conditional lethality of the *sacB* gene makes it a great counterselectable marker for use in genetic modification of microorganisms.

1.4 Homologous Recombination and Neutral Integration Sites

1.4.1 Homologous Recombination

Homologous recombination is a type of genetic exchange between two DNA sequences with extensive regions of sequence similarity, i.e. homology. The process occurs naturally within all cells, although the mechanism might vary somewhat between different species. It is mainly used to accurately repair double-strand breaks resulting from radiation, reactive chemicals, or DNA replication forks that have become stalled or broken. This is essential for every proliferating cell, as DNA replication is an error-prone process. In addition, homologous recombination exchanges homologous DNA regions of different chromosomes, creating new combinations of genes and assisting in accurate chromosome segregation during meiosis (Alberts et al., 2002).

A model for the mechanisms of recombination were proposed by Robin Holliday in 1964. The model described the activity of the numerous enzymes, responsible for cleaving, unwinding, stimulating single-strand invasions of double helices, repairing and joining strands of DNA. An updated version of the model is presented in Figure 1-3, along with a description of the activities of the enzymes involved. The main features of homologous recombination are well established, but specific details remain to be elucidated (Snustad and Simmons, 2012, Reece, 2004).

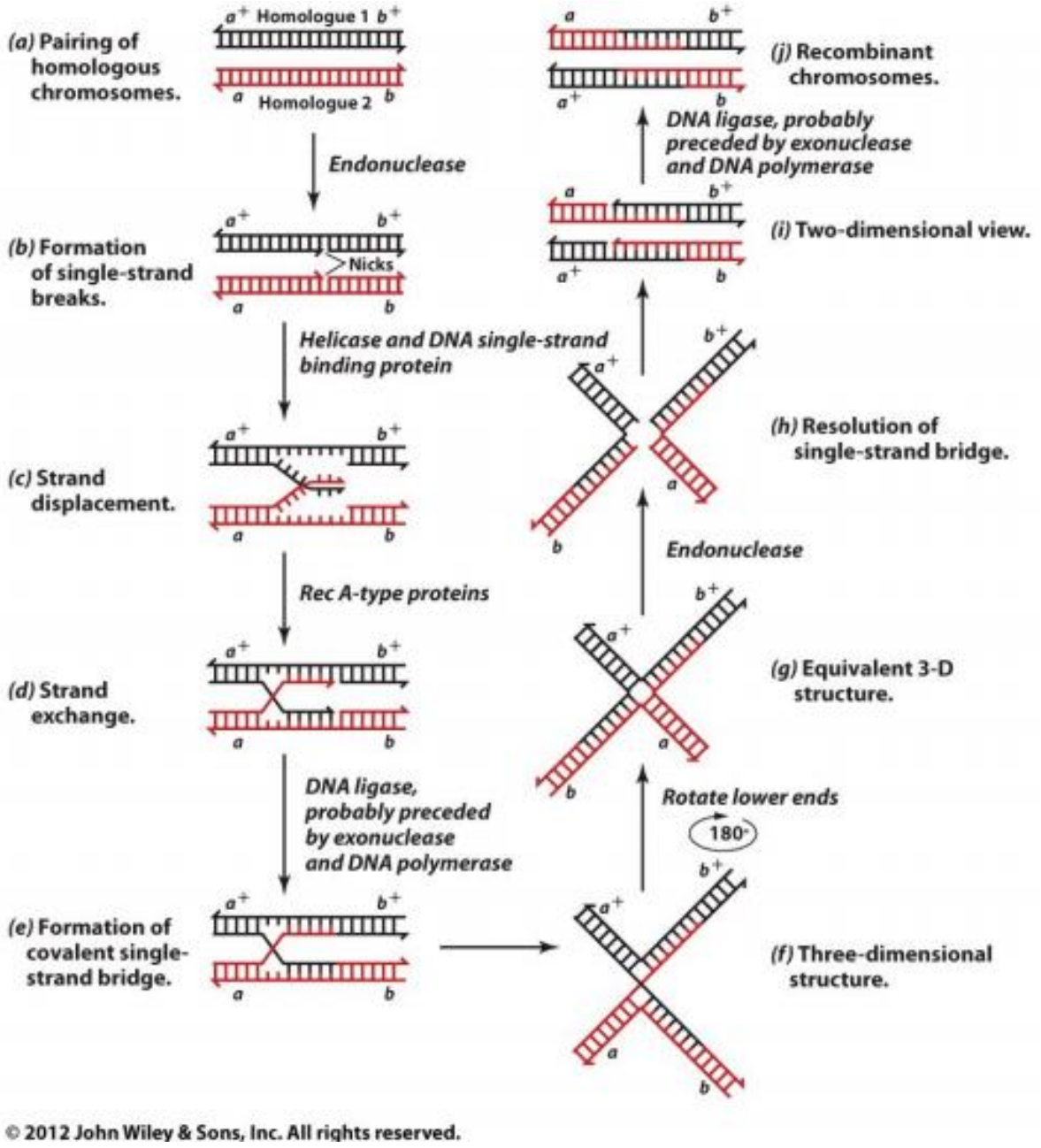

Figure 1-3. A mechanism for recombination between homologous DNA molecules. The pathway shown is based on the model originally proposed by Robin Holliday in 1964. The figure corresponds to figure 13.29 in Genetics (Snustad and Simmons, 2012).

In bacteria, genetic modification by homologous recombination occurs naturally as a part of unidirectional gene transfer. Recombination usually takes place between a fragment from a donor cell chromosome and a region within the chromosome of a recipient cell. To incorporate the fragment into the genome, double crossover must occur. Single crossover destroys the integrity of the circular chromosome, producing a linear DNA molecule that is unable to replicate (Figure 1-4).

This is an important part of bacterial evolution, as the cells do not go through the mitotic and meiotic condensation cycles and recombination events as seen in eukaryotic cells. Since the method is dependent on regions of high similarity, the recombination primarily occurs between closely related species (Snustad and Simmons, 2012).

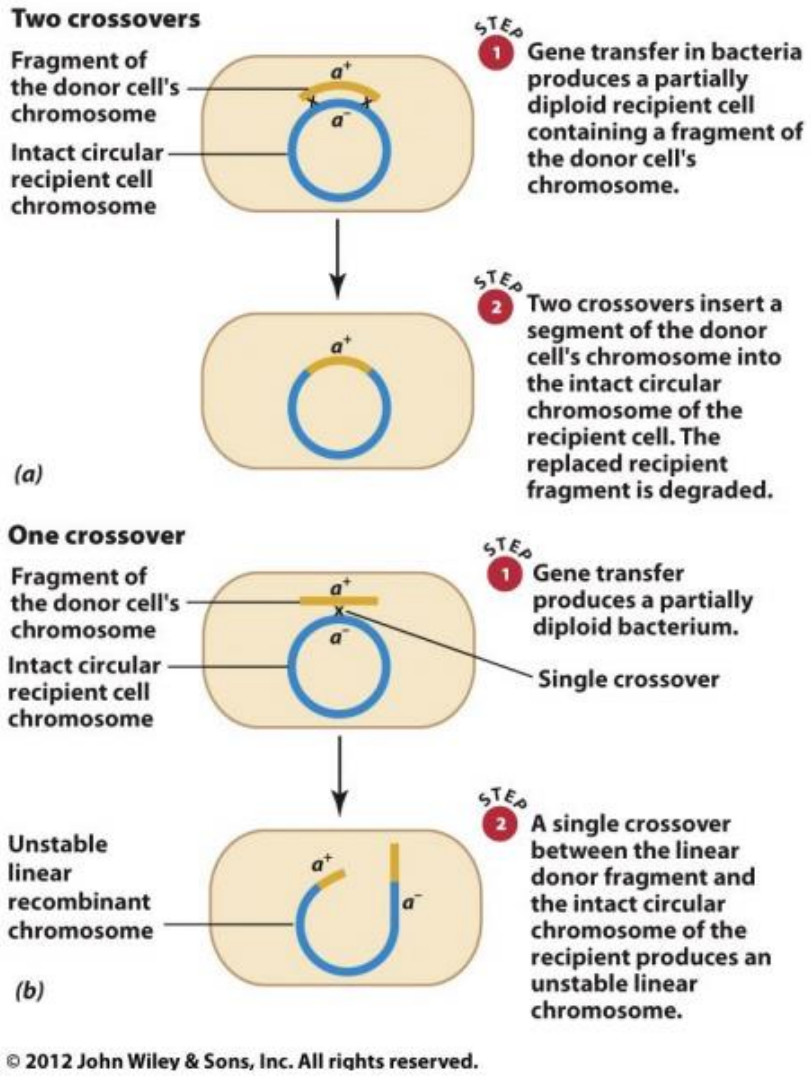

Figure 1-4. Recombination of bacteria. *(a)* To maintain the integrity of the circular chromosomes, crossovers must occur in pairs, inserting segments of the donor chromosomes into the chromosomes of the recipient. *(b)* A single crossover between a fragment of a donor chromosome and a circular recipient chromosome destroys the integrity of the circular chromosome, producing a linear DNA molecule that is unable to replicate and is subsequently degraded. The figure corresponds to figure 8.7 in Genetics (Snustad and Simmons, 2012).

Homologous recombination is also an important tool in synthetic biology, where it is utilized for genetic engineering of cells. As explained in section 1.51.2 and 1.3, cyanobacteria can be genetically modified by transformation and introduction of exogenous DNA into the genome of the cell. Here, homologous recombination facilitates the incorporation of DNA into a target site of the genome by a double crossover event. In order for the recombination to occur at the target site, the DNA sequence to be incorporated needs to contain flanking regions homologous to the target sequence. Furthermore, the target site needs to be a neutral integration site, i.e. a site within the genome that can tolerate insertion with no phenotypical or polar effects of adjacent regions of DNA (Niederholtmeyer et al., 2010, Berla et al., 2013, Reyrat et al., 1998, Viola et al., 2014, Clerico et al., 2007).

1.4.2 Neutral Integration Sites Within the *Synechococcus* Genome

Several neutral integration sites have been discovered in *Synechococcus* sp. PCC 7002. In 1997, Sakamoto et al. utilized the desaturase B (*desB*) site for integration of exogenous DNA. This resulted in disruption of the gene, leading to gene inactivation. The *desB* gene is coding for ω3 acyl-lipid desaturase, an enzyme that help cyanobacteria acclimate to low temperature by desaturating the membrane lipids. Since the gene is only expressed at low temperatures (~22ºC), the site can be utilized as a neutral integration site when the cells are grown under standard laboratory conditions (30-38ºC). As the gene occupies position SYNPCC7002_A0160 within the *Synechococcus* genome, the neutral site is referred to as A0160 for the remainder of this thesis (Sakamoto et al., 1997, Ruffing et al., 2015).

Furthermore, in 2013, M. Begemann et al. introduced exogenous DNA into *glpK* (position SYNPCC7002_A2843). Due to a frame shift mutation, *glpK* is a pseudogene. The site can thereby be utilized as a neutral integration site (Begemann et al., 2013). The neutral integration site is hereafter referred to as A2843.

Finally, in 2014, F. Davies et al. utilized the sequence between two open-reading frames encoding hypothetical proteins (SYNPCC7002_A0935 and SYNPCC7002_A0936) for integration of genes encoding limonene synthase (*MsLS*) and α-bisabolene synthase (*AgBIS*). The enzymes are involved in production of limonene and bisabolene, which are hydrocarbon precursors to a range of

industrially relevant precursors. The genes were transformed as linear double-stranded DNA (dsDNA), with 750 bp flanking regions homologous to the aforementioned sites (Davies et al., 2014, Ruffing et al., 2015). The neutral integration sites are hereafter referred to as A0935.

For all three neutral sites, the mutant strains created were investigated by colony PCR to verify complete segregation. As *Synechococcus* contains 6 copies of the chromosome, the cassettes need to be incorporated into all 6 copies of the neutral sites. Once segregated, the mutations can be stable over long periods of time, even in the absence of selection pressure (Sakamoto et al., 1997, Begemann et al., 2013, Davies et al., 2014, Berla et al., 2013) The neutral sites are presented in Appendix A.

1.5 Molecular Cloning

To facilitate genetic modification of *Synechococcus*, molecular cloning can be utilized to create counterselection cassettes containing a counterselectable marker and an antibiotic resistance gene. Furthermore, the cassettes must contain flanking regions of sequences homologous to the neutral integration site within the genome of *Synechococcus* host cells, utilized for homologous recombination. The procedure is explained in detail in the following text.

Molecular cloning refers to the process where recombinant DNA molecules are created and replicated within a host cell. The method is comprised of four separate stages: (1) isolation of the DNA sequence of interest, (2) incorporation of the isolated DNA into an appropriate cloning vector, (3) introduction of the constructed plasmid into a host organism where it can replicate, and (4) screening for host cells harboring the recombinant vector (Madigan et al., 2012, Reece, 2004).

Step 1 can be accomplished by a variety of methods, as explained in subsection 1.5.1. After isolation, the DNA sample should be investigated to verify that the DNA fragment of interest is present within the sample. This can be achieved by agarose gel electrophorese. Step 2 can be achieved through the digestion of vector and insert DNA by restriction enzymes, and the subsequent joining of fragments by a DNA ligase. Step 3 can be can be performed by transferring the constructed plasmid into an appropriate host, such as *E. coli*. This can be achieved by heat-shock transformation. Finally, step 4 can be carried out by utilizing the antibiotic resistance gene

incorporated into the constructed plasmid. By adding the appropriate antibiotic to the growth media, transformants are selected by positive selection, as only cells harboring the resistance gene are capable of growing under the specified conditions (Madigan et al., 2012, Reece, 2004).

Step 1-3 will be explained in detail in the following subsections. Step 4 will not be included, as positive selection by growth on selective media is emphasized in subsection 2.1.1. In the last part of this section, *E. coli* DH5α will be evaluated as a host for molecular cloning.

1.5.1 Step 1: Isolation of DNA Sequence of Interest

The first step in molecular cloning involves the isolation of a DNA sequence of interest. This can be a gene, an operon, a regulatory element, or any other sequence of interest. The DNA can be prepared in three different ways: (1) by PCR amplification from a plasmid or chromosome, (2) by excising the DNA from a plasmid or chromosome utilizing restriction enzymes (explained in the next subsection), or (3) by synthesizing a DNA fragment in vitro (NEB(a), 2015).

Strategy 1 is emphasized below. Strategy 2 is facilitated by restriction enzymes, which is the focus of the next subsection. The DNA of interest is then excised from a source DNA and investigated by agarose gel electrophoresis. If several bands fragments are present within the sample, the fragment can be excised from the gel and purified. The resulting fragment can be used in cloning in the same way as a fragment amplified by PCR. Finally, as strategy 3 were not utilized in this project, it will not be elucidated in this thesis.

As restriction enzymes are the focus of the next subsection, strategy 2 will be presented there. After the DNA have been isolated, the product needs to be investigated to see if the fragment of interest is present within the sample. This is achieved by agarose gel electrophorese. PCR and agarose gel electrophoresis are explained in detail in the following text.

Amplification of DNA by Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a powerful method utilized in molecular biology to amplify a specific sequence of DNA in vitro. PCR requires two oligonucleotide primers, designed to bind to flanking regions at each side of the DNA sequence to be amplified. In addition, the reaction requires dNTPs, a DNA polymerase, a buffer system, as well as a template DNA containing the target sequence. The reaction mixture is placed in a thermocycler, where the reaction is divided into three separate steps. First, the sample is heated to denature the dsDNA into single strands. The temperature is thereafter lowered, to facilitate annealing of the oligonucleotide primers. Finally, the DNA polymerase binds to the free 3'-end of each oligonucleotide and utilizes dNTPs to synthesize a new DNA strand in the 5'-3' direction. This constitutes the first cycle of PCR, as demonstrated in Figure 1-5. The newly synthesized DNA fragments serves as template in later cycles, allowing exponential amplification of the target DNA. The cycle of denaturation, annealing and extension is repeated 20-30 times, producing millions of copies of the target sequence (Clark and Pazdernik, 2013, Reece, 2004).

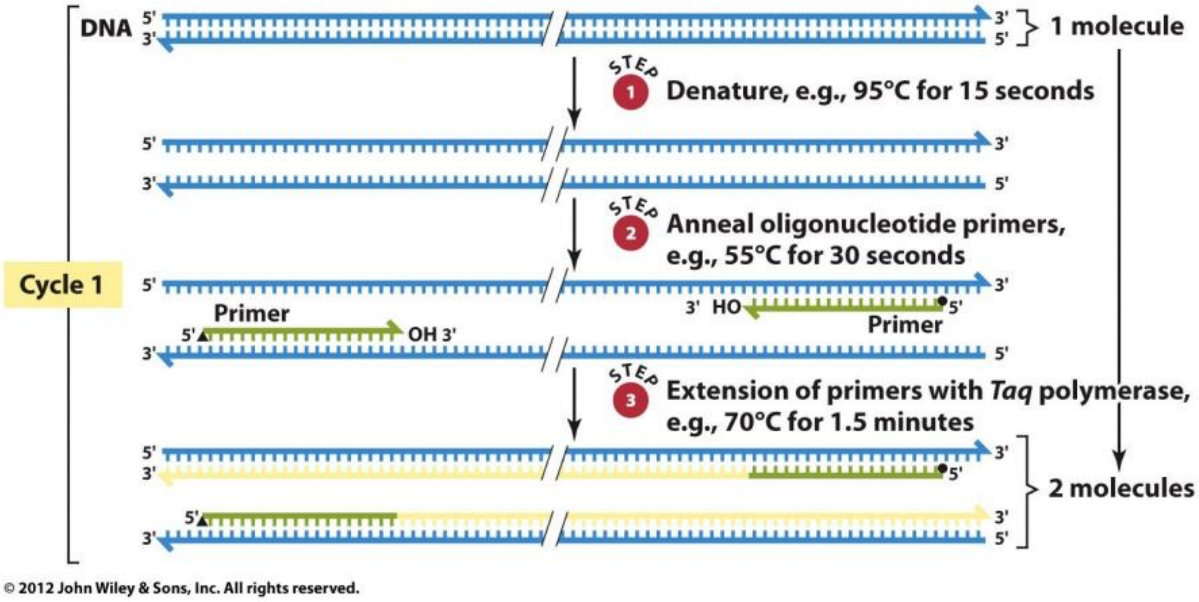

Figure 1-5. The use of PCR to amplify DNA molecules *in vitro*. Each sample of amplification involves three steps: (1) denaturation of the genomic DNA under study, (2) annealing of the denatured DNA with complementary oligonucleotide primer spanning the sequence of interest, and (3) enzymatic replication of the region of interest by a DNA polymerase, such as *Taq*. The newly synthesized DNA fragments serves as template in later cycles, allowing exponential amplification of the target DNA. The figure corresponds to the first part of figure 14.6 in Genetics (Snustad and Simmons, 2012).

The breakthrough in PCR came with the introduction of *Taq* DNA polymerase – a thermostable DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus*. The polymerase is a monomeric enzyme, and it remains functional even after incubation at 95°C. It possesses 5' to 3' polymerase activity but lacks 3' to 5' exonuclease activity (proofreading). This means that the polymerase is error prone, and should not be used in applications where high fidelity is required. However, if the goal of the PCR is to investigate whether or not a DNA fragment is present, then the success of the reaction is not affected by the introduction of errors in the amplified sequence. *Taq* DNA polymerase remains the most popular choice for routine PCR (Longley et al., 1990, Reece, 2004).

Since the discovery of *Taq* DNA polymerase, a number of other thermostable polymerases have been described and utilized in PCR. The different polymerases vary in important characteristics, such as fidelity, extension time and appropriate amplicon size. The DNA polymerase best suited for a reaction is therefore dependent on the application (Reece, 2004). One popular choice for routine cloning applications is Q5® High-Fidelity DNA polymerase. It is a high-fidelity thermostable DNA polymerase, with a 3' to 5' exonuclease activity. With an error rate more than 100 times lower than for *Taq* DNA polymerase, it is ideal for cloning and for amplification of long and difficult amplicons (NEB(d)).

The single-stranded oligonucleotides utilized as PCR primers are usually 17-30 nucleotides in length and have a GC content of approximately 50%. Individual primers should not contain palindromic sequences, and there should be no complementarity between the forward and reverse primer. Several tools exist to help design primers that fulfills all of the above criteria (Reece, 2004). Once the thermocycling is completed, the PCR product should be analyzed by agarose gel electrophoresis, as explained in the following section.

Separation of DNA Fragments by Agarose Gel Electrophoresis

Nucleic acids are highly charged molecules, containing negatively charged phosphate groups. The molecules have a constant charge per unit length, a characteristic that can be utilized to separate the molecules on the basis of size or confirmation. For DNA, this separation is carried out by agarose gel electrophoresis (Clark and Pazdernik, 2013).

Agarose is a linear polysaccharide naturally occurring in seaweed. The polymer is composed of several repeating units of agarobiose, which comprises alternating units of galactose and 3,6-anhydrogalactose. When a mixture of agarose and an aqueous buffer is boiled and subsequently cooled down, the result is a three-dimensional matrix of cross-linked polysaccharides containing small pores filled with buffer (Madigan et al., 2012, Reece, 2004).

After the agarose solution has been heated, it is held at 65ºC until use. During preparation for agarose gel electrophoresis, the solution is first poured into a gel tray with a comb placed at the far end of the gel. This give rise to small wells within the gel. After the solution has solidified, the comb is carefully removed, and the gel placed in an electrophoresis chamber. Once the gel has been submerged in a buffer solution, DNA samples mixed with loading buffer can be loaded into the wells. An electric field is applied to the solution, and the molecules move through the matrix towards the positive electrode. The passage through the matrix creates friction, which result in retention of larger molecules. The fragments are thereby separated in order of size, with small molecules travelling faster than larger ones. Furthermore, the conformation of the DNA influences the rate of passage, due to differences in friction. Supercoiled, circular DNA travels the longest distance, followed by linear DNA. Circular DNA experiences the most friction, and therefore travels the shortest distance. Gels with different concentrations of agarose are used to separate molecules of different sizes, as a higher concentration causes more retention of large molecules (Madigan et al., 2012, Reece, 2004, Alberts et al., 2002).

By running the samples alongside a DNA ladder containing DNA fragments of familiar sizes, the size of the DNA under study can be determined by comparison to the ladder. Once the DNA fragments have been separated, they must be visualized within the gel (Reece, 2004). This can be facilitated by the addition of GelRed – a fluorescent nucleic acid dye that binds to DNA and fluoresces when illuminated with ultraviolet light. GelRed is added to the heated agarose solution prior to making the gels (Crisafuli et al., 2015).

DNA samples that contains fragments with correct sizes can be purified by a PCR purification kit, and utilized in the second step of molecular cloning.

1.5.2 Step 2: Incorporation of Isolated DNA into an Appropriate Cloning Vector

A cloning vector is a small piece of DNA that can self-replicate within a host cell and be used for carrying cloned genes or segments of DNA. A variety of DNA molecules can be utilized as vectors. The DNA should be small and manageable, be easy to move from cell to cell, and it should be straightforward to generate and purify large amounts of vector DNA. Bacterial plasmids are by far the most popular choice, as well as small viral genomes. The plasmid vectors used today are small circular dsDNA, derived from larger plasmids occurring naturally within bacterial cells (Clark and Pazdernik, 2013, Alberts et al., 2002).

Before the DNA segment of interest can be incorporated into the vector backbone, both vector and insert needs to be cut with enzymes. This can be done by digesting both DNA molecules with the same enzyme, or alternatively, with enzymes that create fragments with compatible overhangs. These enzymes are called restriction enzymes, and are emphasized below.

Digestion and Ligation

Enzymes are biological catalysts, usually composed of proteins, that promotes specific reactions or groups of reactions. After their discovery in the 60s, restriction enzymes became the cornerstone of molecular biology. The discovery lead to the award of the 1978 Nobel Prize to W. Arber, D. Nathans, and H. O. Smith (Reece, 2004).

Restriction enzymes, also called restriction endonucleases, are capable of fragmenting DNA at specific sites – a vital part of molecular cloning. The term endonuclease refers to the enzymes ability to cut nucleic acids somewhere within the sequence, rather than digesting the fragments from the ends. The enzymes occur natural in bacterial cells, as a part of a restriction-modification system, present to protect the cell against foreign DNA. They recognize a specific DNA sequence, and cleaves the DNA. The recognition site is extremely specific for each enzyme, and is usually comprised of 4-8 nucleotides. Furthermore, to protect its own DNA, the bacteria needs to be able to distinguish its own DNA from foreign DNA. This is achieved by modifying enzymes, the second component of the restriction-modification system. The modifying enzymes recognize the same sequence as the restriction enzymes within the host DNA, and transfers a methyl group to either adenine or cytosine within the recognition site. Methylated DNA are not cleaved by restriction

enzymes. Thus, only incoming, unmodified DNA will be degraded by the enzymes. Today, more than 900 restriction enzymes have been isolated, from more than 230 species of bacteria. Due to their ability to recognize highly specific sequences of DNA, the enzymes are widely used in synthetic biology as tools for genetic engineering (Clark and Pazdernik, 2013, Reece, 2004).

Restriction enzymes are divided into three groups, on the basis of enzyme complexity, cofactor requirements, and the position of DNA cleavage. In short, type I enzymes makes random cuts in the DNA, more than 1000 bp from the recognition sequence. Type II cut DNA at a specific site within a palindromic recognition sequence, generating specific blunt ends, or so-called sticky ends with 5' or 3' overhangs. Finally, type III enzymes cleaves the DNA 24-26 bp to the 3' side of the recognition sequence (Clark and Pazdernik, 2013, Reece, 2004).

Since type II restriction enzymes is the only type where the exact cleavage position is known, these are the enzymes utilized in genetic engineering. Furthermore, type II enzymes that generates sticky ends are most useful. If a vector and an insert are cut with the same restriction enzyme, or with different enzymes generating compatible sticky ends, the DNA molecules can be bound together by base pairing. This pairing is only temporary, as the sugar-phosphate backbone is nicked. In a subsequent event, the molecules can be covalently linked by the enzyme DNA ligase. The enzyme is originally used to seal nicks in the backbone of dsDNA after replication, or for repairing damaged DNA. This feature has been exploited in genetic engineering, to join DNA fragments from different sources – creating a recombinant DNA molecule (**Figure 1-6**) (Clark and Pazdernik, 2013, Reece, 2004).

Cloning can also be performed with enzymes generating blunt ends. As DNA ligase only finds and ligates DNA fragments that are in contact, this is much harder to achieve. Since fragments with blunt ends cannot hybridize or in other means attach to each other, they mostly drift apart in the solution. To facilitate such cloning, high concentrations of DNA and DNA ligase are needed. Furthermore, as bacterial ligase cannot ligate blunt ends, T4 DNA ligase isolated from bacteriophage T4 is generally utilized (Clark and Pazdernik, 2013, Reece, 2004).

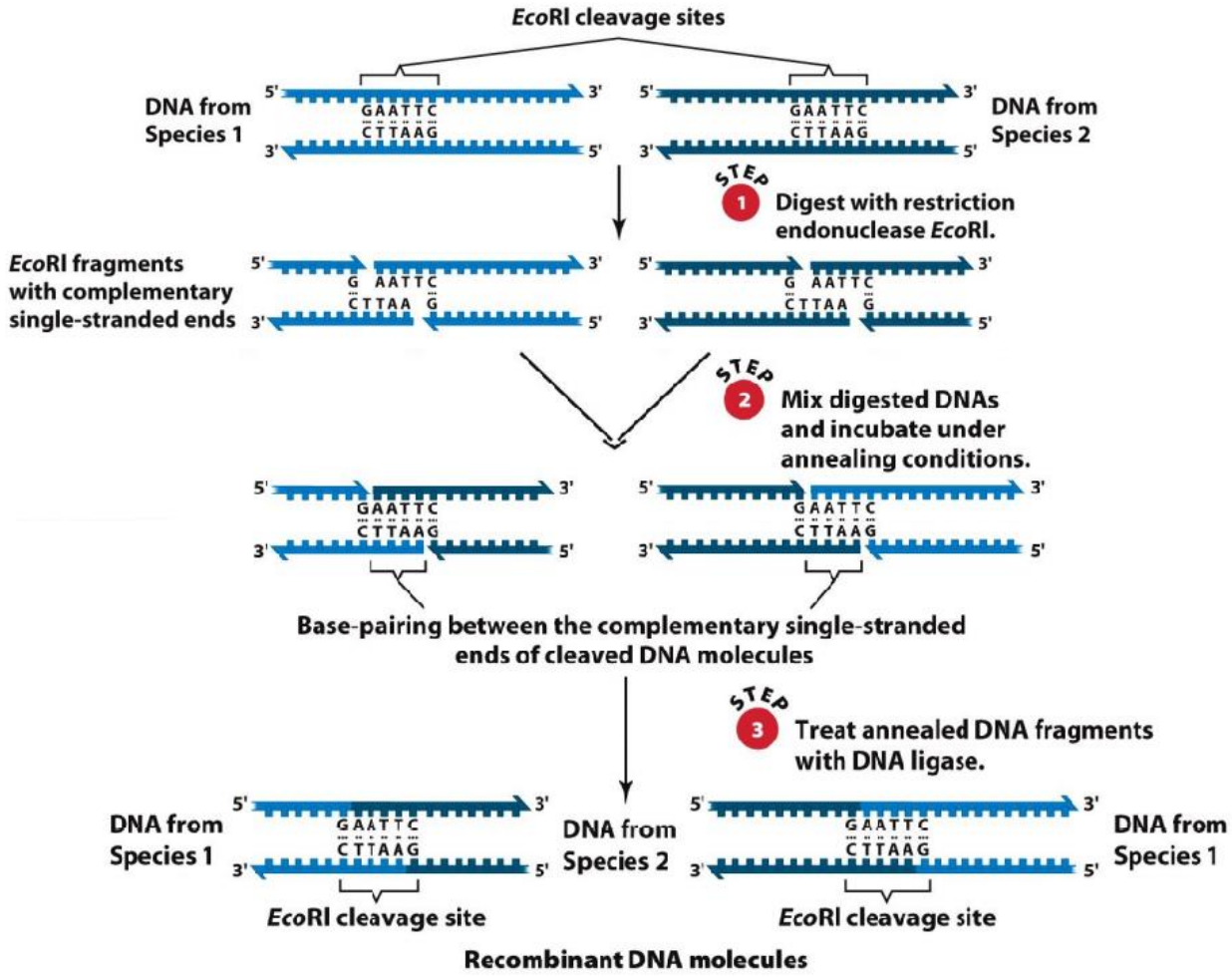

Figure 1-6. The construction of recombinant DNA molecules *in vitro*. DNA molecules isolated from two different sources are cleaved with a restriction enzyme, mixed under annealing conditions, and are covalently joined by treatment with DNA ligase. Digestion of DNA with the restriction enzyme *EcoRI* produces the same complementary single-stranded 5'-AATT-3' ends regardless of the source of the DNA. The figure corresponds to figure 14.2 in Genetics (Snustad and Simmons, 2012).

BioBrick Standard Assembly

In recent years, several advances have been made within the field of synthetic biology. One of the aims it to make standardized and well established genetic tools and solutions. Examples includes, but are not limited to, genetic building blocks (BioBricks) such as promoters, reporters, ribosome-binding sites, genes, origins of replications, and terminators, which can be readily swapped in and out of standardized plasmids. The building blocks, or parts, are flanked by two a standardized DNA sequences called prefix and suffix – containing a specific set of type II restriction enzymes. This facilitates easy assembly of several parts, which can be performed by established cloning

techniques. The method is demonstrated in Figure 1-7a. By performing successive cloning cycles, larger complexes of genes can be created by connecting two parts at a time (Huang et al., 2010, Berla et al., 2013, Taton et al., 2014).

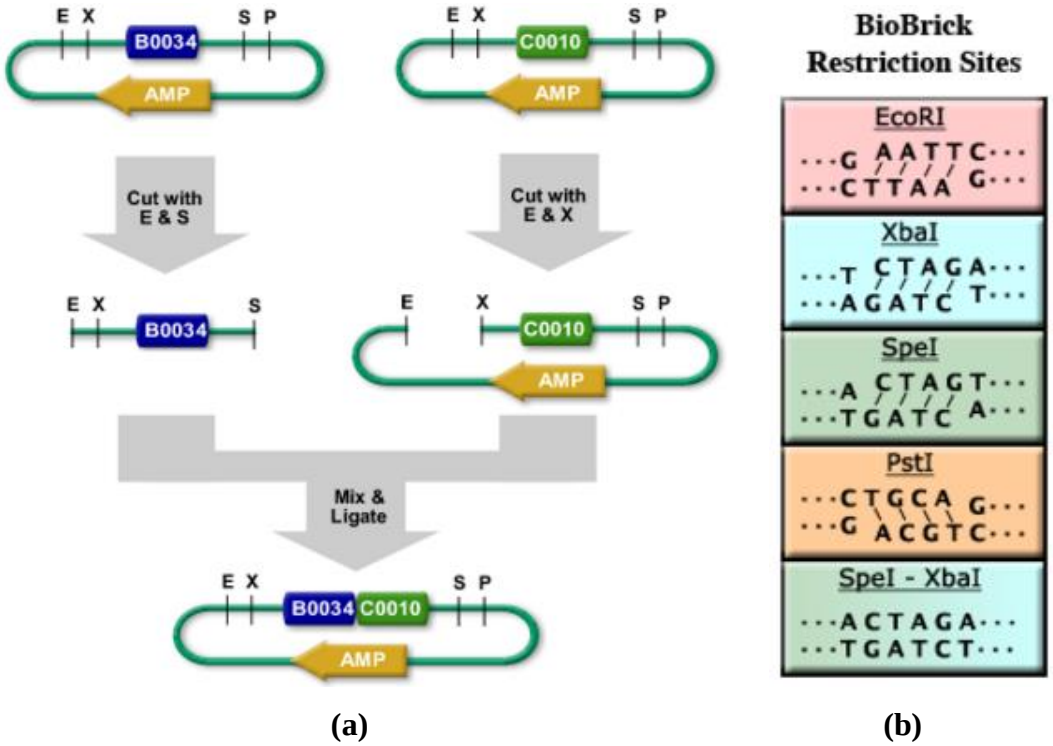

Figure 1-7. Illustration of BioBrick standard assembly. In (a) two genetic parts are connected by BioBrick standard assembly. The insert (blue) is cut with EcoRI and SpeI, and the vector (green) with EcoRI and XbaI. The parts are thereafter mixed and ligated. (b) represents the recognition sequences of the enzymes. As both parts are cut with EcoRI, and since SpeI and XbaI have compatible sticky ends, the genetic parts can be connected with easy, with the insert incorporated in a specific position and orientation. The figures are collected from the BioBrick homepage (BioBrick(a), BioBrick(b)).

The prefix contains the restriction enzymes EcoRI and XbaI, and the suffix SpeI and PstI. The enzymes have specific recognition sequences, and cleaves the DNA in a specific way. The goal is to connect two individual genetic parts into one new. This can be achieved by utilizing two enzymes with compatible sticky ends, but incompatible recognition sequences, such as SpeI and XbaI (see Figure 1-7b). After ligation, the resulting sequence do not match the recognition sequence of either enzyme, leading to a mixed site. The formation of such mixed sites are the basis of BioBrick standard assembly (BioBrick(b)).

The BioBrick standard was originally proposed by Knight in 2003, and today consists of thousands of established BioBicks parts, all available at the Registry of Standard Biological Parts (http://partsregistry.org) (Huang et al., 2010, Berla et al., 2013, Taton et al., 2014).

1.5.3 Step 3: Introduction of the Cloning Vector into a Host Organism

Once the DNA sequence of interest have been successfully incorporated into a vector backbone, the construct should be cloned into an appropriate host cell for amplification. Several methods exist to transport the DNA into bacterial host cells. In heat-shock transformation, the cells are first treated with calcium ions to make them competent for the uptake of DNA. Subsequently, a transformation procedure is performed, where DNA adheres to the surface of the cells and uptake is mediated by a pulsed heat-shock. Furthermore, transformation can be conducted by a process called electroporation. Here, the cells are treated with an electrical pulse, which results in formation of pores within the cell membrane. The DNA must enter the cell through the pores before they spontaneously reseal. Finally, transformation can be facilitated by a device called a gene gun. DNA are then bound to small beads, and the beads subsequently fired at cells. Once the beads have reached the cytoplasm, the DNA dissociates (Reece, 2004).

As heat-shock transformation will be utilized in this project, the other methods will be excluded from the remainder of the thesis. The method will be explained in detail, along with the chemical treatments required to make competent cells.

Heat-Shock Transformation and Competence

The process where free DNA molecules released from one bacterium is taken up by another, is called transformation. Cells capable of taking up free DNA and be transformed are thereby called competent cells. However, not all bacterial species have the ability to do so. Even within transformable genera, only certain strains or species are transformable. Furthermore, in most naturally transformable bacteria, competence is regulated. Special proteins, called competence (Com) proteins, play an important role in the uptake and processing of DNA. Only cells expressing the genes encoding these proteins are thereby naturally transformable. In *E. coli*, the required genes and metabolic machinery required have not evolved. Thus, under natural conditions, transformation does not occur (Madigan et al., 2012, Snustad and Simmons, 2012).

To facilitate use of transformation as a part of genetic engineering, scientists have developed a method to transform *E. coli* cells in the laboratory. The method was discovered by Mandel and Higa (1970), but has later on been improved. It was found that if the cells are treated with high concentrations of calcium ions and thereafter chilled for several minutes, they become more permeable to free DNA. In addition, treatment with cations such as Mg^{2+}, Mn^{2+} and Rb^{+} further increases the transformation efficiency. The cells are subjected to a heat-shock, by briefly transferring the solution from ice to 37-45ºC, and then back to ice again. The cells are thereafter grown in nutrient-rich media, and plated onto selective solid medium. Cells treated in this manner are able to take up dsDNA, such as plasmids. The role of calcium in this process is still unclear. It is thought that calcium affects the bacterial cell wall, and possibly facilitates binding of DNA to the cell surface. The actual uptake of DNA is thought to be a result of the brief heat-shock the cells were subjected to. This process is critical for biotechnology, as *E. coli* remains the organism of choice for most molecular cloning (Madigan et al., 2012, Snustad and Simmons, 2012, Hanahan, 1983, Reece, 2004).

As the plasmids constructed by molecular cloning usually contain an antibiotic resistance gene or another selectable marker, the cells are grown on selective plates to favor growth of cells harboring the construct. Once colonies appear on the selective plates, the transformants can be inoculated in liquid medium, and the constructed plasmids harvested the following day. The constructs should also be investigated by PCR and agarose gel electrophoresis, as explained in subsection 1.5.1. This can be done by colony PCR, i.e. with PCR performed utilizing a cell culture as a source for template DNA, or by regular PCR after plasmids have been harvested. The procedures for preparing selective media, inoculating cells, performing PCR and plasmid isolation is emphasized in the next chapter.

1.5.4 *Escherichia coli* DH5α as a Host for Molecular Cloning

Escherichia coli are Gram-negative, rod-shaped bacteria, with a size of approximately 1 by 2.5 microns (Figure 1-8). The bacteria live in the colon (hence “coli”) of humans and other vertebrates. As previously stated, it is the most widely used model organism in molecular cloning. The bacteria have several advantages that make it a great host for cloning, including a high growth rate

(approximately 30 minutes), a well characterized genome (about 4.6 Mbp), and the ease of cultivating the bacteria under laboratory conditions. Studies conducted with this organism has led to a vast amount of information about the fundamental mechanisms of life, including replication, gene expression, and various other cellular processes – mechanisms that have proved to be highly conserved throughout evolution (Alberts et al., 2002, Reece, 2004, Clark and Pazdernik, 2013).

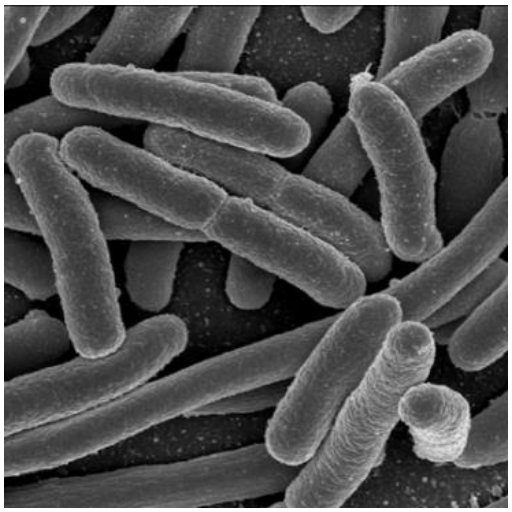

Figure 1-8. *Escherichia coli* photographed by a scanning electron microscope. The picture is collected from physics.org (NIH, 2014).

In addition, the bacteria are relatively easy to transform. As long as the cloning vectors contain an origin of replication compatible with the species, the vectors will self-replicate and be amplified within the host cells. Due to all the information available, the field of synthetic biology is most advanced in regard to this organism. A large amount of standardized tools is available, including BioBrick parts such as promoters, genes, terminators, ribosome-binding sites and origins of replications. Several strains have been engineered that are relatively harmless to humans, making them suitable for use in genetic engineering and molecular cloning (Reece, 2004, Berla et al., 2013, Huang et al., 2010, Taton et al., 2014).

1.6 Aim of Study

This thesis represents a small contribution to the goal of giving *Synechococcus* sp. strain PCC 7002 and other cyanobacteria a toolbox of genetic parts, that can be utilized to establish the organisms as successful biotechnological platforms for production of renewable chemicals and biofuels.

The aim of the study is to construct a genetic system that facilitates the genetic modification of wild-type *Synechococcus* by counterselection. The *sacB* gene from *Bacillus subtilis* is chosen as the counterselectable marker that will be utilized in this project. The gene confers conditional lethality to Gram-negative bacteria harboring the gene, where growth in the presence of 5% sucrose leads to death of the host cells (Gay et al., 1985, Pelicic et al., 1996, Viola et al., 2014).

Various counterselection plasmids will be constructed, with *E. coli* DH5α as a host for molecular cloning. The cloning will be conducted utilizing BioBrick standard assembly, as demonstrated in Figure 1-9 (next page). Each plasmid will contain a counterselection cassette comprised of *sacB* and one or two antibiotic resistance genes, i.e. kanamycin resistance (*kan*^{r}) and chloramphenicol resistance (*Tn9cat*). The cassettes will be surrounded by synthesized sequences identical to the A0160, A0935 or A2843 neutral integration sites within the genome of wild-type *Synechococcus*. The flanking sequences will be utilized to facilitate homologous recombination, and thereby incorporation of the cassettes within the neutral sites of the *Synechococcus* genome. As *Synechococcus* is polyploid, the cassettes need to be incorporated into every copy of the chromosome, i.e. complete segregation. Finally, the functionality of the counterselection cassettes will be assessed within *E. coli* and *Synechococcus* host cells, by growing the cells in the presence of antibiotics and various concentration of sucrose.

Once the system has been implemented in *Synechococcus*, it can be utilized for genetic engineering to facilitate markerless genetic insertions, deletions and replacements within the *Synechococcus* genome. This tool can be used to study gene function by reverse genetics, or to facilitate metabolic engineering to produce products of interest.

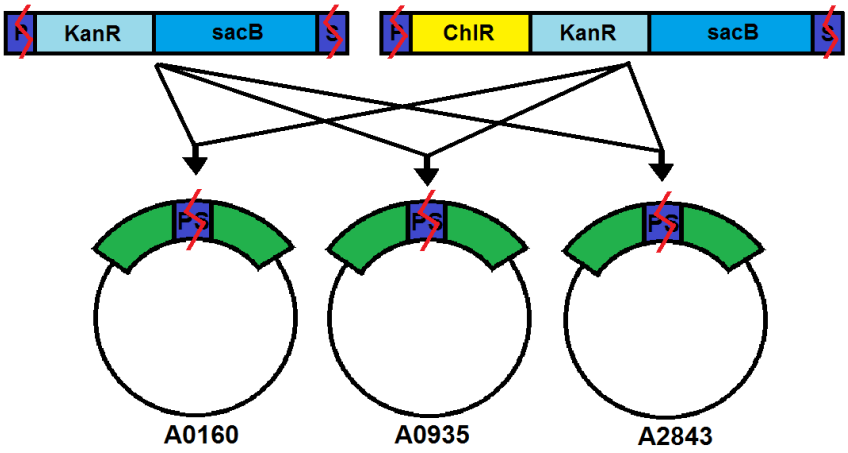

Figure 1-9. Construction strategy of counterselection plasmids. KanR = kanamycin resistance gene (*kan*^{r}). ChlR = chloramphenicol resistance gene (*Tn9cat*). *sacB* is a counterselectable marker. P and S refers to the BioBrick prefix and suffix, utilized in molecular cloning. Red markings represent where the DNA molecules are cut. Green regions correspond to the A0160, A0935 and A2843 synthesized regions with homology to neutral integration sites within the *Synechococcus* sp. PCC 7002 genome.

2 Materials and Methods

This chapter gives a detailed description of the materials and methods utilized in this project. Section 2.1 entail information about the materials used, including culture media and other important solutions, cloning vectors, PCR primers and enzymes. Additional information about DNA Ladders, cloning vectors and primer characteristics are presented in Appendix B, C and D, respectively. Section 2.2 describes the methods utilized in this work, including PCR, agarose gel electrophoresis, molecular cloning, plasmid isolation, sequencing, *sacB* testing, sucrose and NaCl growth experiments, and transformation of *Synechococcus* sp. PCC 7002. Calculations utilized in transformation preparations of *Synechococcus* is presented in Appendix F.

2.1 Materials Utilized in This Project

2.1.1 Culture Media

In the laboratory, culture media supply the nutritional needs of the organism. The particular type, source and amount of nutrients required varies between different organisms. The choice of culture media to be utilized is therefore dependent on the organism under study. The media can be used as a liquid, or can be solidified by the addition of a gelling agent. Solid media is prepared in the same way as liquid media, with the exception that agar is added before sterilization. This immobilizes the cells, and allows them to form visible, isolated colonies – which makes it easier to visualize the composition and presumptive purity of the culture. Before use, the culture medium is sterilized to eliminate possible contaminants. This is usually achieved through autoclavation or filter sterilization. By utilizing aseptic techniques, contamination of the cultures can be prevented (Madigan et al., 2012).

The culture media and solutions utilized in this project to grow *Escherichia coli* DH5α and *Synechococcus* sp. PCC 7002 are presented categorically below.

***Escherichia coli* Strain DH5α**

E. coli cells were grown in Lysogeny Broth (LB), a nutritionally rich medium used for bacterial growth. The medium is based on an article by Giuseppe Bertani (1951), first utilized to optimize *Shigella* growth. LB has since become a popular choice for cultivation of a wide range of bacteria.

The acronym is often, albeit incorrectly, interpreted as Luria Broth, Lennox broth, or Luria-Bertani medium (Bertani, 2004). The medium was prepared according to the directions in Table 2-1. Solid medium containing agar is referred to as Lysogeny Agar (LA).

Table 2-1. Content of Lysogeny Broth (LB), utilized for cultivation of *E. coli* DH5α. The components were dissolved in Milli-Q water, and the solution autoclaved at 121°C for 20 minutes before use.

Compound:	Amount (g/L):
Tryptone	10
NaCl	10
Yeast Extract	5
Bacteriological Agar[1]	15

[1]Only for plates. The media is then referred to as Lysogeny Agar (LA).

Synechococcus sp. Strain PCC 7002

The growth media utilized for *Synechococcus* is called AA+, and is based on an article by Marcus Ludwig and Donald A. Bryant (2011). The medium was prepared as described in Table 2-2. Descriptions on how to prepare the stock solutions is provided in Table 2-3. After autoclaving the solution (20 min, 121°C), the remaining ingredients were added inside the sterile bench. To prevent contamination, the medium was never handled outside the bench.

Table 2-2. Content of AA+ culture media, utilized for cultivation of *Synechococcus* sp. PCC 7002. Components above the black line were dissolved in Milli-Q water, and the solution was autoclaved (20 min, 121°C). The remaining solutions were added inside the sterile bench. Descriptions on preparation of the stock solutions is provided in Table 2-3.

Compound:	Amount:
NaCl	18 g/L
$MgSO_4x7H_2O$	5 g/L
Agar agar[1]	15 g/L
Glycerol	1.1 mL/L
100x Tris pH 8.2	10 mL/L
1000x $FeCl_3x6H_2O$	1 mL/L
1000x Trace mineral solution	1 mL/L
100x AA+ solution	10 mL/L
Vitamin B_{12}[2]	4 µg/L

[1]Only for plates. NB! Not bacteriological agar.
[2]Only add B_{12} if the medium will be used right away. Store remaining medium in a dark place.

Table 2-3. Stock solutions utilized to make AA+ media. The compounds were dissolved in Milli-Q water, and the solutions sterilized according to the descriptions provided.

Solution:	Compound:	Amount (g/L):	Notes:
100x Tris pH 8.2	Tris	99.4	Adjust pH to 8.2 with HCl, and autoclave[1] solution.
1000x $FeCl_2x6H_2O$	$FeCl_3x6H_2O$	4.05	Filter sterilize solution[2].
1000x Trace mineral solution	H_3BO_3	2.86	Autoclave[1] or filter sterilize[2] solution.
	$MnCl_2x4H_2O$	1.81	
	$ZnSO_4x7H_2O$	0.222	
	$Na_2MoO_4x2H_2O$	0.39	
	$CuSO_4x5H_2O$	0.079	
	$Co(NoO_3)_2x6H_2O$	0.0494	
100x AA+ solution	KH_2PO_4	2.5	Autoclave[1] solution.
	Na_2-EDTA$x2H_2O$	3	
	KCl	60	
	$NaNO_3$	100	
	$CaCl_2$	13.3	

[1]Autoclave solutions at 121°C for 20 minutes.
[2]Filter the solution through a sterile syringe filter (25 mm, pore size 0.20 µm).

Selective Media

Culture media can be made selective by the addition of a substance that kills or inhibits the growth of some microorganisms in favor of others. A popular choice is the use of antibiotics. In bacteria, antibiotics frequently target ribosomes, specific cell wall structures, the cytoplasmic membrane, DNA transcription or translation. The effect of the antibiotic depends on the target, and is either bacteriostatic (inhibits growth), bacteriocidal (kills the cell) or bacteriolytic (causes cell lysis). Microorganisms that has acquired the ability to grow in the presence of an antibiotic to which the organism is usually sensitive is said to be resistant to that antibiotic. This is frequently exploited in the laboratory, where antibiotics are used to select for cells harboring a specific resistance gene (Madigan et al., 2012). Another example is the use of sucrose in the culture media of Gram-negative bacteria harboring the *sacB* gene. As explained in section 1.3.2, addition of sucrose in the culture media causes production of levans, which is toxic to the bacteria (Recorbet et al., 1993, Wrobel et al., Gay et al., 1985, Viola et al., 2014, Pelicic et al., 1996).

Under conditions where antibiotic selection was required, kanamycin and/or chloramphenicol were added according to the descriptions in Table 2-4. For LA and AA+ media with agar, antibiotics were added to handwarm solution to avoid degradation. The solutions were subsequently poured into petri dishes. This was performed inside the sterile bench, to prevent contamination of the media.

Table 2-4. Antibiotics utilized in selective LB, LA and AA+ media. Solvent, stock concentration, final concentration and dilution factor is specified. When both antibiotics were added at the same time, half the amount of each were utilized.

Compound:	Abbreviation:	Solvent:	Stock concentration:	Final concentration:	Dilution factor:
Kanamycin[1]	Kan	H_2O[1]	100 mg/mL	50 µg/mL	2000
Chloramphenicol	Chl	Ethanol	30 mg/mL	30 µg/mL	1000

[1]Filter the solution through a sterile syringe filter (25 mm, pore size 0.20 µm).

For *sacB* testing, LA or solid AA+ media containing different concentrations of sucrose were utilized. 40 mL of the Milli-Q water in the culture media was then replaced with filter sterilized sucrose solution. The amount of sucrose utilized is specified in Table 2-5. Culture media were prepared with and without NaCl, as *sacB* testing might be NaCl-sensitive. The sucrose solution was added to autoclaved, handwarm culture medium under sterile conditions, and the medium was subsequently poured into petri dishes.

Table 2-5. Concentration of sucrose in LA or AA+ culture media to conduct *sacB* testing. Sucrose was dissolved in Milli-Q water to a total volume of 40 mL. The solutions were filter sterilized utilizing sterile syringe filters (25 mm, pore size 0.20 µm) and subsequently added to handwarm media.

Percent sucrose (w/v):	Amount sucrose (g/L):
1	10
3	30
5	50
7	70
10	100
15	150

2.1.2 Other solutions

Media and Solutions Utilized to Prepare Competent Cells

Cells capable of taking up free DNA and be transformed are called competent cells. Not all bacteria are naturally transformable, including *E. coli*. However, as explained in subsection 1.5.3, cells can be made competent by treatment of high amounts of calcium ions. In addition, treatment with Mg^{2+}, Mn^{2+} and Rb^{+} further increases the transformation efficiency. Cells treated in this manner are able to take up dsDNA, such as plasmids. This process is critical for biotechnology, as *E. coli* remains

the organism of choice for most molecular cloning (Madigan et al., 2012, Snustad and Simmons, 2012, Hanahan, 1983).

The media and solutions utilized to prepare competent *E. coli* DH5α cells are listed in Table 2-6, and are based on a protocol by Green and Rogers (2013). ψB and SOC (Super Optimal Broth with Catabolic Repressor) are culture media, while TfBI and TfBII are buffers (transformation buffer I and II). SOC is also utilized in heat-shock transformation, to maximize transformation efficiency. 1 M $MgCl_2$, $MgSO_4$ and glucose is then added before use. The solutions were autoclaved or filter sterilized, according to the descriptions provided in the table.

Table 2-6. Media and buffers utilized to make competent *E. coli* DH5α cells. ψB and SOC are growth media, while TfBI and TfBII are buffers (transformation buffers I and II). All solutions were sterilized before use, according to the descriptions provided.

Solution:	Compound:	Amount (g/L):	Notes:
ψB	Tryptone	20	Dissolve components above the black line in Milli-Q water. pH-adjust solution to 7.6 with 1 M KOH, and autoclave[2]. Add 1 M $MgSO_4$ before use.
	Yeast Extract	5	
	KCl	0.76	
	1 M $MgSO_4$[1]	34 mL/L	
TfBI	Potassium acetate	2.94	Dissolve powder in Milli-Q water. Add glycerol. pH-adjust solution to 5.8 with 0.2 M acetic acid. Filter sterilize[3], and store at 4°C in the dark.
	$MnCl_2$	9.90	
	$RbCl_2$	12.10	
	$CaCl_2$	1.48	
	Glycerol	150 mL	
TfBII	MOPS	2.1	Dissolve powder in Milli-Q water. Add glycerol. pH adjust solution to 5.8 with 0.2 M acetic acid. Filtersterilize[3], and store at 4°C in the dark.
	$CaCl_2$	11.0	
	$RbCl_2$	1.2	
	Glycerol	150 mL	
SOC	Tryptone	20 g	Dissolve components above the black line in Milli-Q water. Autoclave[2] solution and store at 4°C in the dark.
	Yeast Extract	5 g	
	NaCl	0.584	
	KCl	0.186	
	1 M $MgCl_2$[1]	10 mL/L	Add 1 M $MgCl_2$, $MgSO_4$ and glucose before the media is used in heat-shock transformation.
	1 M $MgSO_4$[1]	10 mL/L	
	1 M Glucose[1]	20 mL/L	

[1]The solution should be sterilized and used only in the sterile bench.
[2]Autoclave solution at 121°C for 20 minutes.
[3]Filter the solution through a sterile syringe filter (25 mm, pore size 0.20 µm).

dNTP Solution

A deoxynucleoside triphosphate (dNTP) solution is an equimolar mixture of ultrapure nucleotides, i.e. deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), and deoxythymidine triphosphate (dTTP). The solution has several applications, e.g. as a nucleotide source for use in PCR, where the nucleotides are incorporated into new strands of DNA by a DNA polymerase.

The dNTP solution was prepared as described in Table 2-7, with a final concentration of 10 mM. The solution was subsequently stored at -20°C.

Table 2-7. Deoxynucleoside triphosphate (dNTP) solution. The nucleotides were provided by Roche Diagnostics. The solution has a final concentration of 10 mM, and should be stored at -20°C.

Compound:	Concentration (mM):	Amount (µL):
dATP	100	10
dGTP	100	10
dCTP	100	10
dTTP	100	10
H_2O	-	60

60% Glycerol Solution

Glycerol can be utilized to stabilize the freezing of cells, as described in subsection 2.2.5.

60 mL glycerol (bidistilled, 99.5%) was dissolved in 40 mL Milli-Q water, and the solution was autoclaved at 121°C for 20 minutes. The solution was stored on the lab bench, and opened only under sterile conditions to prevent contamination.

Solutions Utilized in Agarose Gel Electrophoresis

Nucleic acids are highly charged molecules, containing negatively charged phosphate groups. The molecules have a constant charge per unit length, a characteristic that can be utilized for separation on the basis of size or confirmation. For DNA, this separation is carried out by agarose gel electrophoresis (Clark and Pazdernik, 2013).

The buffers and solutions utilized in this project are presented in Table 2-8, and the protocol for agarose gel electrophoresis provided in subsection 2.2.3.

Table 2-8. Solutions utilized in agarose gel electrophoresis.

Solution:	Compound:	Amount:	Notes:
50x TAE Buffer	Tris-base[1] 1 M Acetic Acid 0.5 M EDTA (pH 8)	242 g 57.1 mL 100 mL	Mix components and add 500 mL deionized water. Stir solution until all components are dissolved. Add deionized water to a final volume of 1L, and autoclave[2] solution.
1x TAE Buffer	50x TAE dH_2O	400 mL 16.4 L	Dilute 50x TAE in deionized water to a final volume of 20 L.
0.8% Agarose GelRed	Agarose 1x TAE GelRed	3.2 g 400 mL 20 µL	Mix agarose and 1x TAE and boil solution in the microwave until all the agarose powder is dissolved. Add GelRed and mix solution. Store at 65°C until use.
Loading buffer	Gel Loading Dye Purple (6X) dH_2O DNA	 2 µL 7 µL 1 µL	Mix all components and apply 10 µL of each sample into one of the wells in the agarose gel.
DNA Ladder[3]	GeneRuler 1kb DNA Ladder	0.4 µL	Load sample into one of the wells in the agarose gel. Note, can also make a ladder mix, as presented below.
DNA Ladder[3]	GeneRuler 1 kb Plus DNA Ladder	0.4 µL	Load sample into one of the wells in the agarose gel. Note, can also make a ladder mix, as presented below.
DNA Ladder mix	GeneRuler 1kb (Plus) DNA Ladder Gel Loading Dye Purple (6X) dH_2O	0.2 µL 2 µL 7.8 µL	Mix all components and apply 10 µL into each well. This mix gives very nice results when run at 90V for 30-40 minutes.

[1]Tris(hydroxymethyl)aminomethane.
[2]Autoclave solution at 121°C for 20 minutes.
[3]The DNA ladders are visualized in Appendix B.

2.1.3 Cloning Vectors

A cloning vector is a small piece of DNA that can self-replicate within a cell and be used for carrying cloned genes or segments of DNA. A variety of DNA molecules can be utilized as vectors. The DNA should be small and manageable, be easy to move from cell to cell, and it should be straightforward to generate and purify large amounts of vector DNA. Bacterial plasmids are by far the most popular choice, as well as small viral genomes (Clark and Pazdernik, 2013).

The plasmids utilized in this project are presented in Table 2-9, and the cloning vectors visualized in Appendix C. pTA16 was utilized to test the transformation efficiency of competent *E. coli* DH5α cells. pK19mobsacB and pLitmus28i-ChlR were used for amplification of the *sacB* and *Tn9cat* genes, respectively.

The remaining plasmids were utilized as vector backbones in molecular cloning. They are comprised of synthesized sequences homologous to the A0160, A0935 or A2843 neutral integration sites within the chromosome of *Synechococcus*, inserted into a vector backbone of pUC57-Simple. In addition, the synthesized sequences contain a BioBrick cloning site to facilitate incorporation of genes or segments of DNA, as explained in subsection 1.5.2.

Table 2-9. Plasmids utilized in this project. Plasmid characteristics relevant for the project are presented, including size, features of interest and usage of the plasmids. Plasmids used as vectors in molecular cloning are presented schematically in Appendix C.

Plasmid:	Size (bp):	Features of interest:	Usage:
pTA16	8126	Kanamycin resistance	Test the transformation efficiency of competent *E. coli* DH5α cells.
pK19mobsacB	5729	*sacB* gene	Used for amplification of the *sacB* counterselection gene.
pLitmus28i-ChlR	3497	*Tn9cat* gene	Used for amplification of the *Tn9cat* gene, which confers chloramphenicol resistance.
pA0160	4391	A0160, A0935 or A2843 neutral site + BioBrick cloning site	Contains synthesized sequences homologous to the A0160, A0935 or A2843 neutral integration sites within the chromosome of *Synechococcus* sp. PCC 7002, utilized for homologous recombination. In addition, BioBrick cloning sites facilitates molecular cloning.
pA0935	4391		
pA2843	4379		

2.1.4 Enzymes

Enzymes are biological catalysts, usually composed of proteins, that promotes specific reactions or groups of reactions. After their discovery in the 60s, restriction enzymes became the cornerstone of molecular biology. Restriction enzymes are capable of fragmenting DNA at specific sites – a vital part of molecular cloning (Reece, 2004).

The restriction enzymes used in this project are presented in Table 2-10, along with recognition sequence, cut-site, and important reaction conditions. Additional enzymes utilized are presented in Table 2-11. All enzymatic reactions were carried out according to the recommendations from the manufacturer, a summary of which is given in the aforementioned tables.

Table 2-10. Restriction enzymes utilized in this project. Restriction sites are indicated in the sequence by vertical bars (|). Reaction conditions are specified by reaction buffer and optimal temperature. Enzymes capable of digesting 1 µg DNA in 5-15 minutes are denoted as Time-Saver™ Qualified. Conditions required to heat-inactivate enzymes are given where applicable. All enzymes were supplied by New England Biolabs Inc. (NEB).

Enzyme:	Restriction Sequence:	Buffer (activity):	Optimal Temperature:	Time-Saver™ Qualified:	Heat-Inactivation:
DpnI	5'-GA(CH_3)|TC-3' 3'-CT|A(CH_3)G-5'	CutSmart® (100%)	37°C	Yes	80°C, 20 min
NotI-HF	5'-GC|GGCCGC-3' 3'-CGCCGG|CG-5'	CutSmart® (100%)	37°C	Yes	65°C, 20 min
PstI-HF	5'-CTGCA|G-3' 3'-G|ACGTC-5'	CutSmart® (100%)	37°C	Yes	-
SpeI	5'-A|CTAGT-3' 3'-TGATC|A-5'	CutSmart® (100%)	37°C	Yes	80°C, 20 min
XbaI	5'-T|CTAGA-3' 3'-AGATC|T-5'	CutSmart® (100%)	37°C	Yes	65°C, 20 min

Table 2-11. Additional enzymes utilized in this project. The application in described for each enzyme, and the reaction conditions specified by reaction buffer and reaction temperature. Conditions required to heat-inactivate the enzymes is given where applicable. All enzymes were supplied by New England Biolabs Inc. (NEB).

Enzyme:	Application:	Buffer:	Reaction Temperature:	Heat-Inactivation:
CIP[1]	Dephosphorylation of 5´ and 3´ ends of DNA and RNA.	CutSmart® Buffer	37°C	-
T4 DNA Ligase	Formation of phosphodiester bonds between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA.	10X T4 DNA Ligase Buffer	16°C or room temperature[2]	65°C, 10 min
Taq DNA Polymerase	Thermostable DNA Polymerase utilized in PCR	10x Standard Taq Reaction Buffer	Thermocycling	-
Q5® High-Fidelity DNA Polymerase	Thermostable DNA Polymerase utilized in PCR	Q5® High-Fidelity 2x Master Mix or 5X Q5® Reaction Buffer	Thermocycling	-

[1]Alkaline Phosphatase, Calf Intestinal (CIP).
[2]Incubate at 16°C overnight, or for 10 minutes (cohesive ends) to 2 hours (blunt ends or single base overhangs) at room temperature.

2.1.5 Primers Utilized for Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a powerful method utilized in molecular biology to amplify a specific sequence of DNA *in vitro*. PCR requires two oligonucleotide primers, designed to bind flanking regions at each side of the DNA sequence to be amplified (Reece, 2004). A description of PCR and primer characteristics are given in subsection 1.5.1.

The PCR primers utilized in this project have been ordered from Sigma-Aldrich. The complete list of primers, including sequence, target sequence, length, GC-content and calculated melting temperatures (Tm), is provided in Appendix D. The appendix also includes recommended annealing temperatures and expected sizes of selected amplicons. The reaction setup and PCR programs utilized for *Taq* DNA polymerase and Q5® High-Fidelity DNA polymerase is described in subsection 2.2.2.

2.2 Experimental Methods

2.2.1 Preparation of Competent *E. coli* DH5α Cells

The following procedure is adapted from a protocol by Green and Rogers (2013). Some background information is provided in subsection 1.5.3, and the solutions utilized in subsection 2.1.2.

1 µL competent DH5α cells were inoculated in 20 mL SOC[2] in a small Erlenmeyer flask. The solution was incubated at 37°C with 225 rpm shaking overnight. The following day, 3000 µL cell solution was inoculated in 300 mL pre-heated ψB media. The culture was incubated for another two hours, until the optical density at 600 nm (OD_{600}) reached a value of 0.3-0.4.

The cell solution was chilled on ice for 5 minutes, and was thereafter transferred to six chilled 50 mL Falcone tubes. The tubes were centrifuged at 4000 rpm and 4°C for 10 minutes in a tabletop centrifuge. The supernatant was discarded, and the pellets were resuspended in 15 mL chilled TfBI.

[2] $MgCl_2$, $MgSO_4$ and glucose not added.

The tubes were centrifuged for another 10 minutes under the specified conditions. The supernatant was discarded, and the pellets were resuspended in 1 mL chilled TfBII by pipetting carefully up and down with a P1000 pipette with the end of the tip cut off.

Finally, the cell solutions were transferred to sterile 1.5 mL Eppendorf tubes in aliquots of 100 µL, and snap-frozen utilizing liquid nitrogen. The tubes were subsequently stored at -80°C.

The transformation efficiency of the cells was tested by transforming the cells with a plasmid containing an antibiotic resistance gene. The calculations included the number of colony forming units (cfu), the dilutions utilized, and the concentration of transforming DNA. The heat-shock procedure used for transformation is presented in subsection **Feil! Fant ikke referansekilden.**.

2.2.2 Polymerase Chain Reaction (PCR)

PCR was utilized to amplify target DNA sequences, as explained in subsection 1.5.1. A complete list of the PCR primers used in this project, including sequence, target sequence, length, GC-content and calculated melting temperatures (Tm), is provided in Appendix D. The appendix also includes recommended annealing temperatures and expected sizes of selected amplicons. The reaction setup and PCR programs utilized for *Taq* DNA polymerase and Q5 High-Fidelity DNA polymerase are presented categorically below, followed by a section about colony PCR.

The reaction setup is described for 25 µL and 50 µL reactions, but can be converted to any desired amount. General guidelines and recommendations for PCR optimization are available in the online protocols (NEB(b), NEB(e), NEB(f)). If the thermocycler does not contain a heated lid, a layer of mineral oil can be utilized to cover the top of the PCR tubes and thereby prevent evaporation of the sample.

PCR Protocol for *Taq* DNA Polymerase with 10x Standard *Taq* Buffer

The breakthrough in PCR came with the introduction of *Taq* DNA polymerase – a thermostable DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus*. The polymerase lacks proofreading and should therefore not be used in applications where high fidelity is required.

However, if the goal of the PCR is to investigate whether or not a DNA fragment is present, then the success of the reaction is not affected by the introduction of errors in the amplified sequence. *Taq* DNA polymerase remains the most popular choice for routine PCR (Longley et al., 1990, Reece, 2004).

Taq DNA polymerase was utilized in molecular cloning to investigate whether or not the genes to be incorporated into the cloning vectors were present. PCR was performed according to the *Taq* DNA polymerase protocol from New England Biolabs Inc (NEB(b)). The reaction setup is presented in Table 2-12.

All components were assembled on ice. The largest volumes were added first, and the polymerase last. The solution was mixed carefully by pipetting up and down. If necessary, the liquid was collected at the bottom of the tubes with a quick spin. The PCR tubes were thereafter transferred to a thermocycler. The thermocycling conditions utilized are presented in Table 2-13. After the cycling was completed, the PCR product was investigated by agarose gel electrophoresis, as described in subsection 2.2.3.

Table 2-12. Reaction setup utilized for PCR with *Taq* DNA polymerase. All components were assembled on ice, and the solution mixed carefully. If necessary, the liquid was collect at the bottom of the PCR tubes by a quick spin. The tubes were transferred to a thermocycler, and a thermocycling program initiated, as described in Table 2-13. 10x Standard *Taq* reaction buffer and *Taq* DNA Polymerase were provided by New England Biolabs Inc (NEB), and the dNTP solution mix by Roche diagnostics. The primers utilized are presented in Appendix D.

Component:	25 µL reaction:	50 µL reaction:	Final concentration:
10x Standard *Taq* Reaction Buffer	2.5 µL	5 µL	1x
10 mM dNTPs	0.5 µL	1 µL	200 µM
10 µM Forward Primer	0.5 µL	1 µL	0.2 µM[1]
10 µM Reverse Primer	0.5 µL	1 µL	0.2 µM[1]
Taq DNA Polymerase	0.125 µL	0.25 µL	1.25 units/50 µL PCR
Template DNA	Variable	Variable	<1,000 ng
Nuclease-Free Water	to 25 µL	to 50 µL	-

[1]The concentration should be within the range of 0.05-1 µM.

Table 2-13. Thermocycling conditions utilized for PCR with *Taq* DNA polymerase. The annealing temperatures of the primers are calculated by the Tm-calculator from New England Biolabs Inc. (NEB(c)), and are presented in Appendix D. The extension time is dependent on the size of the DNA fragment to be amplified.

Step:		Temperature:	Time:
Initial Denaturation		95°C	30 seconds[1]
Denaturation	30x	95 °C	15-30 seconds
Annealing		45-68°C	15-60 seconds
Extension		68°C	1 minute/kb
Final Extension		68°C	5 minutes
Hold		4-10°C	∞

[1]For colony PCR, the first denaturation step is extended to 6 minutes to destroy the cell walls and release the DNA.

PCR Protocol for Q5® High-Fidelity DNA Polymerase

Since the discovery of *Taq* DNA polymerase, a number of other thermostable polymerases have been described and utilized in PCR. The different polymerases vary in important characteristics, such as fidelity, extension time and appropriate amplicon size (Reece, 2004). Q5® High-Fidelity DNA polymerase is a popular choice for routine PCR, as the polymerase has a short extension time and possesses proofreading capabilities. With an error rate of more than 100 times lower than *Taq* DNA polymerase, it is ideal for cloning and amplification of long and difficult amplicons (NEB(d)).

Q5® High-Fidelity DNA polymerase was utilized in molecular cloning to amplify genes and other DNA fragments of interest. PCR was performed according to two protocols from NEB – one protocol with a reaction setup containing Q5® 2x master mix (NEB(e)), and another containing individual reaction components (NEB(f)). The reaction setup is presented in Table 2-14 and Table 2-15.

All components were assembled on ice. The largest volumes were added first, and the polymerase last. The solution was mixed carefully by pipetting up and down. If necessary, the liquid was collected at the bottom of the tubes with a quick spin. The PCR tubes were thereafter transferred to a thermocycler. The thermocycling conditions utilized are presented in Table 2-16. After the cycling was completed, the PCR product was investigated by agarose gel electrophoresis, as described in subsection 2.2.3.

Table 2-14. Reaction setup for PCR utilizing Q5® High-Fidelity DNA Polymerase with 2x master mix. All components were assembled on ice, and the solution mixed carefully. If necessary, the liquid was collect at the bottom of the PCR tubes by a quick spin. The tubes were transferred to a thermocycler, and a thermocycling program initiated, as described in Table 2-16. Q5® High-Fidelity 2x Master Mix was provided by New England Biolabs Inc (NEB). The primers utilized are presented in Appendix D.

Component:	25 µL reaction:	50 µL reaction:	Final concentration:
Q5 High-Fidelity 2x Master Mix	12.5 µl	25 µl	1x
10 µM Forward Primer	1.25 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	2.5 µl	0.5 µM
Template DNA	Variable	Variable	< 1,000 ng
Nuclease-Free Water	to 25 µl	to 50 µl	-

Table 2-15. Reaction setup for PCR utilizing Q5® High-Fidelity DNA Polymerase. All components were assembled on ice, and the solution mixed carefully. If necessary, the liquid was collect at the bottom of the PCR tubes by a quick spin. The tubes were transferred to a thermocycler, and a thermocycling program initiated, as described in Table 2-16. 5x Q5 Reaction Buffer and Q5 High-Fidelity DNA Polymerase were provided by New England Biolabs Inc (NEB), and the dNTP solution by Roche diagnostics. The primers utilized are presented in Appendix D.

Component:	25 µL reaction:	50 µL reaction:	Final concentration:
5x Q5 Reaction Buffer	5 µl	10 µl	1x
5x Q5 High GC Enhancer (Optional)	(5 µl)	(10 µl)	(1x)
10 mM dNTPs	0.5 µl	1 µl	200 µM
10 µM Forward Primer	1.25 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	2.5 µl	0.5 µM
Q5 High-Fidelity DNA Polymerase	0.25 µl	0.5 µl	0.02 Units/µl
Template DNA	Variable	Variable	< 1,000 ng
Nuclease-Free Water	to 25 µl	to 50 µl	-

Table 2-16. Thermocycling conditions utilized for PCR with Q5® High-Fidelity DNA polymerase. The annealing temperatures of the primers are calculated by the Tm-calculator from New England Biolabs Inc. (NEB(c)), as presented in Appendix D. The extension time is dependent on the size of the DNA fragment to be amplified.

Step:		Temperature:	Time:
Initial Denaturation		98°C	30 seconds
Denaturation	25-35x	98 °C	5-10 seconds
Annealing		50-72°C	10-30 seconds
Extension		72°C	20-30 seconds/kb
Final Extension		72°C	2 minutes
Hold		4-10°C	∞

Colony PCR

Colony PCR is a fast and efficient method for determining the presence or absence of the desired DNA fragment within transformed bacterial cells. Individual colonies are picked from growth plates and used directly in the PCR reaction. By extending the first denaturation step, the cells are lysed and releases DNA from the cells, which can be used directly as a template in the PCR amplification. Alternatively, the cells can be lysed in sterile water prior to the PCR reaction. By utilizing primers designed to target the DNA fragment under study, it can be determined whether or not the desired fragment is present. Furthermore, by utilizing primers targeting the flanking regions, the molecular size of the fragment can be determined. As for regular PCR, the presence or absence and the size of the fragment is verified through agarose gel electrophoresis (NEB(g)).

The method was utilized to examine the contents of transformed bacterial cells. 30 µL Milli-Q water was transferred to sterile Eppendorf tubes. Single colonies of transformed cells were picked with sterile pipette tips and transferred to the water by pipetting carefully up and down. 1 µL of the solution was used as the DNA source in a 25 µL PCR reaction with *Taq* DNA polymerase or Q5® High-Fidelity DNA polymerase, as presented in Table 2-12 to Table 2-16 above. Note, however, that the first denaturation step was extended to 6 minutes to destroy the cell walls and release the DNA. Finally, as for regular PCR, agarose gel electrophoresis was utilized to confirm the presence and/or size of the PCR products.

For *E. coli*, colony PCR was performed in combination with plasmid isolation. The remaining solution was inoculated in 5 mL LB with appropriate antibiotics, and the tubes were incubated overnight at 37°C and 225 rpm shaking, tilted in a 45-degree angle. The PCR results determined which overnight cultures to be harvested the following day.

Colony PCR was also performed with *Synechococcus*, with a minor modification in the protocol. A colony was picked with a sterile pipette tip and streaked onto a new AA+ plate. The same tip was subsequently used to transfer the remaining cells to 30 µL water, by pipetting carefully up and down. As before, 1 µL of the solution was used as the DNA source in the PCR reaction.

2.2.3 Agarose Gel Electrophoresis and Purification of DNA

Agarose Gel Electrophoresis

Agarose gel electrophoresis is a powerful method utilized to separate charged macromolecules, such as DNA, based on size and conformation. The method is emphasized in subsection 1.5.1.

In this project, the method was used to examine PCR products; to verify the presence or absence of a DNA fragment of interest, and to estimate the size of the fragments present in the sample. The solutions utilized were prepared according to the description in subsection 2.1.2. Directions on how to prepare the GeneRuler 1kb DNA ladder and the DNA samples for loading are also provided. Furthermore, the DNA ladders utilized are presented in Appendix B.

Solution containing GelRed and 0.8% agarose was poured into a gel rack with a comb inserted close to one of the ends. The gel was allowed to cool down to solidify (~30 minutes), and was placed in a gel chamber with the wells adjacent to the negative electrode. The chamber was filled with 1x TAE buffer to cover the gel. The comb was gently removed, and the DNA ladder and the samples to be analyzed were loaded into the wells of the gel. The chamber was covered by a lid, and an electrical current of 90-100V was applied to the solution for 25-40 minutes to facilitate the separation of DNA. Once the separation was completed, the DNA was visualized through illumination with ultraviolet light. The gel was photographed utilizing a ChemiDoc™ XRS+ Imaging System, connected to a desktop computer equipped with Image Lab (version 5.2.1.). By comparison to the DNA ladder, the sizes of the DNA fragments in the samples were determined.

DNA Purification

If the PCR products were intended for use in molecular cloning, the samples containing the correct fragments were purified. In addition, PCR purification was carried out after digestion reactions comprising the enzyme Alkaline Phosphatase, Calf Intestinal (CIP), as explained in subsection **Feil! Fant ikke referansekilden.**. In both cases, purification was performed utilizing the QIAquick PCR Purification Kit from Qiagen. The kit can be used for extraction of DNA fragments in the range of 100 bp to 10 kb, with up to 95% DNA recovery. The DNA is purified within a column by a simple bind-wash-elute procedure, with an elution volume of 30-50 µL (Qiagen).

The purification was carried out with an elution volume of 30 µL, to increase the DNA concentration within the eluted sample. The concentration was measured with a NanoDrop ND-1000 spectrophotometer. Apart from this, the procedure was performed exactly as described in the accompanying protocol.

2.2.4 Construction of Recombinant DNA

Recombinant DNA molecules are created by genetic recombination methods, as described in section 1.5. The methods include digestion of DNA from two or more sources with restriction enzymes, ligating the fragments of interest utilizing DNA ligase, and transforming the constructs into an appropriate host cell for amplification. Each step is described in detail in the following text.

Digestion

The DNA of interest was amplified by PCR, purified, and digested by restriction enzymes. The enzymes utilized, reaction conditions, and important characteristics are presented in Table 2-10.

The digestion mixture was prepared in a 1.5 mL Eppendorf tube, as presented in Table 2-17. All reaction components were assembled on ice, with the enzyme(s) added last. The solution was mixed carefully by pipetting up and down, and incubated at the appropriate temperature for 30 minutes. The mixture was subsequently incubated in a heat block for 20 minutes at the appropriate temperature to facilitate heat-inactivation of the enzyme(s). The optimum temperature for the various enzymes, as well as temperatures for heat-inactivation, are provided in Table 2-10.

Table 2-17. Restriction enzyme reaction setup. The components were assembled on ice, with the enzyme(s) added last. The solution was mixed carefully, and incubated for 30 minutes at the appropriate temperature. The enzyme(s) were heat-inactivated before proceeding to ligation. The optimum temperatures and heat-inactivation conditions for the various restriction enzymes are provided in Table 2-10.

Component:	Amount:
10X CutSmart Buffer	1 µL
DNA	200-300 ng
Enzyme 1	0.5 µL
Enzyme 2 (optional)	(0.5 µL)
Nuclease-free water	to 10 µL

When utilizing restriction enzymes for cloning, two reactions were prepared – one for vector DNA and another for insert. The vector was subsequently treated with 1 µL Alkaline Phosphatase, Calf Intestinal (CIP), to prevent religation of linearized vector DNA. As CIP dephosphorylates the ends of DNA, ligation can only occur when the compatible sticky ends of the vector and insert bind, as only the insert DNA contains phosphate groups that can be utilized for ligation. The solution was incubated at 37°C for one hour, followed by DNA purification.

Ligation

After heat-inactivation of the restriction enzyme(s), and purification if utilizing CIP, the DNA could be utilized for ligation. The reaction was catalyzed by T4 DNA Ligase, under the conditions specified in Table 2-11. The amount of vector and insert DNA needed was calculated by utilizing the Ligation Calculator provided by NEB. The tool calculates the mass of insert required at different molar ratios of insert to vector DNA, which is dependent on the insert length, vector length, and vector mass. A molar ratio of 3:1 is usually preferred (NEB(h)).

The ligation mixture was prepared in a 1.5 mL Eppendorf tube, as presented in Table 2-18. All reaction components were assembled on ice, with T4 DNA Ligase added last. The amount of vector and insert DNA to be added was calculated utilizing the NEB Ligation Calculator. Usually, 15-25 ng vector DNA was used, and the amount of insert needed calculated with a molar ratio of 3:1. The solution was mixed carefully by pipetting up and down, and the solution collected at the bottom with a quick spin. The solution was incubated in a 16°C water bath overnight. Finally, the enzyme was heat-inactivated at 65°C for 10 minutes, before proceeding to heat-shock transformation.

Table 2-18. Ligation reaction setup utilizing T4 DNA Ligase. The components were assembled on ice, with the ligase added last. The amount of vector and insert DNA to be added was calculated with the Ligation Calculator from NEB, utilizing a molar ratio of 3:1 insert to vector. The solution was mixed carefully, and incubated at 16°C overnight. The enzyme was heat-inactivated at 65°C for 10 min before proceeding to heat-shock transformation.

Component:	Amount:
10x T4 DNA Ligase Buffer	2 µL
Vector DNA Insert DNA	(3:1)
T4 DNA Ligase	1 µL
Nuclease-free water	to 20 µL

Heat-Shock Transformation

Heat-shock transformation is a method utilized to promote uptake of exogenous DNA in bacterial cells, as described in subsection 1.5.3. The following procedure is adapted from a protocol by Green and Rogers (2013).

Aliquots containing 100 µL competent DH5α cells were thawed on ice for 5-10 minutes. DNA was subsequently added, with the amount dependent on whether the solution contained isolated plasmids (1 µL) or a ligation mix (5-20 µL). The solution was mixed gently by pipetting up and down or flicking the tube, and was incubated on ice for 30 minutes.

The cells were heat-shocked by incubating the solution in a 42°C water bath for 45 seconds, followed by incubation on ice for 3 minutes. 1 mL SOC[3] was added to each tube, and the solutions were incubated at 37°C with 225 rpm shaking for 90 minutes.

The cell solutions were plated on selective LA media in front of a Bunsen burner, utilizing a metal rod sterilized with 70% ethanol. The procedure depended on whether the solution contained isolated plasmids or a ligation mix. For cell solutions containing plasmid DNA, 10-100 µL of the solution was applied to the plate right away. For solutions containing a ligation mix, the tubes were first centrifuged at 6,000 rpm for 2 minutes in a mini centrifuge. Most of the supernatant was discarded, and the pellet was resuspended in the remaining solution. The whole amount (<100 µL) was plated on the selective media. Finally, the plates were inverted and incubated at 37°C overnight.

2.2.5 Inoculation, Plasmid Isolation and Storage of DNA

Plasmid Isolation from *E. coli*

Plasmid isolation is a method developed to extract and purify plasmid DNA from bacterial cells. For *E. coli*, plasmids can be harvested from liquid cultures with the Wizard® *Plus* SV Miniprep DNA Purification System from Promega. The kit provides a simple, fast and reliable method for isolation of plasmids smaller than 20,000 bp in size. The procedure includes concentrating the

[3] $MgCl_2$, $MgSO_4$ and glucose added.

solution, cell lysis, protein degradation by alkaline protease, as well as DNA isolation and purification. The yield may vary depending on a number of factors, including plasmid copy number, cell density in the bacterial culture, and the type of culture medium and bacterial strain utilized to clone the DNA of interest (Promega).

E. coli DH5α cells transformed with plasmids were inoculated in 3-5 mL selective LB media in 13 mL inoculation tubes. This was achieved by picking single colonies with a sterile pipette tip and smearing the cells onto the tube wall right above the liquid. The tubes were incubated overnight at 37°C with 225 rpm shaking, tilted in a 45-degree angle. For new plasmid constructs, the procedure was adapted to include colony PCR, as described in section 2.2.2.

The following day, plasmids were isolated by utilizing the centrifugation protocol of the Wizard® *Plus* SV Minipreps DNA Purification System. In step 1, the tubes were centrifuged at 6,000 rpm in a tabletop centrifuge. The other centrifugation steps were carried out at 13,000 rpm. Apart from this, the procedure was performed exactly as described in the accompanying protocol.

The concentration of plasmid in the solutions was measured with a NanoDrop ND-1000 spectrophotometer, and the solutions subsequently stored at -20°C.

Glycerol Stocks

Freezing of bacteria at the temperature of dry ice (-78.5°C) is a valuable method for preservation. However, this process may damage the cells. By addition of an antifreeze molecule, such as glycerol, the cells can be protected during freezing, thawing, and long term storage in the frozen state (Hollander and Nell, 1954). In this project, glycerol stocks were utilized to store *E. coli* transformed with a variety of plasmids. In this way, fresh cells were always available for growth experiments or for plasmid isolation, without the need for retransformation.

60% glycerol was prepared as described in subsection 2.1.2. 400 µL of an overnight liquid culture was mixed with 800 µL 60% glycerol in a cryogenic tube – performed in front of a Bunsen burner to prevent contamination. The solution was thereafter stored at -80°C.

2.2.6 Sequencing

Molecular cloning is often followed by sequencing to verify that the DNA construct is correct. The constructs to be investigated were sent to GATC Biotech for LightRun sequencing. The method is fast and reliable, and can be utilized for both purified plasmid DNA and purified PCR products. One oligonucleotide primer is required in each reaction to facilitate the sequencing. Each construct was examined by sequencing the fragment of interest from both sides – one sample containing a forward primer, and another containing a reverse primer. The samples were prepared as recommended by the company, as specified in Table 2-19.

Table 2-19. Sample requirements for LightRun sequencing. Each sample should be prepared in a 1.5 mL Eppendorf tube, with a total sample volume of 10 µL. The samples should contain purified DNA, either as a plasmid or a PCR product, and in addition an appropriate primer (Biotech).

Component:	Concentration:	Amount:
DNA	Purified plasmid DNA: 80-100 ng/µL Purified PCR product: 20-80 ng/µL	5µL
Primer	5 µM (5 pmol/µL)	5µL

The sequencing results were analyzed by multiple sequence alignment of the samples compared to a reference DNA sequence. This was achieved by utilizing the Clustal Omega tool (version 1.2.1) from EMBL-EBI. Furthermore, the alignment was downloaded and annotated in Microsoft Word, as presented in Appendix E.

2.2.7 Confirmation of Plasmid Functionality in *Escherichia coli* DH5α host cells

sacB is a counterselection gene, coding for the enzyme levansucrase. Induction of the gene causes production of levan – a polysaccharide toxic to Gram-negative bacteria. This is achieved through the addition of sucrose in the culture media. As described for previously performed experiments, a concentration of 5% sucrose should be sufficient to kill all bacteria harboring the gene (Recorbet et al., 1993, Wrobel et al., Gay et al., 1985, Viola et al., 2014, Pelicic et al., 1996).

Selective LA plates were prepared as described in section 2.1.1, with sucrose concentrations of 0, 5, 10, and 15% (w/v). In addition, selective media without NaCl were prepared with sucrose concentrations of 5% and 10% (w/v).

E. coli DH5α cells were transformed with different gene constructs harboring the *sacB* gene, as explained in subsection **Feil! Fant ikke referansekilden.**. From each set of transformants, six colonies were picked from the LA plates and dissolved in 10 µL Milli-Q water. 1 µL from each cell solution was applied in the same spot in each of the selective plates. The plates were inverted and incubated at 37°C, and monitored over a period of 3 days.

2.2.8 Establishing Selection Conditions for *Synechococcus* sp. PCC 7002

Sucrose Growth Experiment with Wild-Type *Synechococcus*

A growth experiment with wild-type (WT) *Synechococcus* was conducted with varying concentrations of sucrose in the AA+ media, to investigate whether the cells were viable under the conditions utilized for *sacB* testing.

WT *Synechococcus* from a liquid culture was diluted in Milli-Q water and applied on AA+ growth plates with six different concentrations of sucrose (0, 1, 3, 5, 7, and 10 % (w/v) sucrose). The OD of the solution was not measured, but equal amounts of cells were applied to each plate for consistency. 1 µL of solution was applied in each position, with a total of three spots per plate. The plates were covered with parafilm and incubated at 33°C with 73 $\mu Em^{-2}s^{-1}$ for 5 days.

NaCl Growth Experiment with Wild-Type *Synechococcus*

A growth experiment with WT *Synechococcus* was conducted with varying amounts of NaCl in the AA+ media, to investigate whether the cells were viable under these restrictive conditions.

WT *Synechococcus* were grown in 50 mL Falcone tubes at 33°C and 90 $\mu Em^{-2}s^{-1}$, with 300 rpm shaking. The growth media utilized contained 0, 25, 50 and 100% NaCl compared to the value given in Table 2-2. The lids were held loosely in position at the top of the Falcone tubes with 3M™ Micropore™ Surgical Tape, to allow airflow but prevent microorganisms from entering the tubes. The growth was monitored by measuring OD_{730} two times a day over a period of 4 days.

2.2.9 Transformation of *Synechococcus* sp. PCC 7002

Synechococcus sp. PCC 7002 are naturally transformable cells, capable of taking up exogenous dsDNA and incorporating it into its genome by homologous recombination. Background information about transformation of the bacterium and homologous recombination is provided in section 1.2 and 1.4, respectively. The following procedure is adapted from a protocol utilized for transformation of *Synechocystis*, as described by Eaton-Rye (2011).

A starter culture of WT *Synechococcus* was prepared by adding wild-type cells to fresh AA+ liquid media in a cleaned and sterile bubbling flask. 200 µL cells were added to 200 mL growth media, to make a 0.01 % solution. The culture was incubated with bubbling at 33°C and 73 $\mu Em^{-2}s^{-1}$ for 2-3 days, until the OD_{730} exceeded a value of 1.

The starter culture was thereafter utilized to make an overnight culture in a new bubbling flask. When starting the transformation procedure for *Synechococcus*, the OD_{730} of the overnight culture need to be 0.4. If the OD_{730} exceeds this value, the culture cannot be used. It is therefore important to be precise with the setup of the overnight culture. The amount of wildtype cells to be added depends on the OD_{730} of the starter culture used, amount of fresh growth media, planned incubation time, doubling time of the cells, and the end OD_{730} that should be reached. The calculations are demonstrated in equation **(1)** to **(4)** in Appendix F.

Once the OD_{730} reached the expected value of 0.4, two 50 mL Falcone tubes were filled with 50 mL cell solution and centrifuged at 2500 g for 8 minutes at room temperature (22°C). The supernatant was transferred to two new Falcone tubes. About 1 mL was subsequently transferred back and utilized to carefully resuspend the pellets, to reach an OD_{730} of 8.

1 µg DNA was added to 100 µL cell solution in 13 mL inoculation tubes. The tubes were incubated at 30°C with low light (8.3 $\mu Em^{-2}s^{-1}$) for six hours. Towards the end of the incubation time, sterile filters (Nucleopore SN 145318) were placed on non-selective AA+ plates using sterile tweezers. This was performed within the sterile bench, utilizing a Bunsen burner and 70% ethanol to sterilize the tweezers. The whole amount of cells was subsequently transferred to the middle of the filters, and the cell solution spread carefully around with a sterile pipette tip. The plates were left open to

dry in the sterile bench for about 30 minutes, subsequently covered with parafilm, and incubated in the 30°C low light incubator overnight.

After 18-20 hours of incubation[4], the filters were transferred with sterile tweezers to AA+ plates with appropriate antibiotics. The plates were thereafter covered with parafilm, and incubated at 30°C with low light. After two days, the incubation conditions were changed to 33°C and more light (45 $\mu Em^{-2}s^{-1}$). The plates were incubated under the specified conditions until single colonies of transformants started to appear (about two weeks).

Once transformants appeared, single colonies were picked with a sterile pipette tip and transferred to new selective AA+ plates. The procedure was repeated several times followed by colony PCR, to investigate the genetic composition at the neutral integration sites and thereby ensure that complete segregation had occurred.

2.2.10 Confirmation of Counterselection Cassette Functionality in *Synechococcus* sp. PCC 7002

Once transformants were detected harboring the counterselection cassettes, the *sacB* gene needed to be tested once more. The procedure is similar to *sacB* testing in *E. coli*, with a few modifications.

Selective AA+ plates were prepared as described in section 2.1.1, with sucrose concentrations of 0, 1, 3, 5, 7 and 10% (w/v). Culture media were prepared with and without NaCl (0% and 100% compared to the value given in Table 2-2), in case the *sacB* testing was NaCl-sensitive.

For each set of transformants, a small amount of cells was picked from selective AA+ plates with a sterile pipette tip, and dissolved in 1 mL AA+ liquid medium. The solutions were added to 200 mL liquid AA+ medium in sterile bubbling flasks. The same procedure was conducted for WT cells, utilized as control. The cultures were grown at 33°C and 73 $\mu Em^{-2}s^{-1}$ with bubbling, until the OD_{730} exceeded a value of 0.3. Subsequently, the cell solutions were prepared to achieve OD_{730} of

[4] To allow for 3 cell divisions before the filters were transferred to selective plates. Since the cells were grown under less than optimal conditions, the exact doubling time was measured. The cells were dividing approximately every 6.84 hours under the specified conditions.

1, 0.1, and 0.01; The samples were concentrated by transferring 50 mL of each cell solution to sterile Falcone tubes and centrifuging the tubes at 4700 rpm and 22°C for 8 minutes in a tabletop centrifuge. Most of the supernatant was poured off, and the remaining solution (about 3 mL) was utilized to resuspend the cells. The solutions were diluted by adding AA+ liquid medium, to achieve OD_{730} of 1, 0.1 and 0.01 for each set of transformants and the WT.

5 µL from each solution was applied to selective growth plates, with three parallels per solution. The procedure is illustrated in **Feil! Fant ikke referansekilden.**. The plates were covered with parafilm, and grown at 33°C and 73 $\mu Em^{-2}s^{-1}$ for 9 days.

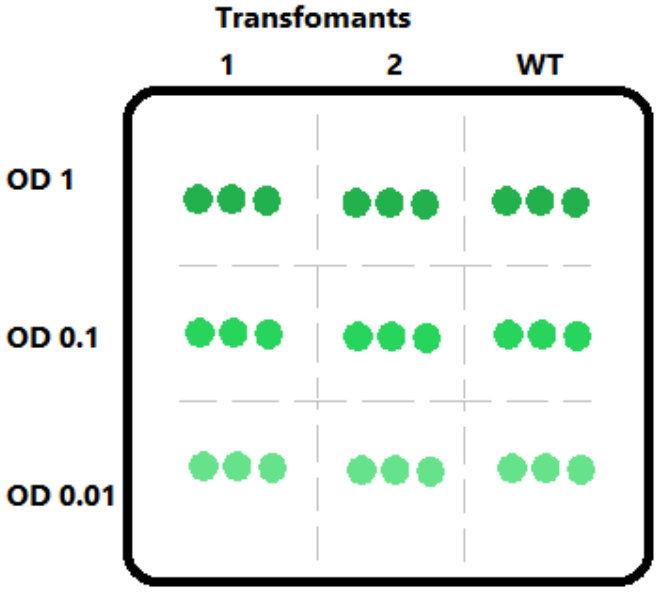

Figure 2-1. *sacB* testing of transformed Synechococcus sp. PCC 7002, including wild-type (WT) *Synechococcus* as a control. 5 µL solution were applied in each position, on selective AA+ growth plates with 0, 1, 3, 5, 7, or 10% (w/v) sucrose.

3 Results and Discussion

This chapter presents the results obtained in this project, followed by an accompanying discussion. Section 3.1 contains experiments performed with *E. coli* DH5α, including preparation of competent cells, molecular cloning to construct counterselection plasmids, and confirmation of plasmid functionality within *E. coli* host cells. Section 3.2 contains experiments performed with *Synechococcus* sp. PCC 7002, including establishment of selection conditions, transformation and verification of segregating, and confirmation of counterselection cassette functionality within *Synechococcus* host cells. Each subsection is initiated by a description of the experimental procedures utilized, followed by the results obtained and a discussion.

3.1 Experiments Performed with *E. coli* DH5α

3.1.1 Transformation Efficiency of Competent E. coli DH5α Cells

To facilitate molecular cloning, competent *E. coli* host cells were needed for amplification and verification of new DNA constructs. For this purpose, competent DH5α cells were prepared according to the descriptions provided in subsection 2.2.1.

The transformation efficiency was tested by transforming the cells with a plasmid of known concentration, grown on selective LA medium. An aliquot of competent cells was transformed with 1 µL pTA16, with a concentration of 111.5 ng/µL. The plasmid contains a kanamycin resistance gene, which could be used to select for transformed cells harboring the plasmid. A dilution series was made as presented in Figure 3-1. A volume of 100 µL of each solution was applied to LA plates with 50 µg/mL kanamycin. In addition, a negative control was included with WT cells, to confirm that only transformed cells were capable of growing in the presence of the antibiotic. The plates were inverted and incubated at 37°C for 17 hours. By counting the number of colonies on each growth plate and taking into account the dilutions and the amount of plasmid DNA in each sample, the transformation efficiency could be estimated. The number of colony forming units (cfu), along with the dilutions utilized, are presented in Table 3-1.

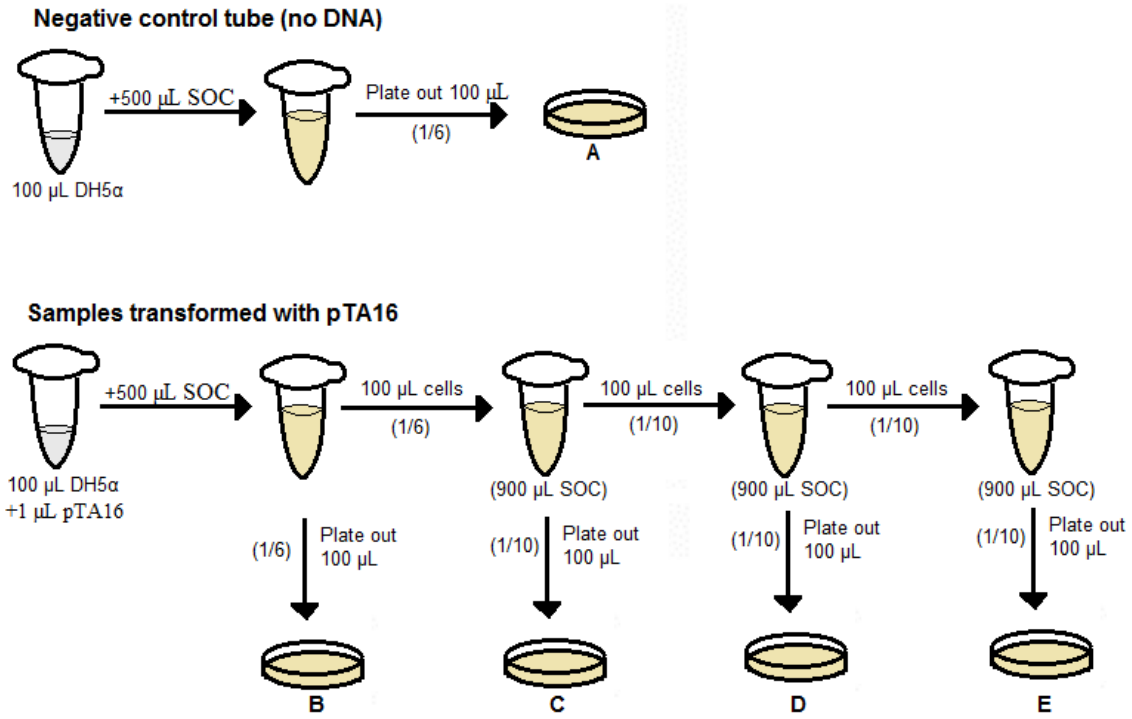

Figure 3-1. Dilution series and plating of competent cells. To calculate the transformation efficiency of competent *E. coli* DH5α cells, an aliquot of cells was transformed with the plasmid pTA16. The plasmid contains a kanamycin resistance gene, which facilitates the growth of cells on LA plates with kanamycin. In addition, a negative control without any DNA added was included, to ensure that only transformants were capable of growing in the presence of the antibiotic. A dilution series was made for the aliquot containing pTA16, to ensure that at least one of the plates had an appropriate amount of colonies to be counted. The growth plates are named A-E, to simplify the presentation of the results. Dilutions are displayed within brackets.

Table 3-1. Number of colony forming units (cfu) of competent *E. coli* DH5α cells grown on selective LA plates. The plates containd 50 µg/mL kanamycin. Plate A represents a negative control, demonstrating that wild-type cells can not grow in the presence of the antibiotic. Plate B-E contains samples with different dilutions of competent cells transformed with the plasmid pTA16, as presented in Figure 3-1. The plasmid contains a kanamycin resistance gene, facilitating the growth of cells on LA plates containing kanamycin.

Plate:	Dilutions:		Colony forming units (cfu):
A	1/6		0
B	1/6		>300
C	1/60	(1/6 x 1/10)	>300
D	1/600	(1/6 x 1/10 x 1/10)	~200
E	1/6000	(1/6 x 1/10 x 1/10 x 1/10)	25

Plate A contained 0 colonies, confirming that wild-type *E. coli* DH5α cells cannot grow in the presence of kanamycin. This suggests that all the cells growing on plate B-E are transformed with the plasmid. As there were too many colonies to count on plate B and C, plate D and E were utilized to calculate the transformation efficiency.

The transformation efficiency was estimated to be 1.2 x 10^6 cfu/µg DNA, as presented in Appendix G. Note, however, that the transformation efficiency varies depending on the size of the plasmid utilized for transformation (Green and Rogers, 2013, Hanahan, 1983). This value is sufficient for routine cloning procedures where the amount of starting DNA is not limiting (ThermoFisher).

3.1.2 Construction of Counterselection Plasmids

The aim of this project is to construct counterselection plasmids in *E. coli* that can be utilized to transform WT *Synechococcus* host cells. As a starting point for the molecular cloning, three cloning vectors provided by the research group were utilized. The vectors were comprised of a synthesized sequence of regions spanning the A0160, A0935 or A2843 neutral integration sites within the genome of *Synechococcus* sp. PCC 7002, inserted into a vector backbone of pUC57-Simple. The neutral sites were present to facilitate homologous recombination within *Synechococcus* host cells. The synthesized fragments included a BioBrick cloning site, containing cut sites for the restriction enzymes EcoRI, XbaI, SpeI, PstI and NotI. The cloning vectors are denoted pA0160, pA0935 and pA2843, and are presented in Appendix C. In silico cloning was conducted to plan all the cloning experiments, facilitated by the DNA program Benchling (available from benchling.com). The program was also used to annotate and visualize all the DNA sequences utilized in this project.

To construct the counterselection plasmids, *sacB* was incorporated into the cloning vectors along with antibiotic resistance genes. The latter provides positive selection of transformants. The genes were incorporated into the synthesized neutral sites, by utilizing the BioBrick cloning site present within the sequence. Two cassettes were constructed, and subsequently incorporated into the three vector backbones. Both cassettes contained the *sacB* gene and a kanamycin resistance gene (hereafter referred to as KanR). In addition, the second cassette also contained a chloramphenicol resistance gene (hereafter referred to as ChlR). The construction strategies and results are presented categorically below. The resulting plasmids are presented at the end of this subsection.

Construction of KanR-sacB Plasmids

Three plasmids were constructed containing *sacB* and the kanamycin resistance gene aminoglycoside 3'-phosphotransferase (*kan*^{r}). The molecular cloning strategy is presented in Figure 3-2.

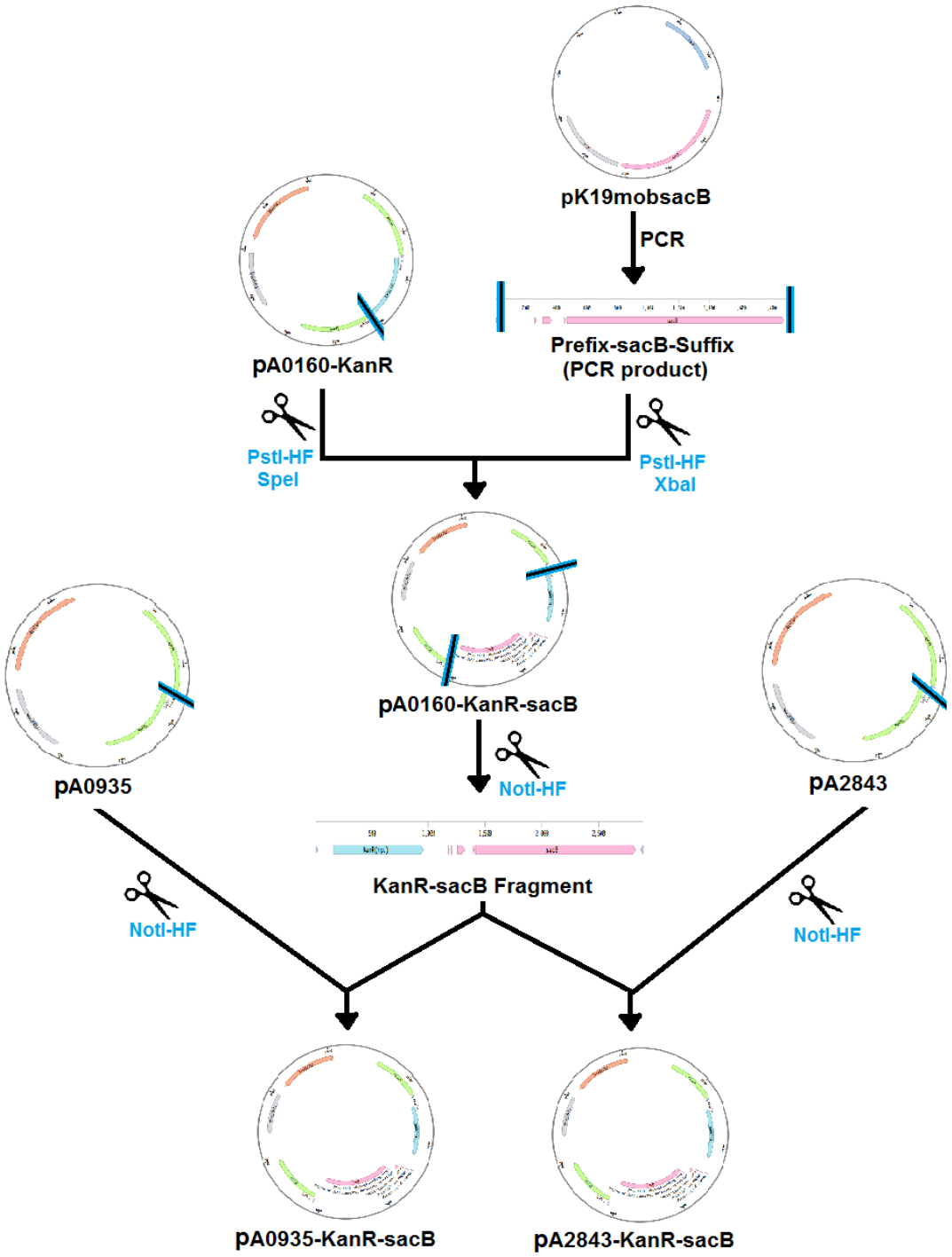

Figure 3-2. Cloning strategy to construct counterselection plasmids containing *sacB* and kanamycin resistance. A0160, A0935 and A2843 (green) refers to DNA fragments identical to three neutral sites within the genome of *Synechococcus* sp. PCC 7002. KanR (blue) refers to the *kan*^{r} gene, providing kanamycin resistance. The *sacB* gene (pink) is a counterselection gene coding for levansucrase. The counterselection cassettes and flanking regions are incorporated into a vector backbone of pUC57-Simple. The *sacB* gene was amplified by PCR from pK19mobsacB and inserted into pA0160-KanR. The KanR-sacB fragment was subsequently inserted into pA0935 and pA2843. Digestion of vectors and DNA fragments are denoted by blue and black lines, and the restriction enzymes utilized indicated in blue. The three KanR-sacB plasmids constructed are presented at the end of this subsection. All plasmid maps are collected from Benchling.

The *sacB* gene was obtained from pK19mobsacB (Appendix C) and incorporated into a cloning vector denoted pA0160-KanR. The vector was provided by the research group, and already contained the *kan*^{r} gene, conferring kanamycin resistance. Amplification of the *sacB* expression cassette was performed by PCR, utilizing Q5 High-Fidelity DNA polymerase. The PCR primers were designed in SECentral and ordered from Sigma-Aldrich, and contained prefix and suffix flanking regions to facilitate BioBrick cloning. Since pK19mobsacB also contains a KanR gene, the PCR product was treated with the restriction enzyme DpnI to make sure that the gene was fragmented. As DpnI solely cuts methylated DNA, only the template DNA was affected. This procedure was necessary to ensure that kanamycin could be utilized as a selectable marker to screen for transformants in a later step.

The PCR product was purified with a PCR purification kit from Qiagen, and the product examined by agarose gel electrophoresis (Figure 3-3). pA0160-KanR (vector) was digested with the restriction enzymes PstI-HF and SpeI, and the PCR product (insert) with PstI-HF and XbaI. The enzymes were subsequently heat-inactivated utilizing a heat block, and the vector treated with CIP to prevent recircularization. Both samples were purified with the PCR purification kit. The insert was ligated into the vector by T4 DNA Ligase, and the enzyme was heat-inactivated. The ligation mix was transformed into competent *E. coli* DH5α host cells by heat-shock transformation, and the transformation solution was plated on LA plates with 50 µg/mL kanamycin.

More than 100 colonies appeared on the selective plates. Six of the colonies were inoculated in LB media with kanamycin (50 µg/mL), and plasmids were harvested the following day with the Miniprep kit from Promega. The constructs were tested by PCR (Figure 3-4), utilizing *Taq* DNA polymerase and a primer pair targeting the BioBrick prefix and suffix. The resulting plasmid is denoted pA0160-KanR-sacB, and is presented in Figure 3-11.

Two bands are visible in Figure 3-3. The most prominent band corresponds to the *sacB* gene, with a expected size of 1.9 kb (see Table D-3 Appendix D). In addition, there is a weak band at about 3 kb. The band is probably be due to unspeciffic binding of the primers.

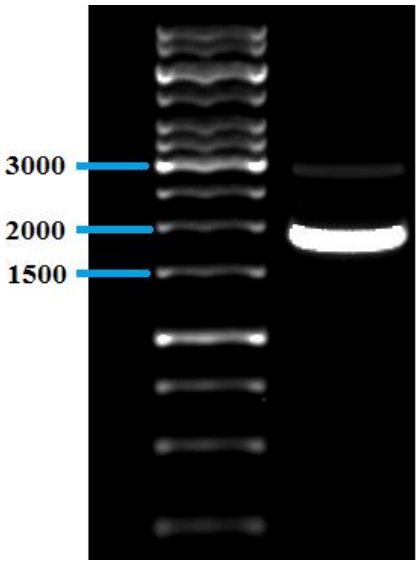

Figure 3-3. Gel image of a PCR sample containing the *sacB* gene. PCR was utilized to amplify *sacB* from pK19mobsacB. The PCR product was analysed on a 0.8% agarose gel containing GelRed, with 100V applied for 35 minutes. The sample was run alongside a GeneRuler 1 kb DNA Ladder. Two bands are visible. The most prominent corresponds to the *sacB* gene, with an expected size of 1.9 kb.

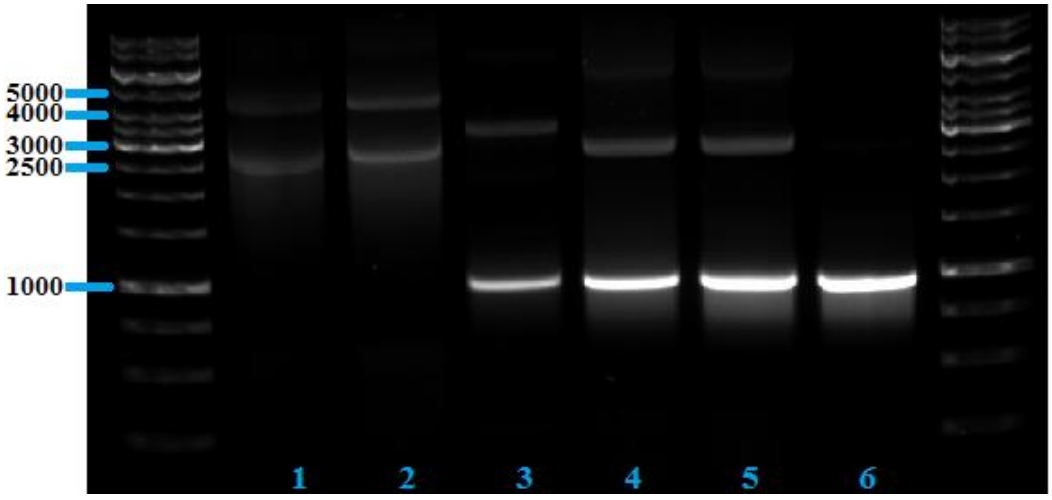

Figure 3-4. Gel image of PCR samples containing pA0160-KanR-sacB. BioBrick primers was utilized to verify the presence of the KanR-sacB fragment within the plasmids. The PCR samples were analysed on a 0.8% agarose gel containing GelRed, with 100V applied for 35 minutes. The samples were run alongside a GeneRuler 1 kb DNA Ladder. Several bands are visible. Sample 1, 2, 4 and 5 seems to contain the correct fragment, with an expected size of 2.9 kb.

There are several bands visible in the samlples in Figure 3-4. Sample 1-5 are PCR samples analyzed for the presence of the KanR-sacB frament. Sample 6 is a control, containing only the KanR fragment, amplified from pA0160-KanR (vector without insert) with an expected size of 1 kb.

The KanR-sacB fragment has an expected size of 2.9 kb, and seems to be present in sample 1, 2, 4 and 5. Sample 1 and 2 also contains a band at about 4.5 kb. This is probably due to unspecific binding of the primers. This hypothesis is strengthened by the presence of a single band at about 3 kb in corresponding PCR samples analyzed at a later stage. The samples were further analyzed by sequencing, as explained in subsection 3.1.3.

Sample 3-5 contain a band at 1 kb, corresponding to the size of the KanR fragment. Furthermore, sample 4 and 5 contains a bands at about 3, 3.5 and 5.5 kb. It seems that the samples utilized for PCR contained both pA0160-KanR (vector) and pA0160-KanR-sacB (vector with insert). Since it is unlikely that a single cell were transformed with more than one plasmid, it is possible that more than one colony were transferred during the inoculation procedure. As the colonies were very small and close together on the plates, it is possible that the pipette tip touched more than one colony. Due to the presence of several products with the wrong size, sample 3-5 were excluded from future use.

The KanR-sacB fragment was also incorporated into the pA0935 and pA2843 plasmids. Both pA0160-KanR-sacB (from sample 1 above) and the two recipient plasmids were digested with NotI-HF. The enzyme has cut sites within the BioBrick prefix and suffix, which is placed right upstream and downstream of the KanR-sacB sequence. After digestion, the enzyme was heat-inactivated utilizing a heat block, and the KanR-sacB fragment (insert) was treated with CIP. This prevents multiple copies from being incorporated into the same vector, and prevents circularization of the fragment. As before, the insert was ligated into the vectors by T4 DNA Ligase, the enzyme heat-inactivated, and the ligation mix transformed into competent *E. coli* DH5α host cells. As only one restriction enzyme was utilized, there are several possible products. However, as ligation can occur only with vector alone or vector plus insert (because of CIP), and since kanamycin is utilized to select for transformants, only cells harboring a plasmid containing both a vector backbone and the KanR-sacB insert are able to grow on the selective medium. Potential transformants were tested

by colony PCR (Figure 3-5), utilizing *Taq* DNA polymerase. The cultures containing host cells with the correct composition of genes were utilized for plasmid isolation the following day. The resulting plasmid are denoted pA0935-KanR-sacB and pA2843-KanR-sacB.

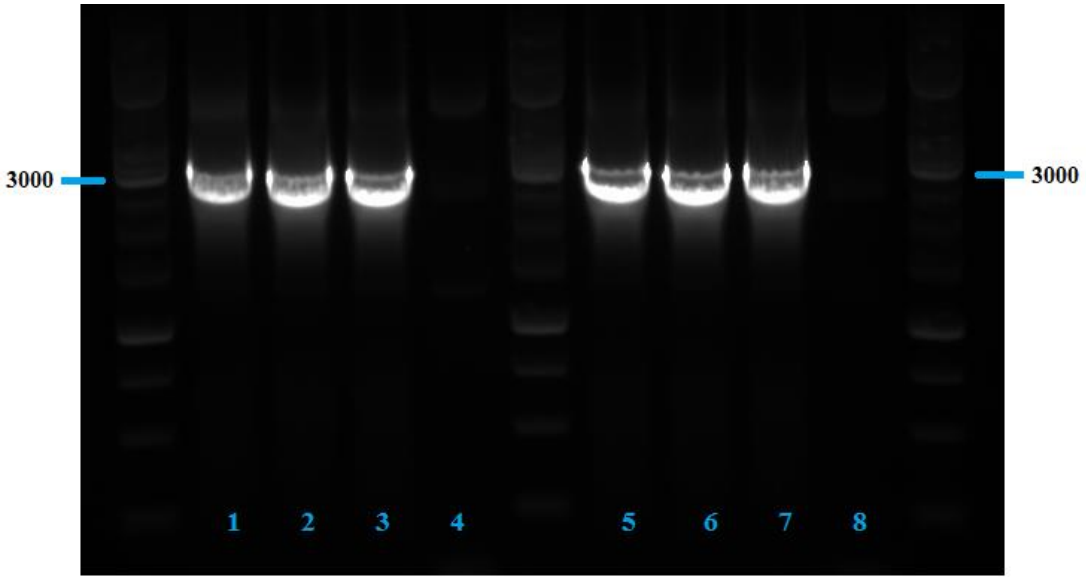

Figure 3-5. Gel image of the PCR products containing pA0935-KanR-sacB and pA2843-KanR-sacB. BioBrick primers was utilized to verify the presence of the KanR-sacB fragment within the plasmids. The PCR samples were analysed on a 0.8% agarose gel containing GelRed, with 100V applied for 40 minutes. The samples were run alongside a GeneRuler 1 kb DNA Ladder. Sample 4 and 8 are controls without the insert. All PCR samples (controls excluded) seems to contain the correct fragment, with an expected size of 2.9 kb.

The gel image in Figure 3-5 verifies the presence of the KanR-sacB fragment within the two plasmid constructs. Sample 1-3 represents pA0935-KanR-sacB, and 5-7 pA2843-KanR-sacB, respectively. All samples contain a band at about 3 kb, which corresponds to the expected size of 2.9 kb. Sample 4 and 8 are controls, containing PCR products of pA0935 and pA2843 without insert.

Incorporation of Chloramphenicol into the KanR-sacB Plasmids

Plasmids were also constructed containing a chloramphenicol resistance gene (ChlR), i.e. the *Tn9cat* gene obtained from the pLitmus28i-ChlR cloning vector (Appendix C). Two construction strategies were utilized, as demonstrated in Figure 3-6 and Figure 3-8. Only the latter was successful, as explained in detail in the text.

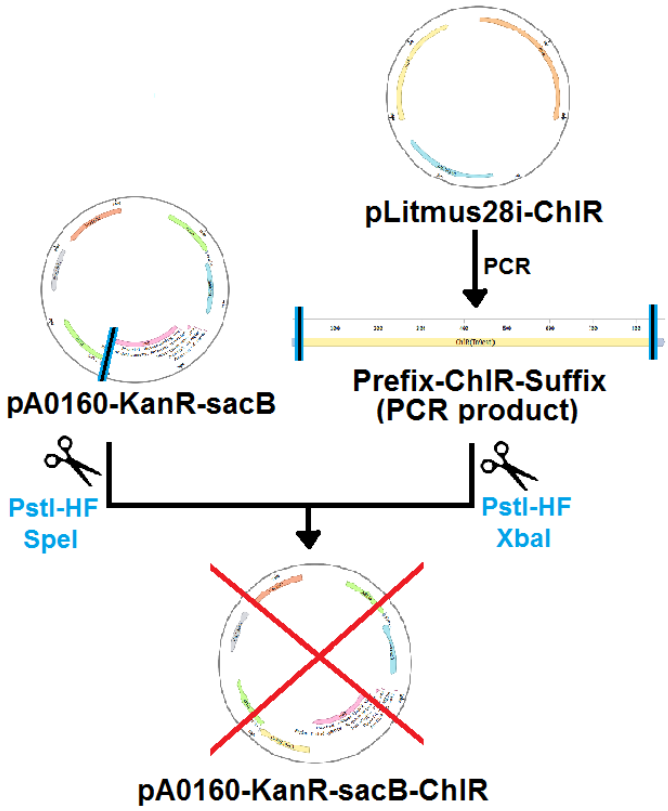

Figure 3-6. Cloning strategy 1 to incorporate chloramphenicol resistance into the KanR-*sacB* counterselection plasmids. A0160, A0935 and A2843 (green) refers to DNA fragments identical to three neutral sites within the genome of *Synechococcus* sp. PCC 7002. KanR (blue) refers to the *kan*^{r} gene, providing kanamycin resistance. ChlR (yellow) refers to the *Tn9cat* gene, providing chloramphenicol resistance. *sacB* (pink) is a counterselection gene coding for levansucrase. The counterselection cassettes and flanking regions are incorporated into a vector backbone of pUC57-Simple. The ChlR gene was amplified by PCR from pLitmus28i-ChlR and inserted into the suffix of pA0160-KanR-sacB. Digestion of the vector and the DNA fragment is denoted by blue and black lines, and the restriction enzymes utilized indicated in blue. Unfortunately, no colonies appeared after transformation. Another construction strategy was utilized to include ChlR, as presented in Figure 3-8. The plasmid maps are collected from Benchling.

Amplification of the *Tn9cat* (ChlR) gene was performed by PCR, utilizing Q5 High-Fidelity DNA polymerase. The primer pair used to copy the gene contained prefix and suffix flanking regions, to facilitate BioBrick cloning. The sequence and characteristics of the primers are provided in Appendix D. The PCR product was purified with the PCR purification kit, and the product examined by agarose gel electrophoresis (Figure 3-7).

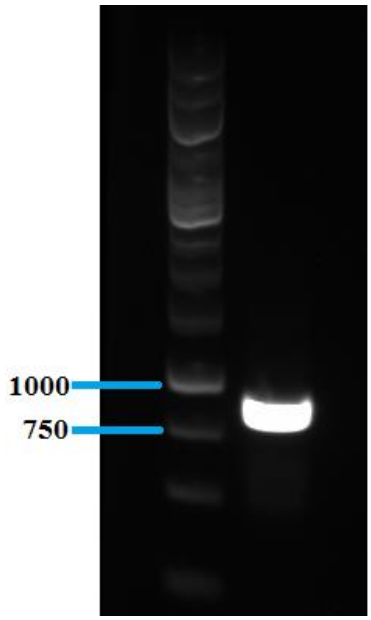

Figure 3-7. Gel image of the PCR product containing the *Tn9cat* (ChlR) gene. PCR was utilized to amplify *Tn9cat* from pLitmus28i-ChlR. The product was analysed on a 0.8% agarose gel containing GelRed, with 100V applied for 30 minutes. The sample was run alongside a GeneRuler 1 kb DNA Ladder. Only one band was present. The fragment corresponds to the *Tn9cat* (ChlR) gene, with an expected size of 0.9 kb.

The gel image in Figure 3-7 verifies that the *Tn9cat* gene has been amplified. The size of the fragment corresponds to the expected size of 0.9 kb.

In cloning strategy 1, the ChlR gene was incorporated into pA0160-KanR-sacB. The gene was incorporated after the *sacB* gene, by utilizing BioBrick cloning sites. pA0160-KanR-sacB (vector) was digested with the restriction enzymes PstI-HF and SpeI, and the PCR product (insert) with PstI-HF and SpeI. The enzymes were subsequently heat-inactivated, and the vector treated with CIP to prevent recircularization. As before, the insert was ligated into the vectors by T4 DNA Ligase and the enzyme subsequently heat-inactivated. The ligation mix was transformed into competent *E. coli* DH5α host cells, and the transformation solution plated on LA medium containing 25 µg/mL kanamycin and 12.5 µg/mL chloramphenicol. Control samples were included with vector without insert, to confer that WT cells or cells containing only vector are not able to grow in the presence of chloramphenicol.

Unfortunately, there was no growth on either of the plates. It is possible that the transformation procedure was not applied successfully, resulting in the cells not taking up the DNA or alternatively that the cells were not viable when applied to the plates. However, this is highly unlikely. As other members of the research group have obtained similar results when utilizing the ChlR gene in molecular cloning, the problem is probably connected to the usage of the gene. It is possible that the gene has a weak promoter, and is not sufficiently expressed when placed downstream of genes with stronger promoters. If the ChlR gene and *sacB* gene have different orientations, the *sacB* mRNA might contain a region identical to the ChlR antisense DNA. If this is the case, a double-stranded RNA molecule could have formed as a result of *sacB* mRNA hybridizing to ChlR mRNA during transcription. This might have terminated transcription of the ChlR, and the gene product might not have been produced in large enough quantities to confer resistance to the antibiotic. To test the hypothesis, another construction strategy was developed, involving incorporating the ChlR gene upstream of the KanR-sacB fragment. The strategy is presented in Figure 3-8.

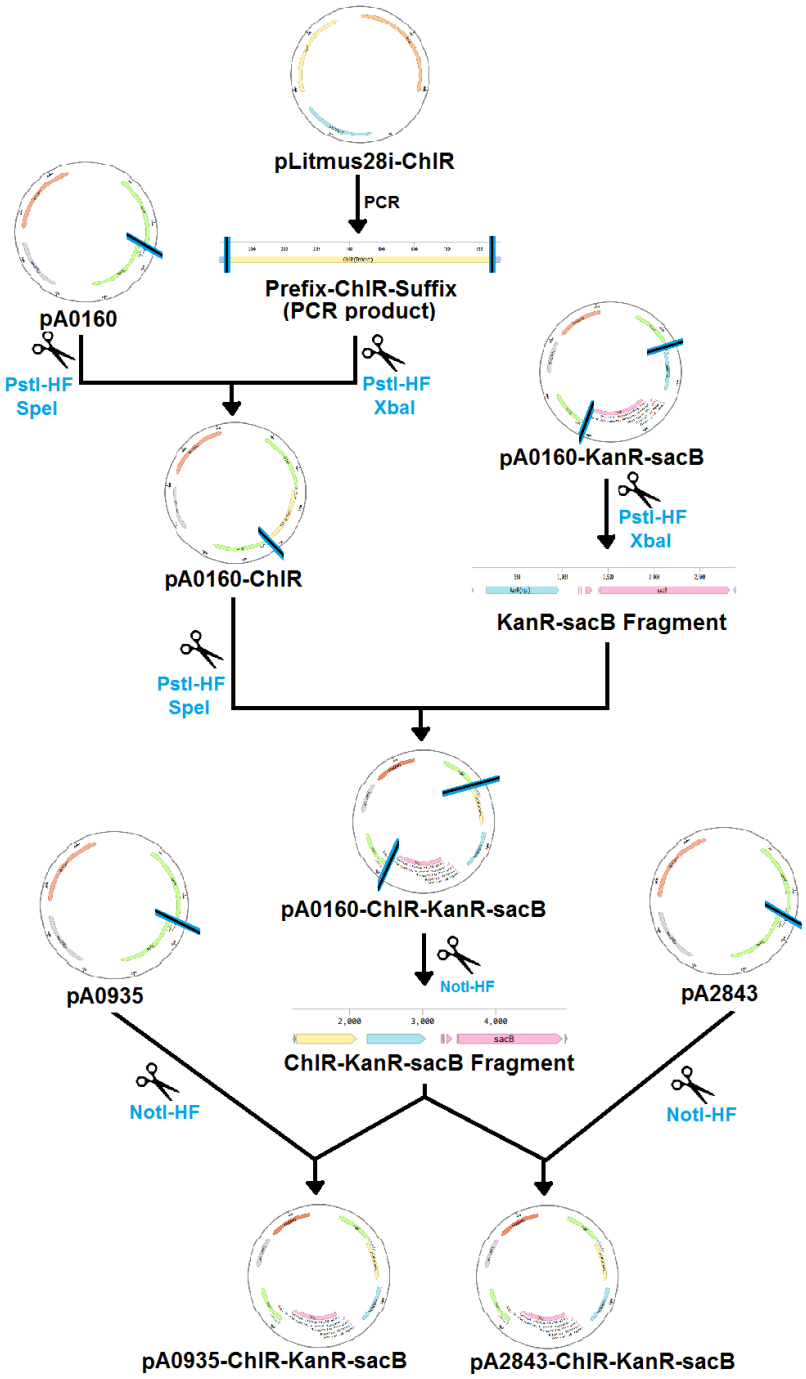

Figure 3-8. Alternative cloning strategy (Strategy 2) to incorporate chloramphenicol resistance into the KanR-sacB counterselection plasmids. A0160, A0935 and A2843 (green) refers to DNA fragments identical to three neutral sites within the genome of *Synechococcus* sp. PCC 7002. KanR (blue) refers to the *kan*^{r} gene, providing kanamycin resistance. ChlR (yellow) refers to the *Tn9cat* gene, providing chloramphenicol resistance. *sacB* (pink) is a counterselection gene coding for levansucrase. The counterselection cassettes and flanking regions are incorporated into a vector backbone of pUC57-Simple. The ChlR gene was amplified by PCR from pLitmus28i-ChlR and inserted into pA0160. The ChlR-KanR-sacB fragment was subsequently inserted into pA0935 and pA2843. Digestion of vectors and DNA fragments are denoted by blue and black lines, and the restriction enzymes utilized indicated in blue. The three ChlR-KanR-sacB plasmids constructed are presented in at the end of this subsection. All plasmid maps are collected from Benchling.

In strategy 2, the ChlR gene was amplified by PCR as described previously, and incorporated into pA0160. As before, pA0160 (vector) was digested with the PstI-HF and SpeI, and the PCR product (insert) with PstI-HF and XbaI. The vector was treated with CIP, and the insert ligated into the vector by T4 DNA Ligase. The enzyme was heat-inactivated, and the ligation mix transformed into competent *E. coli* DH5α host cells. The solution was applied to LA plates containing 25 µg/mL chloramphenicol. The transformants were tested by colony PCR, and cultures containing host cells that indicated the inclusion of the constructed plasmid with insert were utilized for plasmid isolation the following day. The resulting plasmid are denoted pA0160-ChlR.

Furthermore, the KanR-sacB fragment were cut out of pA0160-KanR-sacB, and incorporated into pA0160-ChlR. Once again, BioBrick restriction enzymes were utilized. pA0160-ChlR (vector) was digested with the PstI-HF and SpeI, and KanR-sacB (insert) was excised from pA0160-KanR-sacB by PstI-HF and XbaI. The vector was treated with CIP, and the insert ligated into the vector by T4 DNA Ligase. The enzyme was heat-inactivated, and the ligation mix transformed into competent *E. coli* DH5α host cells. The solution was applied to LA plates containing 25 µg/mL kanamycin and 12.5 µg/mL chloramphenicol. The transformants were tested by colony PCR (Figure 3-9), utilizing *Taq* DNA polymerase. Cultures containing host cells that indicated the inclusion of the constructed plasmid with insert were utilized for plasmid isolation the following day. The resulting plasmid are denoted pA0160-ChlR-KanR-sacB.

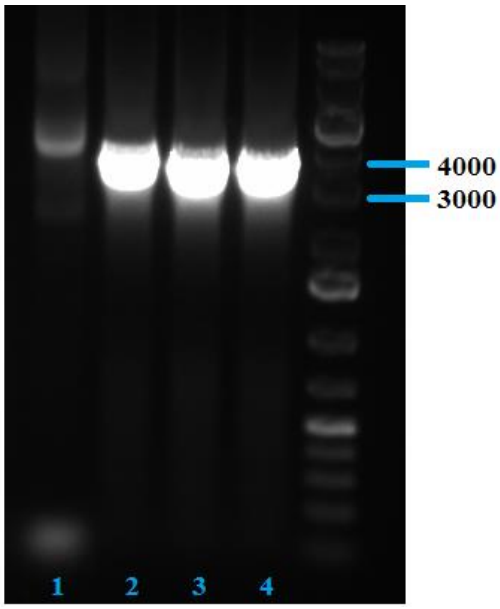

Figure 3-9. Gel image of the PCR products containing pA0160-ChlR-KanR-sacB. BioBrick primers was utilized to verify the presence of the ChlR-KanR-sacB fragment within the plasmids. The PCR samples were analysed on a 0.8% agarose gel containing GelRed, with 100V applied for 30 minutes. The samples were run alongside a GeneRuler 1 kb Plus DNA Ladder. Sample 1 is a control without of pA0160. The remaining samples seems to contain the aforementioned fragment, with an expected size of 3.7 kb.

The gel image in Figure 3-9 verifies the presence of the ChlR-KanR-sacB fragment within the construct. Sample 2-4 contain a fragment of 3-4 kb, which corresponds to the ChlR-KanR-sacB fragment with an expected size of 3.7 kb. Sample 1 is a control, containing the PCR product of pA0160. A few bands are present within the control sample, most likely due to unspecific binding of the primers.

The ChlR-KanR-sacB fragment was also incorporated into pA0935 and pA2843. Both pA0160-ChlR-KanR-sacB and the two recipient plasmids were digested with NotI-HF. The enzyme was heat-inactivated, and the ChlR-KanR-sacB fragment (insert) was treated with CIP. This prevents multiple copies from being incorporated into the same vector, and prevents circularization of the fragment. As before, the insert was ligated into the vectors by T4 DNA Ligase and the enzyme subsequently heat-inactivated. The ligation mix was transformed into competent *E. coli* DH5α host cells, and plated on LA plates containing 25 µg/mL kanamycin and 12.5 µg/mL chloramphenicol. The transformants were tested by colony PCR (Figure 3-10), utilizing *Taq* DNA polymerase. Cultures containing host cells that indicated the inclusion of the constructed plasmid with insert

were utilized for plasmid isolation the following day. The resulting plasmid are denoted pA0935-ChlR-KanR-sacB and pA2843-ChlR-KanR-sacB.

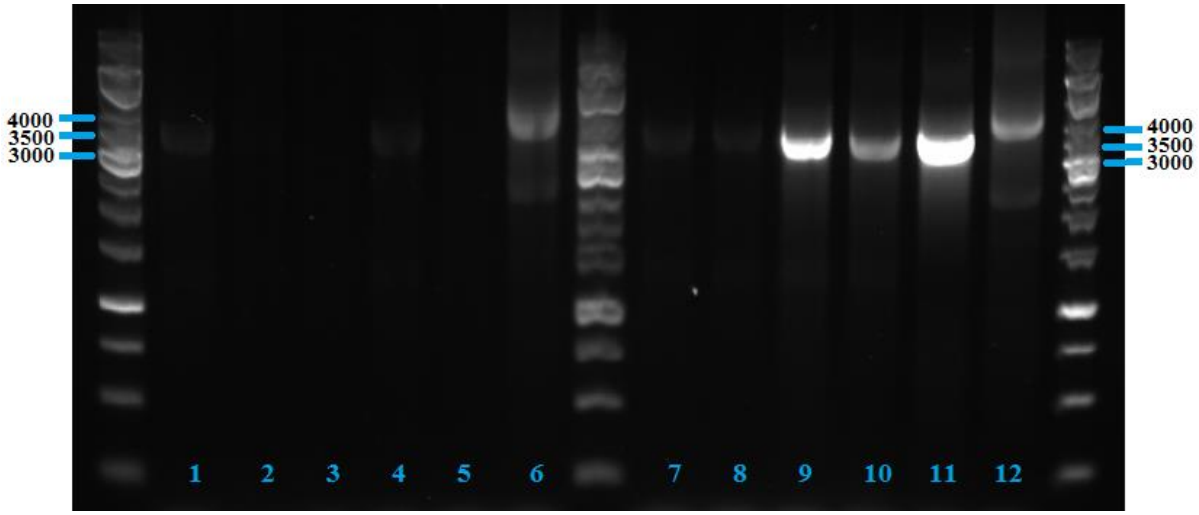

Figure 3-10. Gel image of the PCR product containing pA0935-ChlR-KanR-sacB and pA2843-ChlR-KanR-sacB. BioBrick primers was utilized to verify the presence of the Chl-KanR-sacB fragment within the plasmids. The PCR samples were analysed on a 0.8% agarose gel containing GelRed, with 100V applied for 30 minutes. The samples were run alongside a GeneRuler 1 kb DNA Ladder. Sample 6 and 12 are controls without insert. Sample 1, 4 and 7-11 seems to contain the correct fragment, with an expected size of 3.7 kb. This verifies that the fragment has been incorporated into pA0935 and pA2843.

The gel image in Figure 3-10 verifies the presence of the ChlR-KanR-sacB fragment within the two plasmid constructs. Sample 1-5 represents pA0935-ChlR-KanR-sacB, and 7-11 pA2843-ChlR-KanR-sacB, respectively. Sample 1, 4 and 7-11 seems to contain the correct fragment, with an expected size of 3.7 kb. Sample 6 and 12 are controls, containing PCR products of pA0935 and pA2843 without insert.

Counterselection Plasmids Created

To sum up, six counterselection plasmids were constructed – three containing the KanR-sacB cassette, and three containing the ChlR-KanR-sacB cassette. The two cassettes were incorporated into the three cloning vectors pA0160, pA0935 and pA2843, which contained synthesized regions of the aforementioned neutral sites. These regions are essential to facilitate homologous recombination within *Synechococcus* sp. PCC 7002 host cells. The constructs are visualized in Figure 3-11. The plasmid maps were constructed in the DNA program Benchling.

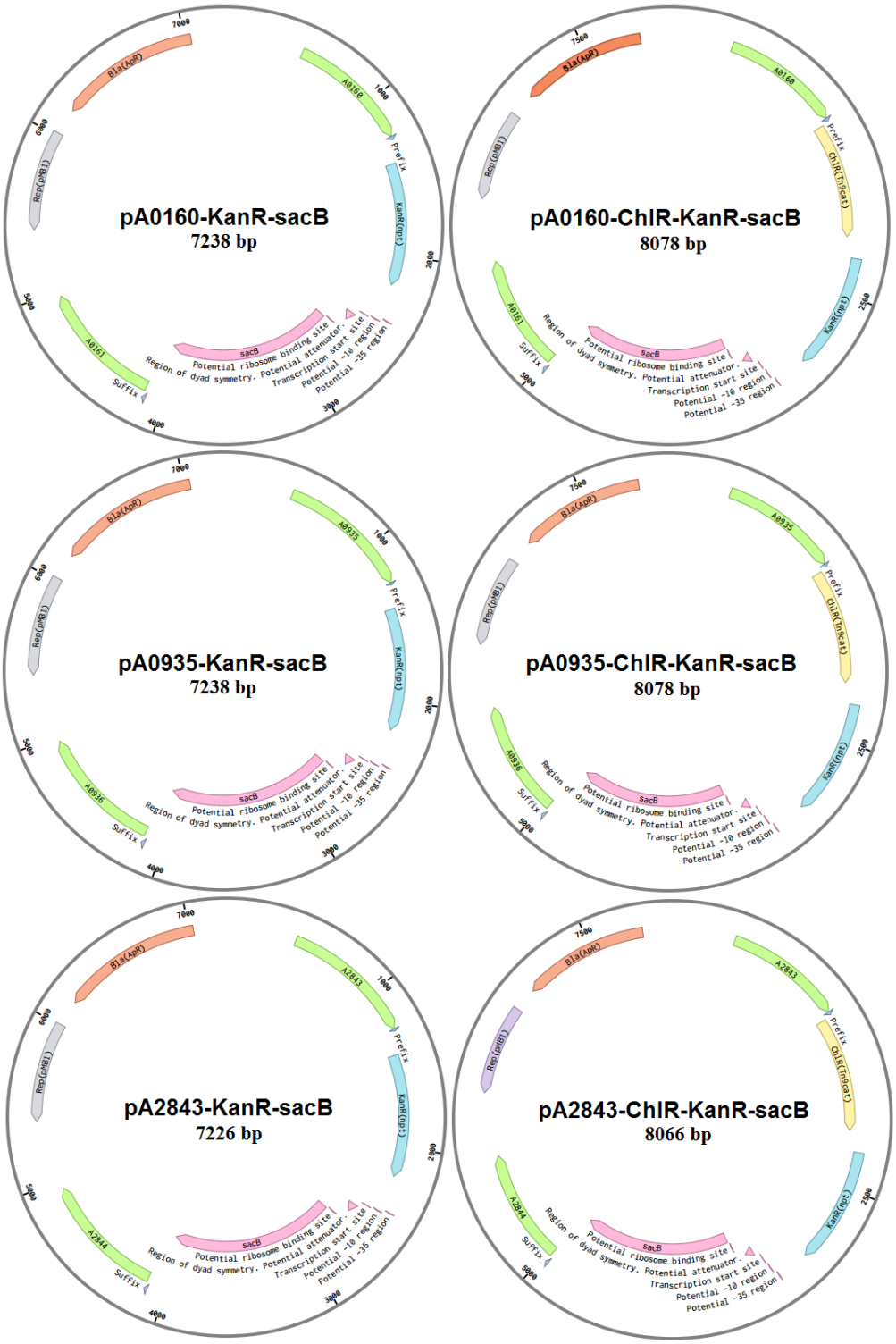

Figure 3-11. Plasmid maps of the counterselection plasmids created in this project. A0160, A0935 and A2843 (green) refers to DNA fragments identical to three neutral sites within the genome of *Synechococcus* sp. PCC 7002. KanR (blue) refers to the *kan*^{r} gene, providing kanamycin resistance. ChlR (yellow) refers to the *Tn9cat* gene, providing chloramphenicol resistance. *sacB* (pink) is a counterselection gene coding for levansucrase. The counterselection cassettes are incorporated into a vector backbone of pUC57-Simple. Two of the structures in the backbone are annotated, i.e. the *bla* gene (red) providing ampicillin resistance, and the origin of replication from the pMB1 plasmid (grey). The plasmid maps were constructed in the DNA program Benchling.

3.1.3 Sequencing Results of pA0160-KanR-sacB

Sample 1 and 2 from Figure 3-4 were sent to GATC Biotech for LightRun sequencing. Each construct was examined by sequencing the fragment of interest from both sides – one sample containing a prefix forward primer targeting the KanR gene, and another containing a suffix reverse primer targeting the end of the *sacB* gene. The samples were prepared as recommended by the company, as described in Table 2-18.

The sequencing results were analyzed by multiple sequence alignment of the samples compared to a reference DNA sequence. An annotated sequence of pA0160-KanR-sacB was utilized as a reference, produced by in silico cloning in Benchling. A multiple sequence alignment was performed by the online tool Clustal Omega (version 1.2.1), provided by EMBL-EBI (available from http://www.ebi.ac.uk/Tools/msa/clustalo/). Furthermore, the alignment was downloaded and annotated in Microsoft Word. The sequence of pA0160-KanR-sacB from Benchling was utilized to localize the KanR and *sacB* genes within the downloaded sequences.

Both samples contained the KanR-sacB fragment, as expected. The sequences were nearly identical to the theoretical sequence, as presented for sample 1 in Appendix E. The forward primer covered the entire KanR gene, verifying that the gene is intact. The reverse primer only covered the last three quarters of the *sacB* coding sequence. To cover the entire sequence of the cassette, several more primers should be utilized. However, the sequence covered by the primer seems to be intact. This is supported by the fact that PCR products contains fragments of the correct size. As both genes appear to be incorporated correctly into the construct, only one experiment in *E. coli* remains: verification of plasmid functionality within host cells.

3.1.4 Confirmation of Plasmid Functionality within *E. coli* Host Cells, and Establishment of Selection Conditions

Before the counterselection plasmids could be utilized in transformation of *Synechococcus* sp. PCC 7002, the functionality needed to be tested. This was achieved by growing *E. coli* host cells harboring the plasmids on selective media. Two gradients were utilized: a sucrose gradient to test the *sacB* gene, and a NaCl gradient in case the testing was NaCl-sensitive. Since several studies involving *sacB* reports NaCl-sensitivity during testing of the counterselection gene (Blomfield et

al., 1991, Recorbet et al., 1993), it was investigated whether this was also the case with the gene obtained from pK19mobsacB. In addition, the LA plates contained antibiotics, to test the resistance genes within the constructs. This subsection is divided in two, according to the counterselection cassettes present within the three cloning vectors. Plasmids containing the KanR-sacB cassette will be examined first, followed by a section about plasmids containing the ChlR-KanR-sacB cassette.

Testing of KanR-sacB plasmids

Testing of counterselection cassette functionality was conducted by growing transformed *E. coli* host cells on LA media containing antibiotics and various concentrations of sucrose. As explained in subsection 2.2.7, single colonies of transformants were picked from selective LA plates and dissolved in 10 µL MilliQ-water. A volume of 1 µL from each cell solution was applied in the same spot on several agar plates containing kanamycin (50 µg/mL) and sucrose (0-15% (w/v)).

The experiment was initially conducted utilizing plates containing NaCl, where cells were growing on all plates. After several failed attempts at verifying *sacB* functionality in both the counterselection plasmids and pK19mobsacB, it was believed that the testing was indeed NaCl-sensitive. To test the hypothesis, cells harboring the plasmids were grown on selective media containing kanamycin and sucrose, but without any NaCl added. The new experimental setup was successful, as counterselection cassette functionality was observed.

The results of the experiments conducted with and without NaCl are presented in Figure 3-12 and Figure 3-13, respectively. Plasmid functionality and other observations are discussed in detail in the text.

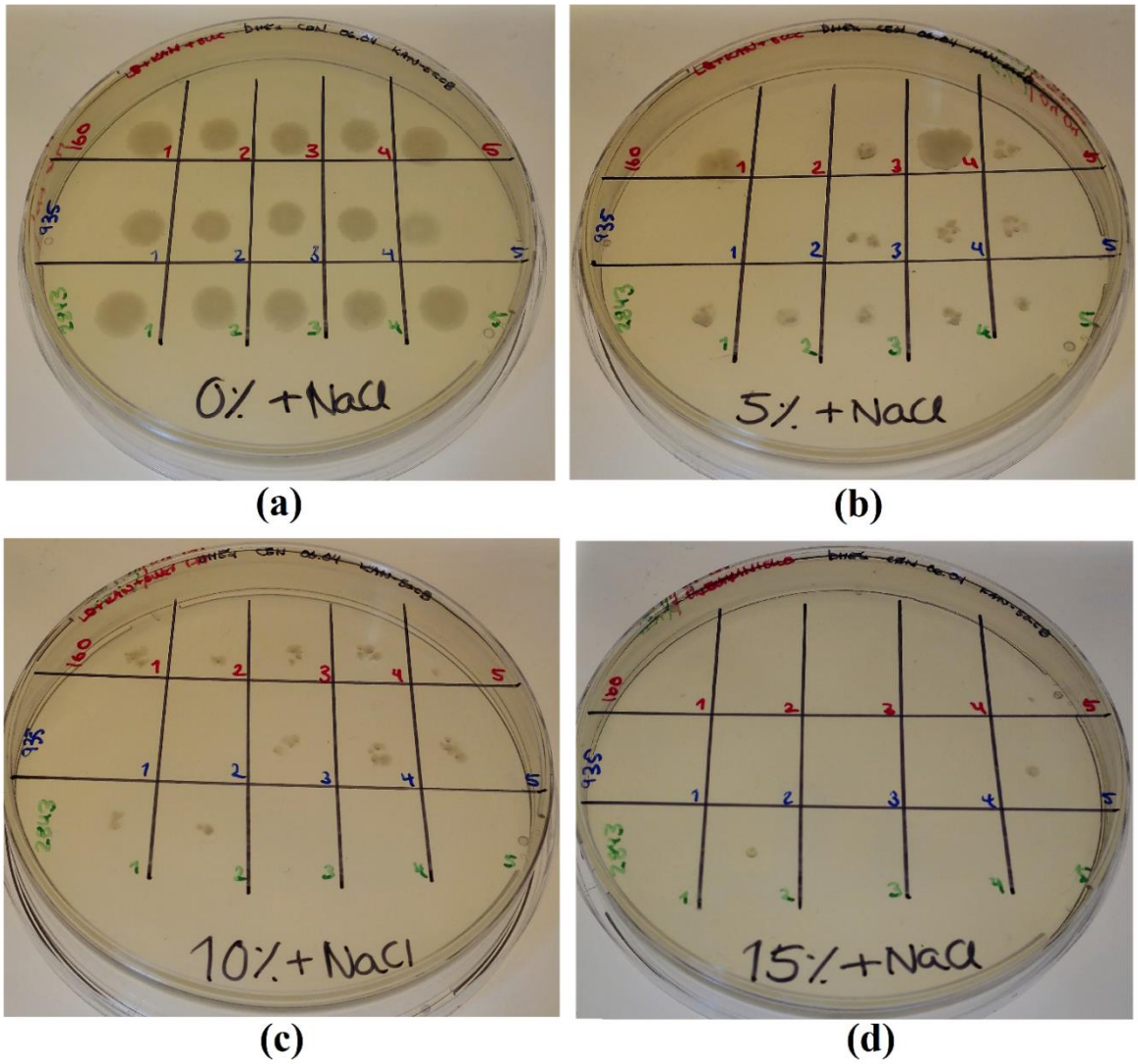

Figure 3-12. Testing of KanR-sacB counterselection plasmids on growth plates containing NaCl. The testing was conducted on LA plates containing kanamycin (50 µg/mL), sucrose (0-15% (w/v)) and NaCl. Growth is visible on all plates, with growth rate negatively correlated to sucrose concentration. As no growth were expected on plates containing sucrose concentration of 5% or higher, a stringent selection was not achieved.

From the literature, it is apparent that a concentration of 5% sucrose should be sufficient to kill Gram-negative bacteria harboring the *sacB* gene (Recorbet et al., 1993, Wrobel et al., Gay et al., 1985, Viola et al., 2014, Pelicic et al., 1996). As presented in Figure 3-12, this is clearly not the case in this specific instance. Cells are growing on all the plates, with growth rate negatively correlated to sucrose concentration. Although the growth experiments on plates containing LA (with NaCl) indicate a sensitivity to growth on sucrose, more stringent selection conditions are required to use sucrose as an efficient counterselection agent.

The same cell solutions were applied to LA plates without NaCl, to test whether or not sodium chloride was affecting the outcome of the experiment.

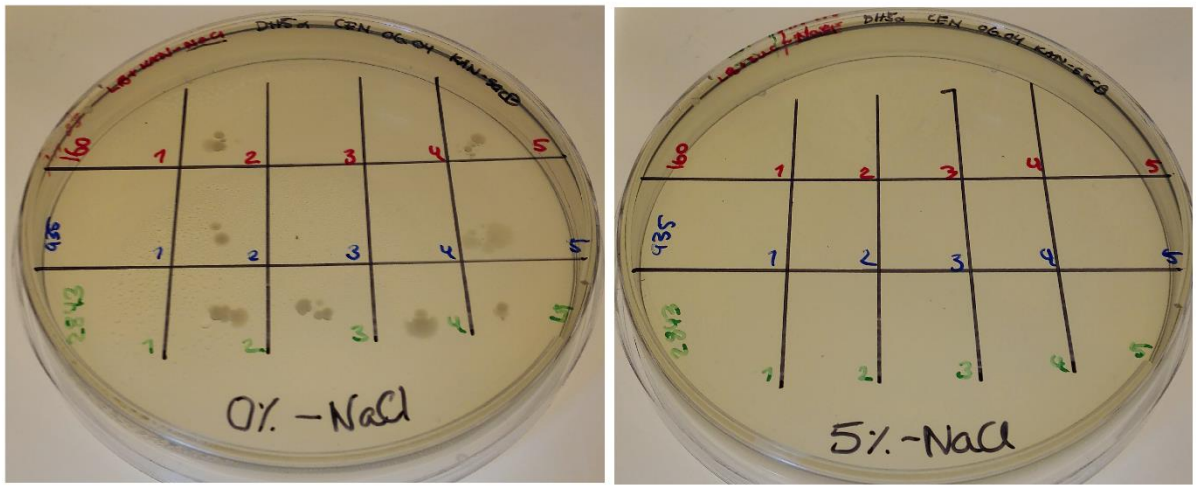

Figure 3-13. Testing of KanR-sacB counterselection plasmids on growth plates without NaCl. The testing was conducted on LA plates containing kanamycin (50 µg/mL), and sucrose (0 and 5% (w/v)). The cells were grown on media without any NaCl added, as the presence of NaCl may interfere with counterselection. Since growth is observed on the plate with 0% sucrose, but not on the plate with a sucrose concentration of 5%, suitable counterselection conditions were established for *E. coli* carrying the KanR-sacB plasmids.

The *sacB* gene conferred counterselection functionality when the cells were grown on plates without NaCl. As presented in Figure 3-13, there is visible growth on the 0% sucrose plate (control), but no growth on the 5% (w/v) sucrose plate. Since cells harboring the three KanR-sacB plasmids are capable of growing in the presence of kanamycin but not in the presence of 5% sucrose, the KanR and *sacB* genes seems to be expressed, conferring counterselection functionality. This verifies the functionality of the counterselection plasmids within *E. coli* host cells.

Note, however, that the *sacB*-mediated counterselection is NaCl-sensitive. It is hard to say what caused this sensitivity. Blomfield et al. observed the same phenomenon in *E. coli* carrying the *sacB* gene, and suggested that NaCl might alter *sacB* expression by influencing DNA supercoiling (Blomfield et al., 1991). Furthermore, if the *sacB* sequence differ from the original sequence published by Shimotsu and Henner (Shimotsu and Henner, 1986), it is possible that the levansucrase have a small change in composition, resulting in formation of complexes between the

enzyme and NaCl. A bioinformatic analysis of the *sacB* sequence should be conducted, to examine the sequence at the DNA level. Nevertheless, the constructed plasmids can be utilized to achieve efficient counterselection when NaCl is excluded from the growth medium.

Furthermore, it was observed that cells harboring the *sacB*-constructs was growing slower than wild-type cells, and slower still on media without NaCl. As no colonies appeared after overnight-incubation, the plates were left in the incubator for three days before they were photographed. The slow growth rate might be due to the presence of the *sacB* gene. According to Shimotsu and Henner, transcription from the *sacB* promoter is constitutive. However, only in the presence of sucrose do transcription extend past a termination structure in the sequence (Shimotsu and Henner, 1986). Although the amount of transcription in the absence of sucrose is too low to quantify, it is possible that a small amount of levansucrase is produced, halting the growth of the cells.

***sacB* testing of ChlR-KanR-sacB plasmids**

Testing of *sacB*-mediated counterselection conferred by the ChlR-KanR-sacB plasmids was conducted in much the same way as for the KanR-sacB plasmids. However, the addition of antibiotics in the growth plates were altered, to confirm that the cells were capable of growing in the presence of both kanamycin (25 µg/mL) and chloramphenicol (12.5 µg/mL). As before, cells harboring the counterselection plasmids were grown on media with and without NaCl (0% and 100% (w/v)), containing antibiotics and different concentration of sucrose. The results are presented in Figure 3-14 and Figure 3-15.

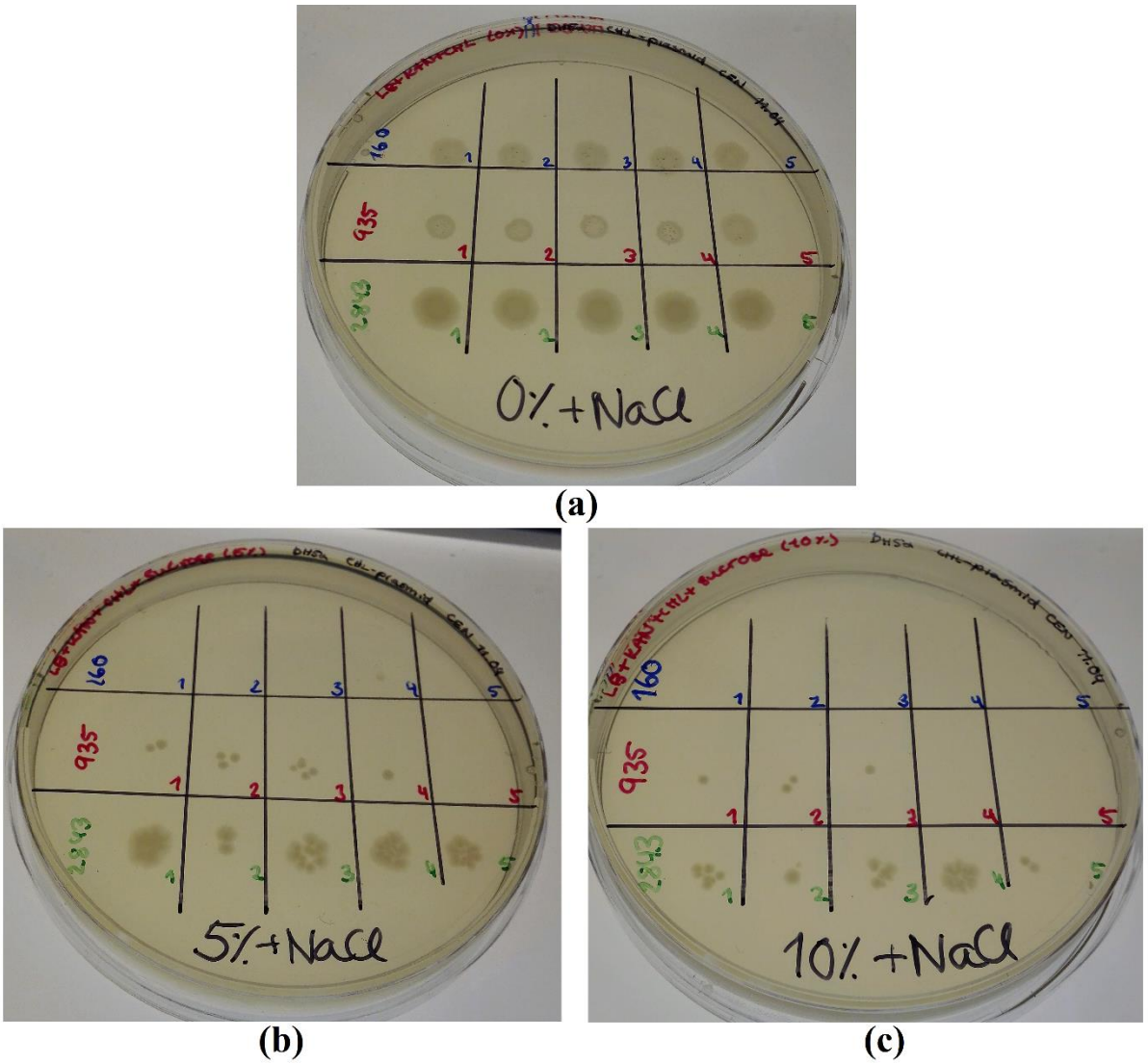

Figure 3-14. Testing of ChlR-KanR-sacB counterselection plasmids on growth plates containing NaCl. The testing was conducted on LA plates containing kanamycin (25 µg/mL), chloramphenicol (12.5 µg/mL), sucrose (0-15% (w/v)) and NaCl. Growth is visible on all plates, with growth rate negatively correlated to sucrose concentration. As no growth was expected on plates containing sucrose concentration of 5% or higher, a stringent selection was not achieved.

Cells harboring the ChlR-KanR-sacB plasmids are growing on plates with NaCl and sucrose concentration of 5% and above. Growth is visible on all the plates, with growth rate negatively correlated to sucrose concentration. This concurs with the results obtained for cells harboring the KanR-sacB plasmids, when grown on plates containing NaCl.

It was observed that cells harboring pA0160-ChlR-KanR-sacB were more affected by sucrose then cells harboring pA0935-ChlR-KanR-sacB and pA2843-ChlR-KanR-sacB. However, this might be due to a smaller amount of cells present within the cell solutions utilized for the testing. To check whether exclusion of NaCl can improve counterselection, the cell solutions were also applied to LA plates without NaCl, as presented in Figure 3-15.

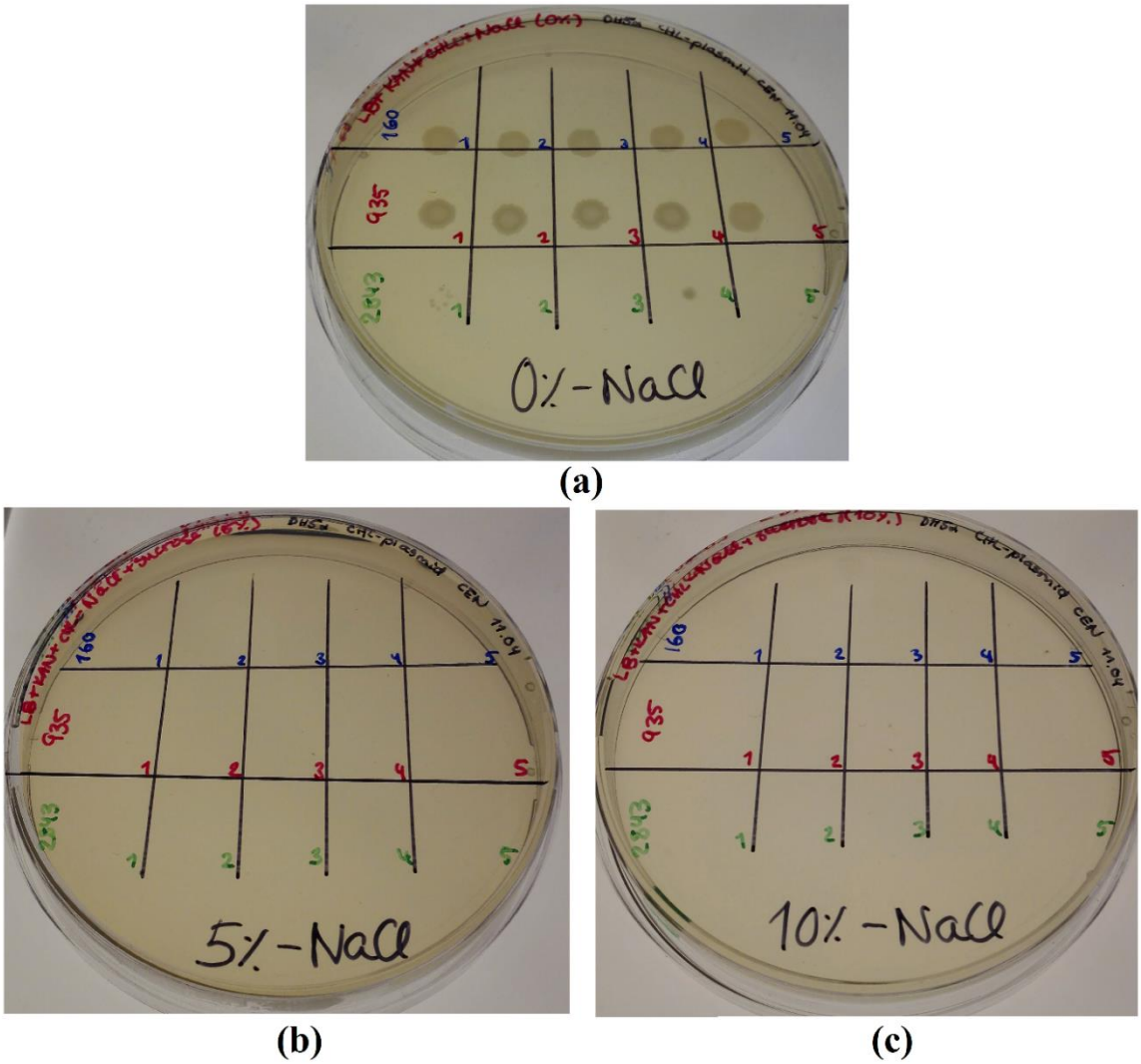

Figure 3-15. Testing of ChlR-KanR-sacB counterselection plasmids on growth plates without NaCl. The testing was conducted on LA plates containing kanamycin (25 µg/mL), chloramphenicol (12.5 µg/mL), and sucrose (0 and 5% (w/v)). The cells were grown on media without any NaCl added, as the presence of NaCl may interfere with counterselection. Since growth is observed on the plate with 0% sucrose, but not on the plate with a sucrose concentration of 5%, the ChlR, KanR and sacB genes seems to be expressed, thereby conferring counterselectivity. This verifies the functionality of the counterselection plasmids within *E. coli* host cells.

The results obtained in Figure 3-15 are in accordance with the results obtained for the KanR-sacB plasmids grown on plates without NaCl. Cells harboring the ChlR-KanR-sacB plasmids are growing on the 0% plate, but no growth is observed on plates with sucrose concentration of 5% or higher. This implies that the ChlR, KanR and *sacB* gene are present and expressed. Stringent counterselectivity by ChlR-KanR-sacB plasmids within *E. coli* host cells can be achieved by excluding NaCl from the growth medium.

It was observed that cells harboring pA2843-ChlR-KanR-sacB were present at much smaller quantities than pA0160-ChlR-KanR-sacB and pA0935-ChlR-KanR-sacB. It is possible that there was a smaller amount of cells present within the cell solutions utilized for the testing. This is unlikely, as the same samples contained the largest amount of growth of the three plasmids when applied to medium containing 0% sucrose and NaCl added (Figure 3-14). However, the latter could be caused by different antibiotics present in the growth media during testing.

3.2 Experiments Performed with *Synechococcus* sp. PCC 7002

3.2.1 Transformation and Verification of Segregation

WT *Synechococcus* sp. PCC 7002 were transformed with the counterselection plasmids created by molecular cloning, as presented in the previous section. As *Synechococcus* takes up linear dsDNA, the counterselection cassettes were amplified by PCR, utilizing Q5 High-Fidelity DNA polymerase. The amplicons also contained flanking regions of the three neutral sites, to facilitate homologous recombination within *Synechococcus* host cells. The transformation procedure is explained in detail in subsection 2.2.9. Once transformants appeared on selection plates, the cells were tested by colony PCR to verify that segregation had occurred. Amplification of DNA, transformation, and verification of segregation is presented categorically below, dependent on which counterselection plasmid was utilized in the transformation. Important primer characteristics, annealing temperatures, and expected sizes of PCR products are presented in Appendix D.

Transformation conducted with pA0160-KanR-sacB

Amplification of KanR-sacB and flanking regions

The KanR-sacB cassette with A0160 flanking regions was amplified from pA0160-KanR-sacB, by utilizing the A0160-400 and A0160-800 PCR primer pairs. Gel images of the amplicons are presented in Figure 3-16and Figure 3-18.

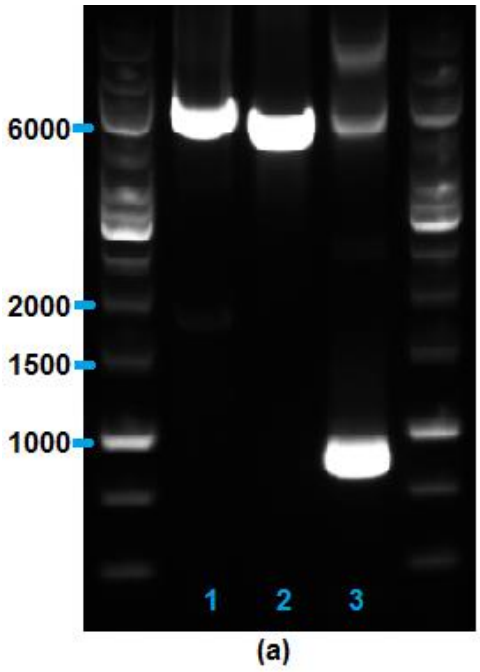

Figure 3-16. Gel image of amplicons containing the KanR-sacB cassette with 400 bp A0160 flanking regions. The cassette and flanking regions were amplified by A0160-400 primers, from pA0160-KanR-sacB (1) and pA0160-ChlR-KanR-sacB (2), respectively. A control sample of pA0160 was also included (3). The PCR samples were analyzed on a 0.8% agarose gel containing GelRed, with 100V applied for 40 minutes. The samples were run alongside a GeneRuler 1 kb DNA ladder. As evident in sample 1, no bands were detected with the expected size of 3.7 kb. The most prominent band has a size of about 6 kb.

As evident in sample 1 in Figure 3-16, the amplified fragment was larger than expected. The gel image displays a fragment at about 6 kb, and another with lower concentration at about 1.8 kb. The latter is probably due to unspecific binding of primers, as no fragment was expected with the aforementioned size. The control sample (sample 3) contains a band at about 0.8 kb, corresponding to the expected size of the vector without insert. Furthermore, the sample contains weaker bands at about 10 kb and 1.8 kb. The two fragments are most likely due to unspecific binding of primers.

As pA0160-KanR-sacB is the sole focus of this section, sample 2 will be excluded from further discussion.

Since the 400-KanR-sacB fragment has an expected size of 3.7 kb, the results are very peculiar. To investigate the fragment, sample 1 was purified with the PCR purification kit, and the isolated DNA utilized as a template in a new PCR reaction. The DNA was investigated with BioBrick prefix and suffix primers, as presented in Figure 3-17.

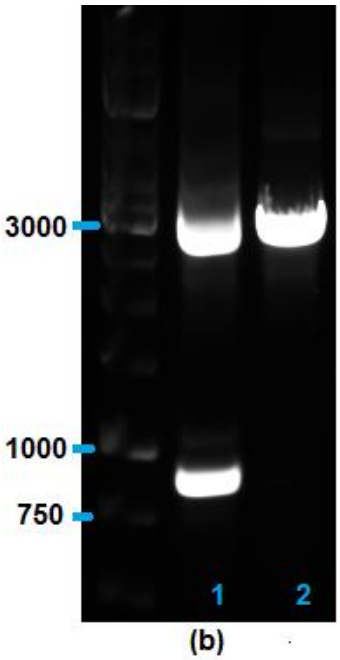

Figure 3-17. Gel image of PCR products containing the KanR-sacB cassette. BioBrick primers were utilized to verify the presence of the KanR-sacB cassette within the 6 kb fragment of sample 1 in Figure 3-16. A control sample of pA0160-KanR-sacB was also included, for size comparison and to verify that the cassette was present in the plasmids used for amplification (3). The PCR samples were analyzed on a 0.8% agarose gel containing GelRed, with 100V applied for 35 minutes. The samples were run alongside a GeneRuler 1 kb DNA ladder. As presented in sample 1, a band at about 3 kb was observed, corresponding to the size of the KanR-sacB cassette of 2.9 kb. The control contains a corresponding band at about 3 kb. This implies that the KanR-sacB is present within the 6 kb fragment under study.

As presented in sample 1, a band at about 3 kb was observed, corresponding to the size of the KanR-sacB cassette of 2.9 kb. The control in sample 2 contains an equivalent band at about 3 kb. This implies that the KanR-sacB is present within the 6 kb fragment under study. Sample 1

also contains a band at about 0.8 kb. This is probably due to unspecific binding of the primers. Alternatively, the fragment might be a product of BioBrick amplification of the 1.8 kb fragment present in sample 1 in Figure 3-16. It is hard to determine what this fragment is composed of. The size corresponds to the expected size of the KanR gene, but as no equivalent band is present in sample 2 in Figure 3-17, it is unlikely that a plasmid with only KanR was present within the DNA sample utilized for amplification. Nevertheless, as the 6 kb fragment seems to contain the KanR-sacB cassette, the sample can be utilized for transformation of WT *Synechococcus.*

The KanR-sacB cassette was also amplified by utilizing the A0160-800 primer pair. The result is presented in Figure 3-18.

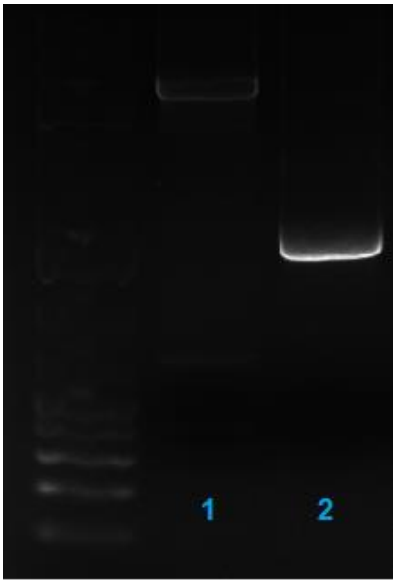

Figure 3-18. Gel image of amplicon containing the KanR-sacB cassette with 800 bp A0160 flanking regions. The cassette and flanking regions were amplified from pA0160-KanR-sacB (1). A sample of pA0160 was also included as a control (2). The PCR samples were analyzed on a 0.8% agarose gel containing GelRed, with 100V applied for 30 minutes. The samples were run alongside a GeneRuler 1 kb Plus DNA ladder. Since the ladder is a bit blurred, the exact size of the 800-KanR-sacB fragment within sample 1 cannot be determined.

Since the ladder is a bit blurred, the exact size of the 800-KanR-sacB fragment cannot be determined. However, the fragment seems to be larger than the expected size of 4.5 kb. Furthermore, the fragment is larger than the fragment observed in sample 2, implying that the fragment contains an insert between the BioBrick prefix and suffix. Because of restrictive time, the

presence of KanR-sacB within the fragment were not verified before the transformation procedure was initiated.

Amplification of the cassette was also attempted with A0160-200 and A0160-100 primers. All amplicons contained fragments with a size larger than expected. Furthermore, when tested with BioBrick primers, all samples seemed to contain the KanR-sacB cassette. It is hard to assess what causes these peculiar results, especially since the problem does not occur when the BioBrick primers are utilized. One possibility is that the flanking regions are larger than expected. When pA0160-KanR-sacB was sequenced, the BioBrick primers were used as sequencing primers. Therefore, the flanking regions were never investigated. However, as the control samples of the corresponding plasmid without insert obtained the expected size, this is highly unlikely.

Another possibility is that the counterselection cassette sequence is causing a secondary structure of the adjacent DNA that affects PCR amplification of the flanking regions. This can be caused by either the KanR or the *sacB* gene. To investigate this hypothesis, A0160-800 and A0160-400 primers were utilized to amplify pA0160-KanR. If the samples contain products with the expected sizes, this would indicate that the problem involves the *sacB* gene. However, if the products once again are larger than expected, this might indicate that there is a problem with the KanR gene, or alternatively with the combination of KanR and sacB within the construct. The result is presented in Figure 3-19.

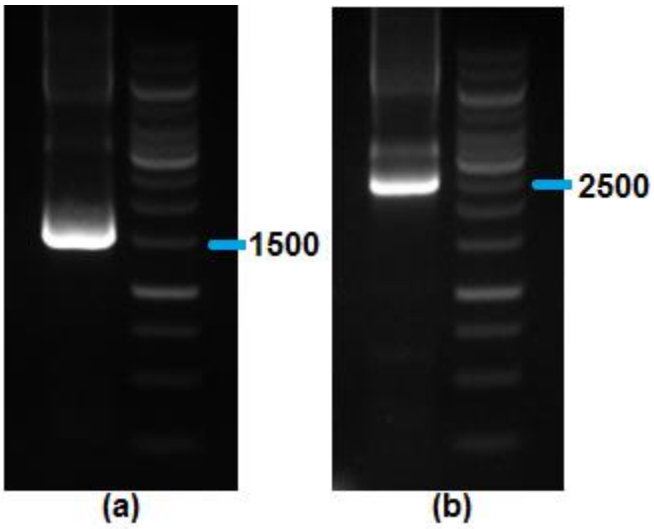

Figure 3-19. Gel image of PCR products attained by A0160-400 and A0160-800 amplification of WT *Synechococcus.* The PCR samples were analyzed on a 0.8% agarose gel containing GelRed, with 100V applied for 30 minutes. The samples were run alongside a GeneRuler 1 kb DNA ladder. The PCR products have sizes corresponding to the expected values of 1.6 kb (a) and 2.4 kb (b), respectively.

As evident in Figure 3-19, the PCR products have sizes corresponding to the expected values of 1.6 kb and 2.4 kb. Since pA0160 and pA0160-KanR amplification yields fragments of correct size, this indicates that the problem is connected to the *sacB* gene. To investigate the reason for this, bioinformatic analyzes should be conducted in the future to explore the sequence at the DNA level.

Transformation

Even though amplification of pA0160-KanR-sacB was somewhat problematic, the counterselection cassette within the fragments seemed to be intact. The DNA samples could therefore be utilized for transformation of WT *Synechococcus*. Transformations were conducted with both A0160(400)-KanR-sacB and A0160(800)-KanR-sacB, according to the transformation procedure described in subsection 2.2.9. After 2-3 weeks of incubation, colonies started to appear on both plates. The results are presented in Figure 3-20.

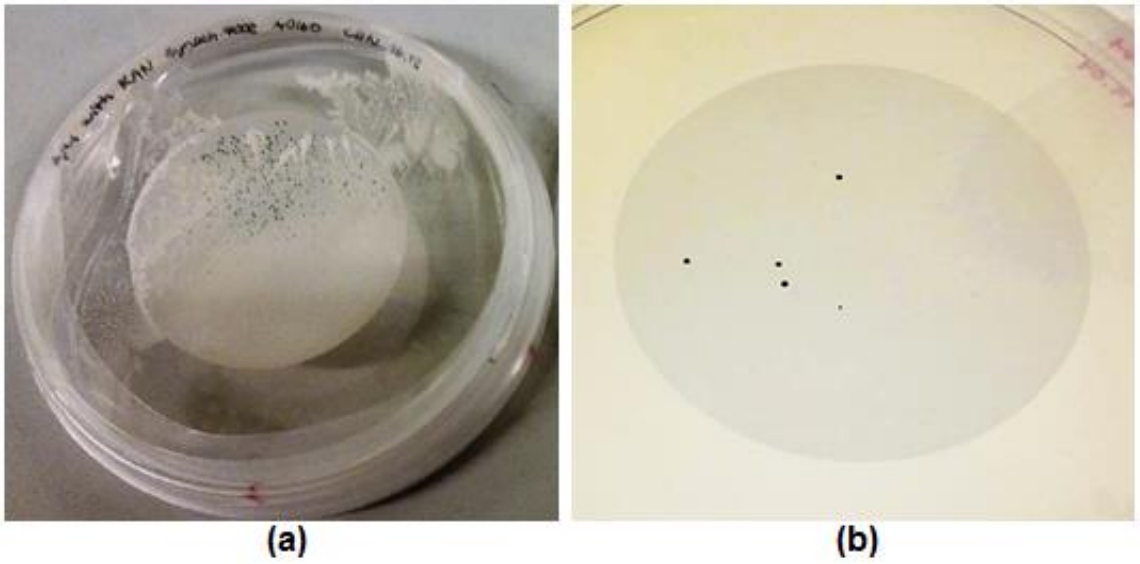

Figure 3-20. *Synechococcus* sp. PCC 7002 transformed with A0160-KanR-sacB. The KanR-sacB counterselection cassette is incorporated into the A0160 neutral site of the *Synechococcus* chromosome, by utilizing A0160 flanking regions for homologous recombination. Two different primer pairs were utilized for PCR amplification of the fragments, i.e. A0160-800 and A0160-400, which resulted in flanking regions of different sizes. Transformation with the first amplicon incorporated is presented in (a), and the latter in (b). Transformants were observed on both plates, albeit in different quantities.

Both transformations (A0160(400)-KanR-sacB and A0160(800)-KanR-sacB) were successful, as transformants were observed on both plates. The number of colonies present do however differ. Transformation performed with 800-KanR-sacB (a) have a much higher transformation efficiency than transformation with 400-KanR-sacB (b). A possible explanation is that the transformation procedures were conducted in a somewhat different manner, as the transformations did not occur at the same time. Alternatively, the transformation efficiency is correlated with the size of the flanking regions. The last theory is the most probable, as other members of the PhotoSynLab research group have obtained similar results.

Unfortunately, the plate in (a) dried and could not be used in further experiments. Verification of segregation, and subsequently sacB testing, was therefore conducted only for transformants from plate (b).

Colony PCR to test segregation

The five colonies from plate (b) were transferred to new selective AA+ plates, followed by colony PCR. BioBrick primers were utilized to verify the presence of the KanR-sacB fragment within *Synechococcus* host cells (Figure 3-21), and various A0160 primers were thereafter used to verify segregation. As amplification by A0160 primer pairs have proven to be problematic, a combination of BioBrick primers and A0160 primers were tested. The most successful outcome was obtained with A0160-400 forward primer in combination with BioBrick reverse primer. The result is presented in Figure 3-22.

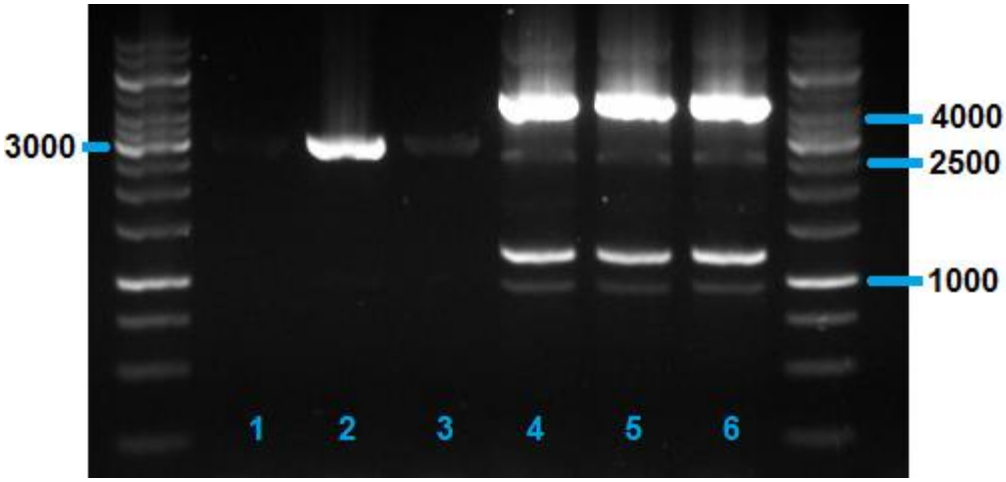

Figure 3-21. Confirmation of KanR-sacB presence within *Synechococcus* host cells. Three samples of transformants were investigated by colony PCR, utilizing BioBrick prefix and suffix primers (1-3). Three controls were included, containing pA0160 (4-6). The PCR samples were analyzed on a 0.8% agarose gel containing GelRed, with 90V applied for 40 minutes. The samples were run alongside a GeneRuler 1 kb DNA ladder. Sample 1-3 contains a single band of about 3 kb. This corresponds to the expected size of the KanR-sacB fragment of 2.9 kb. Several bands are present in the controls, but neither possess the same size as the fragment in the three samples of transformants. This implies that the KanR-sacB fragment is present within the A0160 neutral site of *Synechococcus* host cells.

As evident in Figure 3-21, the KanR-sacB fragment seems to be present within the A0160 neutral site of *Synechococcus* host cells. Only one fragment is observed in the three samples (1-3), all corresponding with the expected size of KanR-sacB of 2.9 kb.

Three samples of pA0160 were included as a control (4-6), each containing 5 bands of different sizes. The bands are most likely due to unspecific binding of the primers, as no PCR product was expected. The most prominent band is about 4.5 kb, and might correspond to the whole plasmid. It

is possible that amplification proceeded around the whole plasmid, instead of comprising only the BioBrick cloning site. Nevertheless, none of the bands in the controls possesses the same size as the band in the three samples of transformants, implying that the band in the latter corresponds to the KanR-sacB cassette.

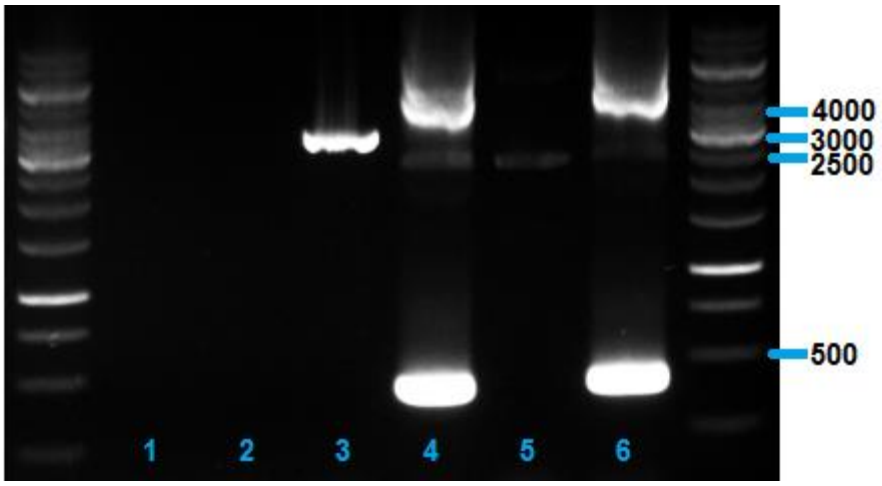

Figure 3-22. Confirmation of KanR-sacB segregation within *Synechococcus* host cells. Three samples of transformants were investigated by colony PCR, utilizing A0160-400 as a forward primer and BioBrick suffix as the reverse primer (1-3). Three controls were included, comprised of pA0160 (4-6). The PCR samples were analyzed on a 0.8% agarose gel containing GelRed, with 90V applied for 40 minutes. The samples were run alongside a GeneRuler 1 kb DNA ladder. No product was detected in sample 1 and 2. The band in sample 3 corresponds to the expected fragment of 400-KanR-sacB-Suffix of 3.3 kb. Furthermore, the three controls contain several bands, but neither possesses the same size as the fragment in sample 3. The most prominent band corresponds to plasmid without insert, with an expected size of 0.4 kb. As this band is not present in sample 3, this implies that the KanR-sacB fragment is segregated within the A0160 neutral site of *Synechococcus* host cells.

The gel image in Figure 3-22 implies that the KanR-sacB cassette is segregated within the A0160 neutral site of the *Synechococcus* chromosome. This conclusion is based on the fact that only one band is present in sample 3, with a size differing from any of the bands in the controls. The 400-KanR-sacB fragment has an expected size of 3.3 kb, which corresponds to the size of the fragment observed in the sample. Unfortunately, no product was detected in sample 1 and 2.

Furthermore, the controls contain three distinct bands, of about 4.5 kb, 2.5 kb, and 0.5 kb, respectively. The two largest fragments are probably due to unspecific binding of primers, while the smallest corresponds to the size of the vector without insert (0.4 kb). As this band is not present

in sample 3, this implies that the KanR-sacB fragment is segregated within the A0160 neutral site of *Synechococcus* host cells.

As segregation is verified, sample 3 can be utilized to test counterselection cassette functionality within *Synechococcus* host cells.

Transformations Conducted with pA0935-KanR-sacB and pA2843-KanR-sacB

Amplification of KanR-sacB and flanking regions

The KanR-sacB cassette with A0935 or A2843 flanking regions was amplified by utilizing A0935-800 primers and A2843-800 primers, respectively. The results are presented in Figure 3-23.

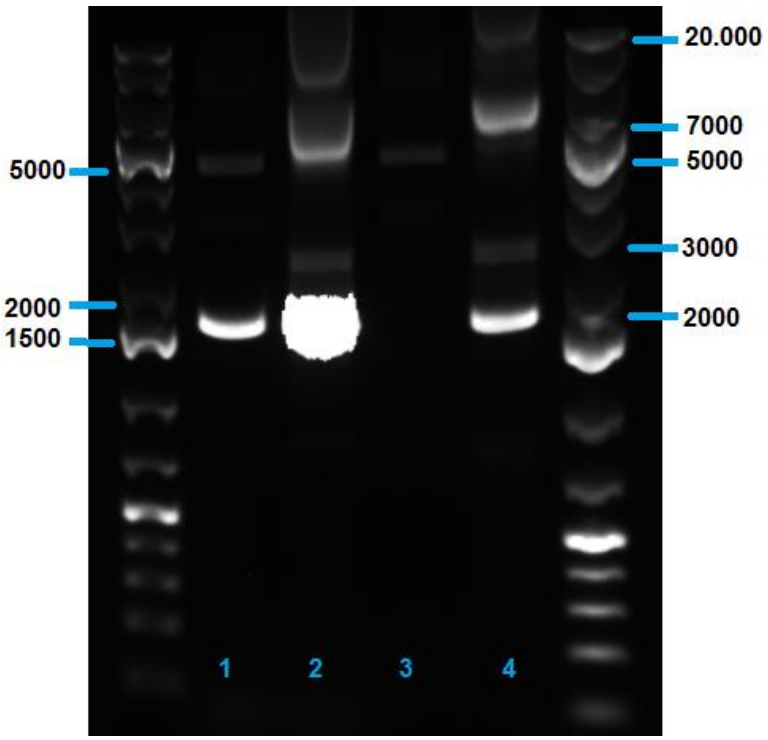

Figure 3-23. Gel image of amplicons containing KanR-sacB and flanking regions of A0935 or A2843. The KanR-sacB cassette with A0935 flanking regions was amplified from pA0935-KanR-sacB by A0935-800 primers (1). Furthermore, KanR-sacB with A2843 flanking regions was amplified from pA2843-KanR-sacB by A2843-800 primers (3). Two controls were included, containing pA0935 (2) and pA2843 (4). The PCR samples were analyzed on a 0.8% agarose gel containing GelRed, with 100V applied for 30 minutes. The samples were run alongside a GeneRuler 1 kb Plus DNA ladder. Sample 1 and 3 contains a band at 5 kb, probably corresponding to the KanR-sacB cassette with an expected size of 4.5 kb.

Sample 1 and 3 are PCR samples of KanR-sacB amplified from pA0935-KanR-sacB (1) and pA2843-KanR-sacB (3), respectively. Sample 2 and 4 are controls, containing the corresponding vectors without insert. As evident in Figure 3-23, sample 1 and 3 both contain a band at about 5 kb. This fragment probably corresponds to the KanR-sacB cassette, although the fragment is somewhat large (expected size 4.5 kb). This might be due to problematic amplification by the 800-primers, as observed for pA0160-KanR-sacB in Figure 3-18.

Sample 1 also contains a band at about 1.5 kb. The fragment might correspond to vector without insert, as the same band is observed in the control (2). Since the pA0935-KanR-sacB sample was investigated only by BioBrick primers, it is possible that some plasmids without insert are present, not detected as the amplicon would be too small. However, this is highly unlikely, as *E. coli* DH5α host cells containing the plasmid verified that the cassette was present and functioning. The band is therefore most likely a result of unspecific binding of the primers.

Because of restrictive time, the presence of KanR-sacB within the 5 kb fragment was not confirmed before the transformation procedure was initiated. Nevertheless, sample 1 and 3 were utilized to transform WT *Synechococcus* cells, as presented in the following subsection.

Due to unsuccessful transformation of KanR-sacB from sample 3 targeting the A2843 neutral site (explained in detail in the two following sections), another round of transformation of the aforementioned fragment was performed. This time, the A2843-400 primer pair was utilized for amplification, resulting in a fragment of about 6 kb (gel image not shown). As explained for A0160, the sample was purified and used as a template in another PCR amplification, utilizing BioBrick primers. The resulting PCR products verified the presence of KanR-sacB within the 6 kb fragment (gel image not shown). Unfortunately, no colonies appeared after transformation. Therefore, this experiment will be excluded from further discussion.

Transformation

Purified DNA from sample 1 and 3 (Figure 3-23) were utilized to transform WT *Synechococcus* cells. Due to restricted time and somewhat low yields of purified DNA, the transformations were

carried out with 0.86 µg and 0.36 µg DNA, respectively. After 2-3 weeks of incubation, colonies started to appear on both plates. The results are presented in Figure 3-24.

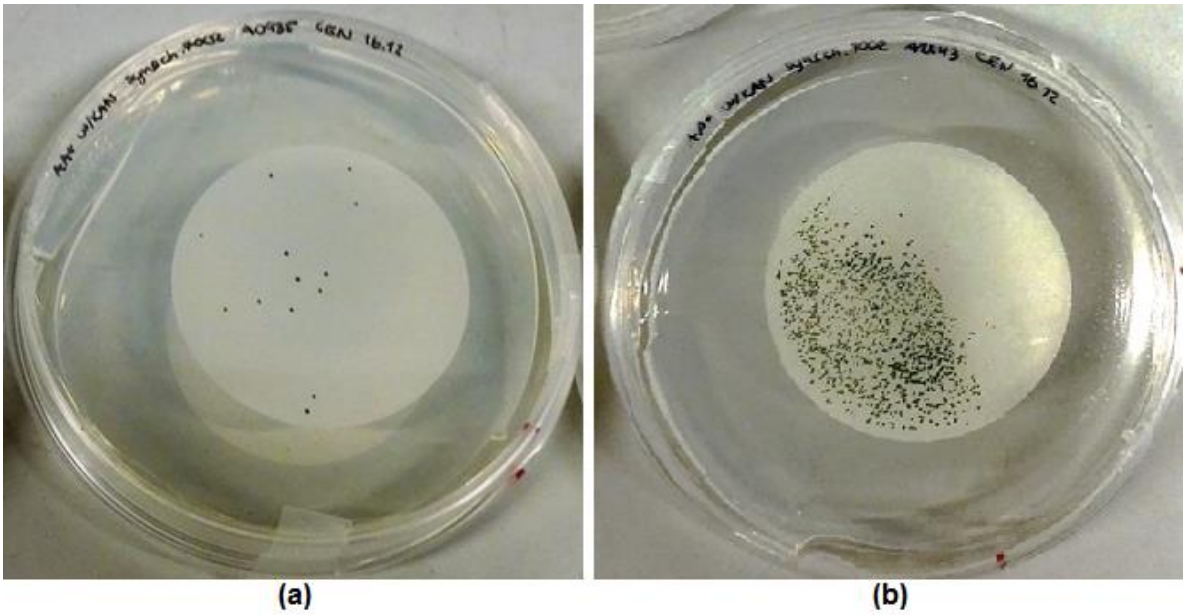

Figure 3-24. *Synechococcus* sp. PCC 7002 transformed with A0935-KanR-sacB (a) and A2843-KanR-sacB (b). The counterselection cassettes are incorporated into the A0935 or A2843 neutral sites of the *Synechococcus* chromosome, by utilizing A0935 or A2843 flanking regions for homologous recombination. Two primer pairs were utilized for PCR amplification of the fragments, i.e. A0935-800 and A2843-800. Transformation with the first amplicon is presented in (a), and the latter in (b). Transformants were observed on both plates, albeit in different quantities.

Both transformations were successful, as transformants were observed on both plates. The number of colonies present do however differ. Transformation conducted with 800-KanR-sacB with A0935 flanking regions (a) was observed to have a much lower transformation efficiency than transformation conducted with 800-KanR-sacB with A2843 flanking regions (b). A possible explanation is that the transformation procedures were conducted in a somewhat different manner, due to different amounts of DNA applied. Alternatively, the neutral sites have different susceptibilities for insertion of DNA. It is possible that the inserted DNA might have affected the adjacent DNA regions, or possibly that homologous recombination occurred at a lower frequency. The first might have caused inhibition of growth of *Synechococcus* host cells, and the latter might have caused cell death since the cells were grown on selective media. The last hypothesis involving different susceptibilities of neutral sites is the most likely, as similar results have been obtained by other members of the PhotoSynLab research group.

Transformants with the KanR-sacB counterselection cassette incorporated into A0935 (a) and A2843 (b) were transferred to new selective AA+ plates, and segregation was subsequently tested by colony PCR.

Colony PCR to test segregation

Six colonies from each plate were transferred to new selective AA+ plates, followed by colony PCR. BioBrick prefix and suffix primers, as well as various A0935- and A2843-primers, were utilized to investigate whether the cassettes had been incorporated within the A0935 and A2843 neutral sites. A number of different combinations of primers were tested, several of which giving peculiar results or no results at all. A selection of gel images is discussed in the following text, as presented in Figure 3-25 to Figure 3-27.

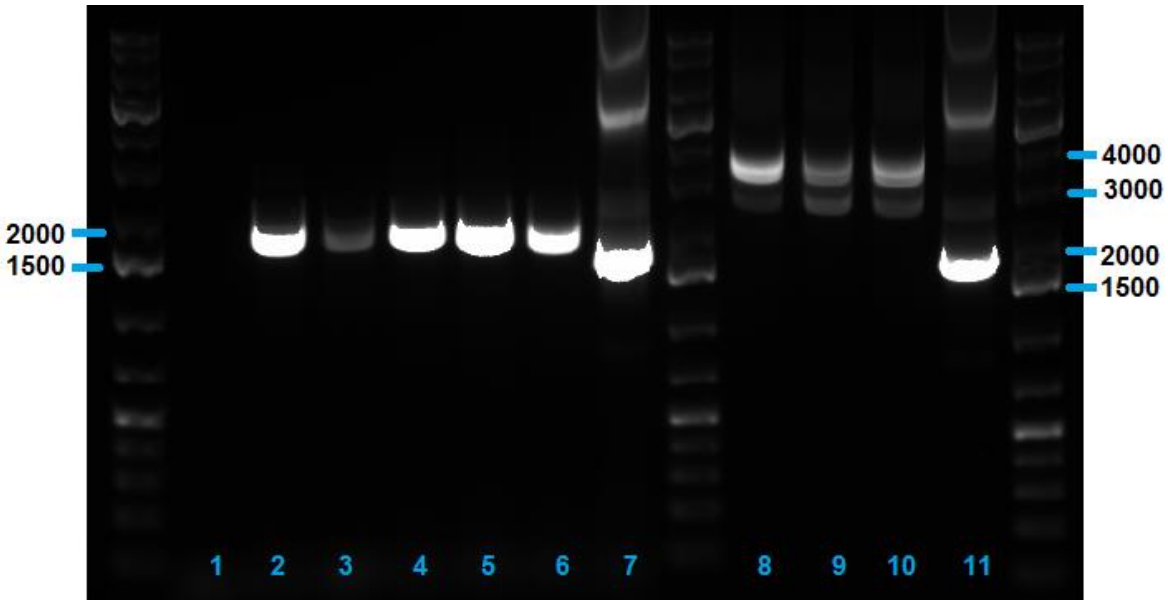

Figure 3-25. Investigation of KanR-sacB presence within *Synechococcus* host cells. Sample 1-6 represents host cells transformed with the KanR-sacB cassette with A0935 flanking regions, and sample 8-10 the equivalent with A2832 flanking regions. Two control samples are included, of the corresponding vectors without insert. Sample 7 represents pA0935 and sample 11 pA2843, respectively. The transformants were investigated by colony PCR, utilizing A0935-800 and A2843-800 primers. The PCR samples were analyzed on a 0.8% agarose gel containing GelRed, with 100V applied for 30 minutes. The samples were run alongside a GeneRuler 1 kb Plus DNA ladder. All samples contained bands of peculiar sizes, except the controls (expected sizes of 1.6 kb).

As evident in Figure 3-25, all samples of transformants contained bands of peculiar sizes. The KanR-sacB cassette amplified by 800-primers have an expected size of 4.5 kb, but the fragments observed in the samples are only about 2 kb. Furthermore, the most prominent bands in the controls corresponds to the expected sizes of 1.6 kb, indicating that there was no problem with the PCR setup. To investigate the 2 kb fragments, the samples were purified, and the isolated DNA utilized as a template in a new PCR reaction. The presence of KanR-sacB were tested, by using BioBrick prefix and suffix primers. The result is presented in Figure 3-26.

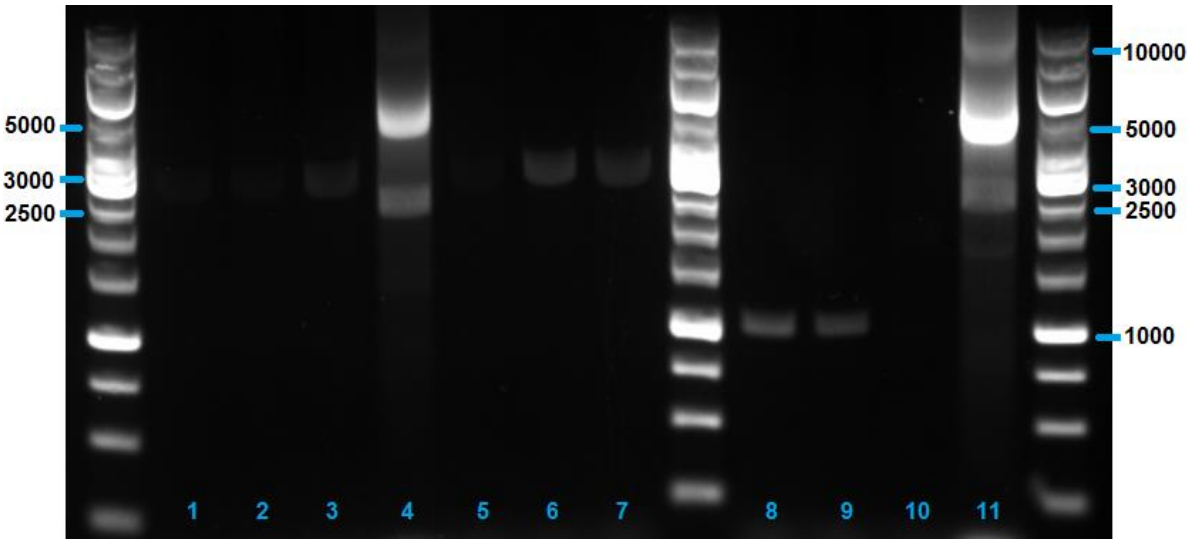

Figure 3-26. Confirmation of KanR-sacB presence within *Synechococcus* host cells. Sample 1-3 and 5-7 represents host cells transformed with the KanR-sacB cassette containing A0935 flanking regions. Sample 7-10 represents the equivalent with A2832 flanking regions. Two control samples are included, of the corresponding vectors without insert. Sample 7 represents pA0935 and sample 11 pA2843, respectively. The transformants were investigated by colony PCR, utilizing BioBrick prefix and suffix primers. The PCR samples were analyzed on a 0.8% agarose gel containing GelRed, with 100V applied for 25 minutes. The samples were run alongside a GeneRuler 1 kb DNA ladder. All A0935 samples (1-3 and 5-7) contained a band at about 3 kb, corresponding to the expected size of the KanR-sacB cassette of 2.9 kb. Furthermore, two of the A2843 samples (8 and 9) contained a sole band of 1 kb. This implies that the KanR-sacB cassette is present within the A0935 samples, but not in the A2843 samples.

As presented in Figure 3-26, all A0935 samples (1-3 and 5-7) contained a band at about 3 kb, corresponding to the expected size of the KanR-sacB cassette of 2.9 kb. This implies that the cassette has been incorporated within the A0935 neutral site of *Synechococcus* host cells, even though amplification with A0935-800 primers resulted in fragments of peculiar sizes. To verify segregation, colony PCR was repeated with other primer pairs. Amplification was performed with A0935-100 primers, as presented in Figure 3-27. In addition, several combinations of BioBrick

primers together with A0935 primers were tested. Unfortunately, no product was observed in the PCR samples.

Two of the A2843 samples (8 and 9) contain a single band of 1 kb. The size of the fragment almost corresponds to the size of the KanR-gene (expected size of 0.8 kb). It is unexpected that the gene is present without the sacB gene, as a single band of 2.9 kb have been observed when pA2843-KanR-sacB has been previously amplified with the BioBrick primer pair. However, as the cells are capable of growing in the presence of kanamycin, this indicates that the *Synechococcus* cells have incorporated the KanR gene into their genome. One possible explanation for this is that something unexpected happened during the amplification of 800-KanR-sacB previous to the transformation. As the composition of the 5 kb fragment in Figure 3-23 was not investigated with BioBrick primers, it is possible that the fragment only contained the KanR gene. Although the transformants did not seem to contain the intact KanR-sacB cassette, the cells were included in the sucrose experiments conducted to test cassette functionality, as described in subsection 3.2.3.

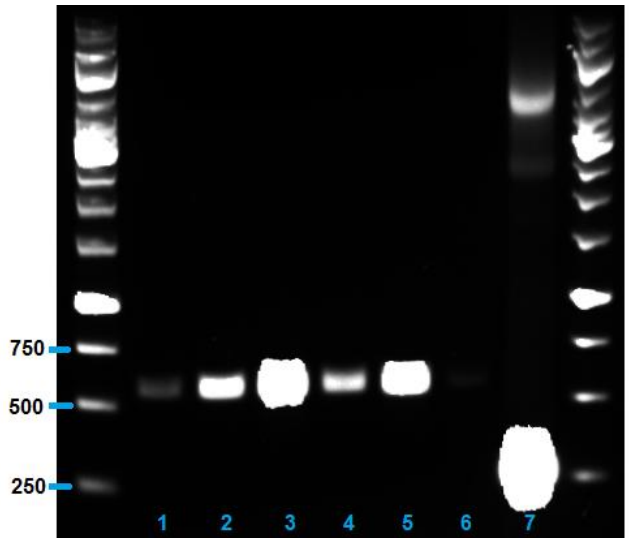

Figure 3-27. Investigation of KanR-sacB presence within *Synechococcus* host cells. Sample 1-6 represents host cells transformed with the KanR-sacB cassette with A0935 flanking regions. A control sample is included, of the corresponding vector without insert, i.e. pA0935. The transformants were investigated by colony PCR, utilizing A0935-100 primers. The PCR samples were analyzed on a 0.8% agarose gel containing GelRed, with 100V applied for 35 minutes. The samples were run alongside a GeneRuler 1 kb DNA ladder. All samples contained bands of peculiar sizes, except the control (expected size of 0.2 kb).

As demonstrated in Figure 3-27, the PCR product of the transformants once again contained fragments of peculiar sizes. The bands are about 0.6 kb, although they should be closer to 3.3 kb. Presumably, the transformants still contain the intact KanR-sacB cassette, although verification of segregation has proven difficult. This hypothesis is supported by the fact that the size of BioBrick amplicons corresponds with the expected size of the cassette.

Furthermore, since A0935-800 and A0935-100 amplicons are larger than the fragments observed in the corresponding controls, the samples most likely contain inserts. And since no additional band is observed with the same size as the fragment within the controls, this implies that no chromosomes without an insert is detected. The aforementioned observations together indicate that the KanR-sacB cassette is segregated within the A0935 neutral site of *Synechococcus* host cells, despite of the peculiar fragments observed during amplification. This hypothesis will be further tested through verification of counterselection cassette functionality, as presented in subsection 3.2.3.

Transformations Conducted with ChlR-KanR-sacB Counterselection Plasmid

Transformations were also planned for counterselection plasmids containing chloramphenicol. Unfortunately, none of the transformations were successful. pA0160-ChlR-KanR-sacB amplification was easily achievable, with correct size and high concentration of the cassette and flanking regions. However, no colonies appeared on the selective plates after performing the transformation protocol. Furthermore, pA0935-ChlR-KanR-sacB was excluded due to the observed reduction in transformation efficiency. The neutral site does not seem to be as neutral as first expected, since so few cells are capable of growing on selective medium. This might be caused by unsuccessful incorporation of the transforming DNA, or possibly polar effects exerted by the insert. Finally, pA2843-ChlR-KanR-sacB proved difficult to amplify. Several primer pairs were tested, but the amplification always resulted in several bands, none of which containing the expected size.

In the future, the ChlR-KanR-sacB counterselection plasmids should be sequenced to see if the constructs are correct, and transformation should be repeated to see if the cassettes are functioning within *Synechococcus* host cells.

3.2.2 Establishing Selection Conditions for Wild-Type Synechococcus

Before counterselection cassette functionality within transformants could be tested, the selection conditions needed to be established for WT cells. Two growth experiments were conducted: one to test sucrose susceptibility, and another to confirm that WT cells can grow in media without NaCl. The first ensures that death of transformants during counterselection functionality testing can be connected to expression of the *sacB* gene, and not to sucrose toxicity. The latter facilitates *sacB* testing itself, as the testing were observed to be NaCl-sensitive when the constructs were tested within *E. coli* DH5α host cells. The experiments are described in detail in the following text.

Sucrose Growth Experiment with Wild-Type *Synechococcus*

A growth experiment with WT *Synechococcus* was conducted with varying amounts of sucrose in the AA+ media, to investigate whether the cells were viable under the conditions utilized for *sacB* counterselection. The experimental procedure is described in subsection 2.2.8, and the result presented in Figure 3-28.

Growth were detected on all the plates, in roughly equal amounts. As the experiment was performed in a qualitative manner, the quantity of cells on each plate is unknown. Therefore, a potential small variation of growth between plates remains undetected. Nevertheless, the experiment verifies that WT cells are capable of growing under the conditions utilized to test counterselection cassette functionality

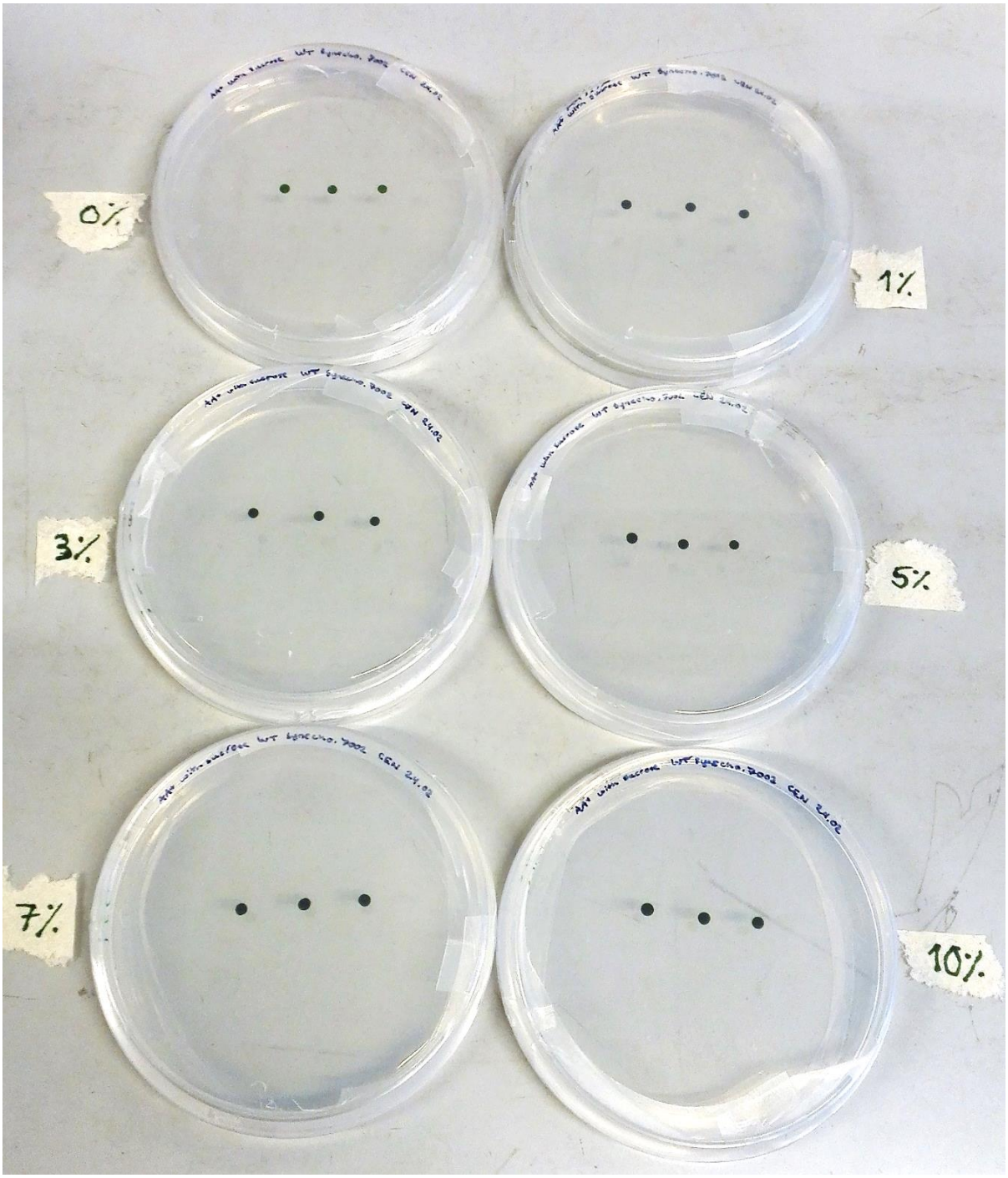

Figure 3-28. Result of sucrose growth experiment of wild-type *Synechococcus* sp. PCC 7002. AA+ plates were prepared containing 0, 1, 3, 5, 7 and 10% (w/v) sucrose. 1 µL of WT cells from the same culture were applied to each of the plates. Three parallels were included. The plates were incubated at 33°C with 73 $\mu Em^{-2}s^{-1}$ for 5 days. Growth were detected on all the plates, in roughly equal amounts. This verifies that WT cells are capable of growing in the presence of sucrose.

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NaCl Growth Experiment with Wild-Type *Synechococcus*

As *sacB* testing of the counterselection plasmids within *E. coli* DH5α host cells was observed to be NaCl-sensitive, a growth experiment was conducted to test if WT Synechococcus cells were able to grow under these restrictive conditions. The experiment was performed as described in subsection 2.2.8. The growth media utilized contained 0, 25, 50 and 100% NaCl compared to the value given in Table 2-2.

The experiment was primarily conducted with 200 rpm shaking. As the cells were growing very slowly, the experiment needed to be repeated. The slow growth rate was probably due insufficient aeration of the sample, caused by inadequate shaking. Alternatively, the B_{12} utilized in the media might have been degraded. Nevertheless, the experiment was conducted once more with 300 rpm shaking and fresh media. The OD_{730} measurements of the last experiment is presented in Figure 3-29.

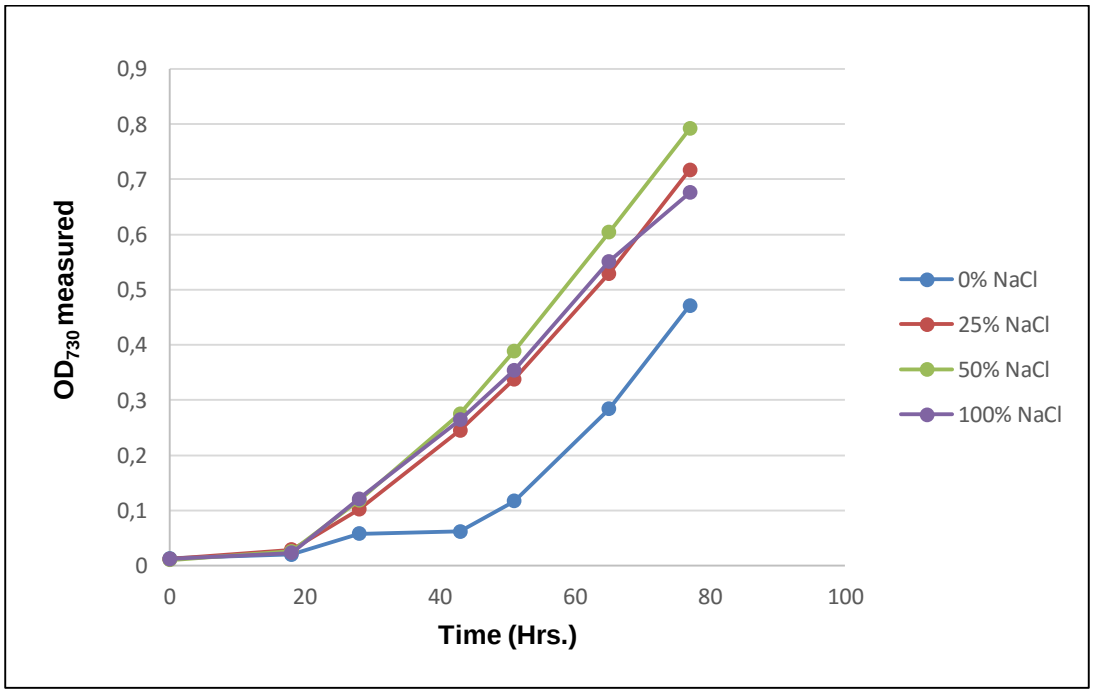

Figure 3-29. OD_{730} measurements obtained in NaCl growth experiment. The experiment was conducted with wild-type *Synechococcus* sp. PCC 7002, grown in liquid AA+ media with different concentrations of NaCl. The liquid cultures were incubated at 33°C and 90 $\mu Em^{-2}s^{-1}$, with 300 rpm shaking.

The results obtained implies that NaCl is not required for growth of WT *Synechococcus* cells. The growth is somewhat slower in the 0% NaCl sample compared to the other three, but the cells are still growing in a satisfactory rate. Surprisingly, the 25% and 50% samples obtained a higher OD_{730} value than the 100% sample, suggesting that the AA+ media does not actually require such large quantities of NaCl. Note, however, that the measurements were only conducted with one parallel. As the sample size is inadequate for statistical analysis, the numbers were merely used qualitatively as a means to evaluate if the cells were capable of growing without NaCl.

3.2.3 Confirmation of Counterselection Cassette Functionality within *Synechococcus* host cells

Confirmation of counterselection cassette functionality was conducted as described in subsection 2.2.10. Three sets of transformants were tested. All contained the KanR-sacB counterselection cassette, incorporated into the A0160, A0935 or A2843 neutral sites.

Functionality in A0935-KanR-sacB and A2843-KanR-sacB transformants was primarily investigated, utilizing AA+ media containing kanamycin (50µg/mL) and 0, 1, 3, 5, 7, or 10% (w/v) sucrose. Roughly equal quantities of growth were observed on all plates, deeming the experiment unsuccessful (not shown). The KanR gene seemed to be present and working since the cells were able to grow in the presence of kanamycin. The *sacB* gene, however, was either not present or not expressed successfully.

The experiment was repeated at a later stage, including A0160-KanR-sacB. This time growth media was prepared with and without NaCl (0% and 100% (w/v)), due to the observed NaCl-sensitivity in *E. coli* DH5α host cells during *sacB* testing. Kanamycin was excluded, to ensure that WT *Synechococcus* could be included as a control. As before, sucrose concentrations of 0, 1, 3, 5, 7, and 10% (w/v) were utilized. Unfortunately, the A2843-KanR-sacB transformants could not be included as the strain maintenance plate dried out. The experiment was therefore conducted with only A0160-KanR-sacB and A0935-KanR-sacB. Solutions was prepared with OD_{730} of 1, 0.1 and 0.01 for the two samples of transformants and the wild-type control. The exact OD_{730} values measured and applied to the growth plates are presented in Table 3-2.

Table 3-2. Exact OD-values of samples utilized for counterselection cassette testing. A0160-KanR-sacB and A0935-KanR-sacB refers to *Synechococcus* cells transformed with the KanR-sacB counterselection cassettes, incorporated into the neutral sites A0160 and A0935 within the genome of host cells. The OD_{730} is measured for the three cell cultures in the bubbling flasks, as well as for samples prepared with OD_{730} of 1, 0.1 and 0.01.

	A0160-KanR-sacB	**A0935-KanR-sacB**	**Wild-Type**
OD_{730} of cell culture	0.518	0.331	0.350
OD_{730} 1[1]	0.98	1.01	0.98
OD_{730} 0.1	0.099	0.102	0.099
OD_{730} 0.01	0.009	0.009	0.012

[1]Measurements performed with 1/10 dilution with fresh AA+ media.

After 8 days of incubation, the growth plates were photographed. The results of plates with 100% NaCl is presented in Figure 3-30, and with 0% NaCl in Figure 3-31, respectively.

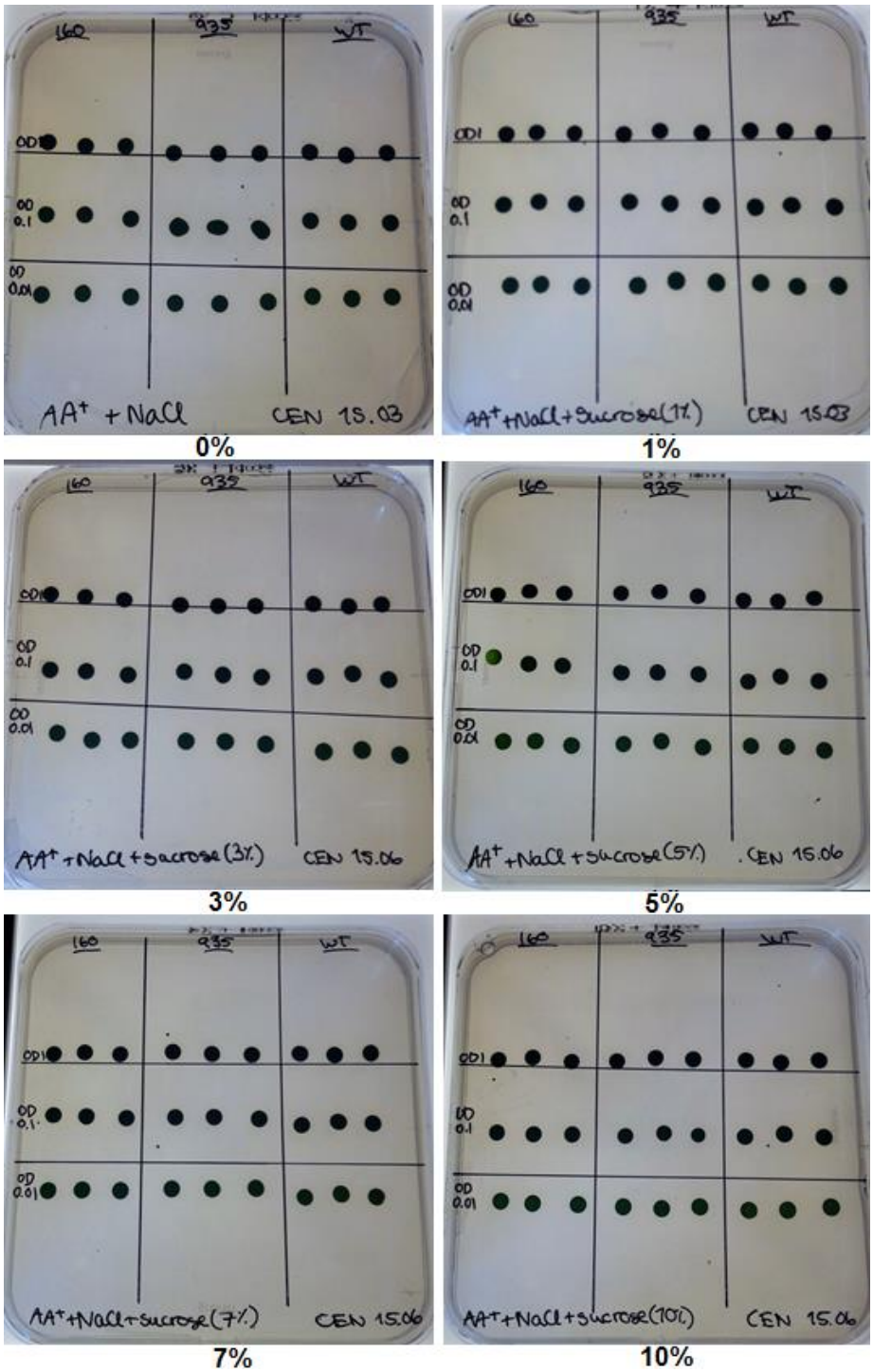

Figure 3-30. Testing of KanR-sacB counterselection cassette on growth plates containing NaCl. The testing was conducted on AA+ plates containing sucrose (0-10% (w/v)) and NaCl. Prior to testing, the cassette was incorporated into the A0160 (left) or A0935 (middle) neutral sites within the chromosome of *Synechococcus* sp. PCC 7002. A control sample was included, containing WT *Synechococcus* (right). Each sample is prepared to achieve OD_{730} of 1 (top), 0.1 (middle), and 0.01 (bottom). Three parallels were applied from each solution. Growth is visible on all plates, in roughly equal amounts. Since no growth was expected on plates containing a sucrose concentration of 5% or higher, counterselection cassette functionality could not be verified.

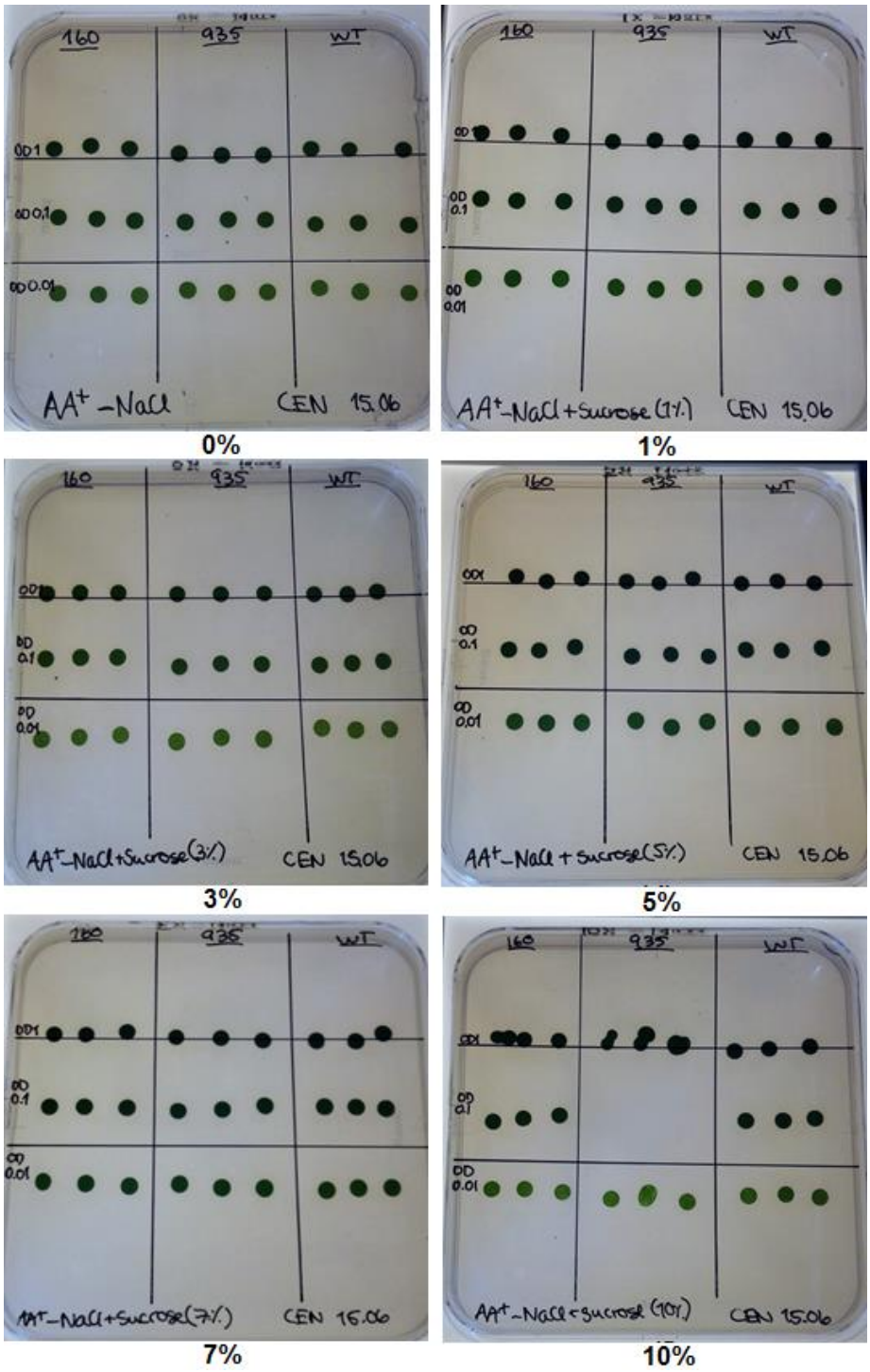

Figure 3-31. Testing of KanR-sacB counterselection cassette on growth plates without NaCl. The testing was conducted on AA+ plates containing sucrose (0-10% (w/v)). No NaCl was added to the media. Prior to testing, the cassette was incorporated into the A0160 (left) or A0935 (middle) neutral sites within the chromosome of *Synechococcus* sp. PCC 7002. A control sample was included, containing WT *Synechococcus* (right). Each sample is prepared to achieve OD_{730} of 1 (top), 0.1 (middle), and 0.01 (bottom). Three parallels were applied from each solution. Growth is visible on all plates, in roughly equal amounts. Since no growth was expected on plates containing a sucrose concentration of 5% or higher, counterselection cassette functionality could not be verified.

Growth of the *sacB*-containing strain was observed on all the plates, both on media containing NaCl (Figure 3-30) and media without any NaCl added (Figure 3-31). One exception is observed in the middle square of the 10% plate without NaCl. This is probably caused by forgetting to applying the samples, as all the spotting was done manually.

The results imply that the presumptive presence of the *sacB* gene did not lead to accumulation of the toxic biopolymer under the specified conditions. This might be due to other salts present in the AA+ media. Even though NaCl was excluded from the media, sodium and chloride are still present as part of other compounds, i.e. $FeCl_3$, $MnCl_3$, Na_2MoO_4, Na_2-EDTA, KCl, $NaNO_3$, and $CaCl_2$. The experiment could be repeated without the aforementioned compounds added in the growth media, but this is difficult to achieve since the compounds are part of stock solutions essential to the cells. By removing the compounds, it is possible that the cells will not receive the nutrients required for growth and reproduction.

Another possibility is that mistakes were made during the preparation of the growth media, or alternatively, that the cassette was lost due to removal of selection pressure. As the liquid media utilized to grow the transformants prior to the counter-selection cassette testing did not contain kanamycin, it is possible that the loss of selection pressure resulted in loss of the cassette. This is, however, highly unlikely as the cassette is incorporated into the *Synechococcus* genome, and segregation has been demonstrated by PCR. Once segregated, such cassette incorporations should be stable over long time periods even in the absence of selective presence (Berla et al., 2013).

Furthermore, it is possible that *Synechococcus* sp. PCC 7002 becomes stressed without salt (hypo-osmotic stress), resulting in no expression of the *sacB* gene. Or alternatively, that sucrose is not transported into the cells under these conditions.

In addition, it is possible that *sacB* expression do not cause the expected response as observed in other Gram-negative bacteria. Even though cyanobacteria are classified as Gram-negative, the cells possess envelopes with a combination of Gram-negative and Gram-positive features. Despite the overall Gram-negative structure, the peptidoglycan layer is considerable thicker than expected. In unicellular cyanobacteria such as *Synechococcus*, the peptidoglycan layer is about 10 nm,

compared to the expected value of 2-6 nm. Furthermore, cyanobacteria possess other characteristics that are similar to Gram-positive bacteria, including the composition and degree of cross-linking of peptidoglycan, and the composition of lipopolysaccharide and carotenoids within the outer membrane (Hoiczyk and Hansel, 2000). It is therefore possible that levan synthesis is not as toxic for *Synechococcus* as observed for other Gram-negative bacteria. However, as *sacB* expression is observed to be lethal in the presence of 5% sucrose for the closely related cyanobacteria *Synechocystis* sp. strain PCC 6803 harboring the gene, this is highly unlikely (Viola et al., 2014).

The experiment should be repeated with new media, to see if the same results will be obtained. Furthermore, the transformants should be re-tested by colony PCR to see if the cassette is still present, despite of the observed growth in the presence of sucrose. If the cassette is present but not functioning, the sequence of the cassette should be investigated by sequencing followed by bioinformatic analysis, to determine if the problem is connected to the sequence of the cassette. Due to close proximity of the deadline for delivering the thesis, none of the suggested measures were performed.

If further testing would imply that NaCl-sensitivity was the problem related to the *sacB*-testing, a modified version of the *Synechococcus* growth media should be developed to facilitate testing. As this is a complex and time consuming task, it might be better to construct new counterselection-plasmids with *sacB* from another source. It would be more convenient to attain an equivalent system where counterselection cassette testing is not NaCl-sensitive.

4 Conclusion

This study aimed at constructing a genetic system that facilitates the genetic modification of wild-type *Synechococcus* sp. PCC 7002 by counterselection. Various counterselection plasmids were constructed, containing a counterselection cassette comprised of *sacB* and one or two antibiotic resistance genes, i.e. kanamycin resistance (*kan*^{r}), and chloramphenicol resistance (*Tn9cat*). Six plasmids were constructed, three containing the KanR-sacB cassette, and another three containing the ChlR-KanR-sacB cassette. Each cassette was surrounded by synthesized sequences identical to the A0160, A0935 or A2843 neutral sites within the genome of wild-type *Synechococcus*. The designed plasmids were denoted pA0160-KanR-sacB, pA0935-KanR-sacB, pA2843-KanR-sacB, pA0160-ChlR-KanR-sacB, pA0935-ChlR-KanR-sacB and pA2843-ChlR-KanR-sacB, respectively. One of the constructed plasmids, pA0160-KanR-sacB, was investigated by sequencing. The results verified the presence of *sacB* and the *kan*^{r} gene, and confirmed that the genes were incorporated in the expected place and in the right orientation within the constructs.

Counterselection cassette functionality was tested by transforming the designed plasmids into *E. coli* DH5α host cells. The cells were grown on LA media containing kanamycin and various concentrations of sucrose (0-15%, w/v). Testing of cassettes containing *Tn9cat* also required addition of chloramphenicol into the growth medium. The functionality of the antibiotic resistance genes was verified, as only transformants were capable of growing in the presence of the antibiotics. The functionality of *sacB* proved to be more problematic, as growth was observed on all plates – even on plates containing 15% sucrose. However, growth was observed to be NaCl-dependent. When testing was conducted on growth plates without any NaCl added, the gene functioned perfectly. In the absence of NaCl, no growth was observed at concentrations of 5% sucrose or higher. This verifies that a sucrose concentration of 5% is lethal for Gram-negative bacteria harboring the *sacB* gene (Gay et al., 1985, Pelicic et al., 1996, Viola et al., 2014).

Wild-type *Synechococcus* host cells were transformed with the counterselection cassettes by utilizing the previously reported neutral integration sites A0160, A0935 or A2843 as flanking regions to facilitate homologous recombination (Sakamoto et al., 1997, Begemann et al., 2013, Davies et al., 2014). Prior to the transformation, the cassettes were amplified by PCR with 800 and 400 bp flanking regions. Transformation by A0160(800)-KanR-sacB, A0160(400)-KanR-sacB, A0935(800)-KanR-sacB and A2843(800)-KanR-sacB resulted in transformants appearing on the

AA+ growth plates supplemented with kanamycin. It was observed that transformation performed with 800 bp flanking regions gave rise to more transformants than when the corresponding transformation was performed with 400 bp flanking regions. In addition, A0935 was observed to be the neutral integration site with lowest transformation efficiency. This might indicate that A0935 is not as neutral as first expected. However, more experiments need to be conducted to verify if this is truly the case. Furthermore, segregation of the KanR-sacB cassette within the A0160 neutral site of the *Synechococcus* chromosome was verified by colony PCR. Segregation proved more difficult to verify for the cassette within the A0935 and A2843 neutral sites, as the PCR amplification resulted in fragments of peculiar sizes. However, the results indicated that the neutral sites within all copies of the chromosome contained an insert – indicating that the cassette was segregated.

In order to assess if the constructs are able to confer counterselection in *Synechococcus*, transformants were grown on AA+ plates containing kanamycin and sucrose (0-10%, w/v). Prior to testing, the suitable sucrose and NaCl selection conditions were established for wild-type cells. As for *E. coli* host cells harboring the cassettes, *sacB*-mediated counterselection proved to be problematic for *Synechococcus* host cells. Growth was observed on all plates, regardless of the concentration of sucrose. *sacB*-mediated counterselection was conducted once more without NaCl in the media, but the same results were observed – sucrose did not inhibit growth in *Synechococcus* that contained the *sacB* gene. Although NaCl was removed from the growth media, sodium and chloride were present as part of other media constituents, which may impair counterselection. Therefore, *sacB*-mediated counterselection could not be confirmed within *Synechococcus* host cells.

While *sacB*-mediated counterselection shows great promise, several problems need to be examined and addressed before the system can be implemented in *Synechococcus*. All constructs should be sequenced, both as plasmids and as incorporated parts within the *Synechococcus* genome. Furthermore, the *sacB* gene needs to be further investigated, in order to determine why the testing is observed to be NaCl-sensitive. Finally, the *Synechococcus* culture media may require optimization to facilitate *sacB*-mediated counterselection. If this proves difficult, one could consider replacing the *sacB* gene with another *sacB* variant from another source, or alternatively, to utilize another counterselectable marker altogether.

5 Future Perspectives

As previously stated in the results and discussion section, and as summarized in the conclusion, several problems presented itself during the work on this thesis. Foremost, *sacB*-mediated counterselection was observed to be NaCl-sensitive. In *E. coli* DH5α host cells this problem could be circumvented by excluding NaCl from the LA growth plates. However, for *Synechococcus* sp. PCC 7002 host cells, the problem was more difficult to circumvent. Although NaCl was removed from the AA+ growth media, sodium and chloride were present as part of other media constituents, which might have been the cause of the impaired counterselection. The *sacB* gene needs to be further investigated, in order to determine why the testing is observed to be NaCl-sensitive. Furthermore, the *Synechococcus* culture media may require optimization to facilitate *sacB*-mediated counterselection. If this proves difficult, one could consider replacing the *sacB* gene with another *sacB* variant from another source, or alternatively, to utilize another counterselectable marker altogether.

In addition, several measures can be taken to improve the credibility of the genetic system. All constructs should be sequenced, both as plasmids and as incorporated parts within the *Synechococcus* genome. Furthermore, transformation should be repeated and optimized to facilitate incorporation of cassettes from all the constructed counterselection plasmids. Several lengths of flanking regions should be tested, to find the optimal length to facilitate homologous recombination. A more quantitative transformation procedure can also be utilized to investigate the difference in transformation efficiency between the three neutral integration sites A0160, A0935 and A2843. Finally, the colony PCR procedure used to verify segregation of cassettes should be optimized. It should be investigated why amplification by BioBrick primers results in fragments with the expected sizes, while amplification by 800, 400, 200 and 100 primers spanning the three neutral sites gives rise to fragments of peculiar sizes. If necessary, new PCR primers can be designed to facilitate verification of segregation.

Despite the aforementioned problems, the genetic system proposed shows great promise. When the problems have been examined and addressed, the system can be taken to the next level. Once the system has been established in *Synechococcus*, a gene or DNA sequence of interest can be incorporated into the genome of *Synechococcus* during a second transformation step. The

counterselection cassette is then excised, giving rise to a markerless mutant strain. The system can be utilized to substitute a non-functional gene, or to incorporate a new gene of interest. Alternatively, the system can be used to create knockout mutation of genes already present. This can be utilized to investigate the function of a gene or to create a desired phenotype (Berla et al., 2013, Jacobsen et al., 2011, Snustad and Simmons, 2012, Reece, 2004).

The system is important to learn more about the genome of *Synechococcus* sp. stain PCC 7002 to establish the organism as a model. Furthermore, as the antibiotic selection marker will be excised together with the counterselectable marker, this creates a markerless mutant strain, limiting the opportunities of transferring resistance to pathogenic bacteria. Finally, the possibility to create insertions, deletions and replacements within the *Synechococcus* genome can be utilized to create a strain that is optimized as a production platform for specific chemical compounds.

Cyanobacteria have recently gained a great deal of attention as hosts for metabolic engineering, due to their innate ability to convert CO_2 and sunlight into a chemical product of interest. They are of special interest as hosts for production of biofuels, which are important for combating global climate change. Cyanobacteria, eukaryotic algae and plants are all possible hosts for this production, but due to numerous favorable properties, cyanobacteria are considered of great interest. Compared to eukaryotic algae and plants, cyanobacteria are much easier to genetically engineer, and they grow faster. Furthermore, cyanobacteria are capable of growing under a wide variety of temperatures, light condition and salinities, and culturing of the bacteria does not compete with the food industry for arable land. Cyanobacterial model strains have been engineered to produce a wide variety of products, including sucrose, fatty acids, long-chain alcohols, alkanes, ethylene, polyhydroxybutyrate, 2,3-butanediol, and ethanol. However, the production rate is slow, and the price of commodity goods generated by cyanobacteria is too high to make the production economically competitive. More research and synthetic biology tools are needed to overcome these limitations (Liu et al., 2011, Berla et al., 2013, Taton et al., 2014, Markley et al., 2014).

This thesis represents a small contribution to the goal of giving *Synechococcus* and other cyanobacteria a toolbox of genetic parts, that can be utilized to establish the organisms as successful biotechnological platforms for production of renewable chemicals and fuels.

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APPENDIX A – Visualization of *Synechococcus* sp. PCC 7002 Neutral Integration Sites Utilized in this Project

Neutral integration site are sites within a genome that can tolerate insertion of DNA with no phenotypical or polar effects of adjacent regions of DNA (Niederholtmeyer et al., 2010).

Three such integration sites were utilized in this project to genetically engineer *Synechococcus* sp. PCC 7002, denoted A0160, A0935 and A2843. The sites were discovered by other scientists, and proved to be amenable to incorporation of DNA (Sakamoto et al., 1997, Begemann et al., 2013, Davies et al., 2014).

PCR primers were designed and ordered from Sigma-Aldrich, targeting flanking regions of the aforementioned sites within the *Synechococcus* genome, as well as homologous sequences in constructed counterselection plasmids. In this way, counterselection cassettes could be amplified in preparation for transformation, and integration of exogenous DNA within the *Synechococcus* genome could be verified.

The neutral integration sites and regions utilized to facilitate homologous recombination are demonstrated in Figure A-1. In colony PCR, forward primers target regions denoted neutral site 1 (NS1), and reverse primers neutral site 2 (NS2). Incorporation of exogenous DNA leads to deletion of sequences naturally occurring between NS1 and NS2 (blue).

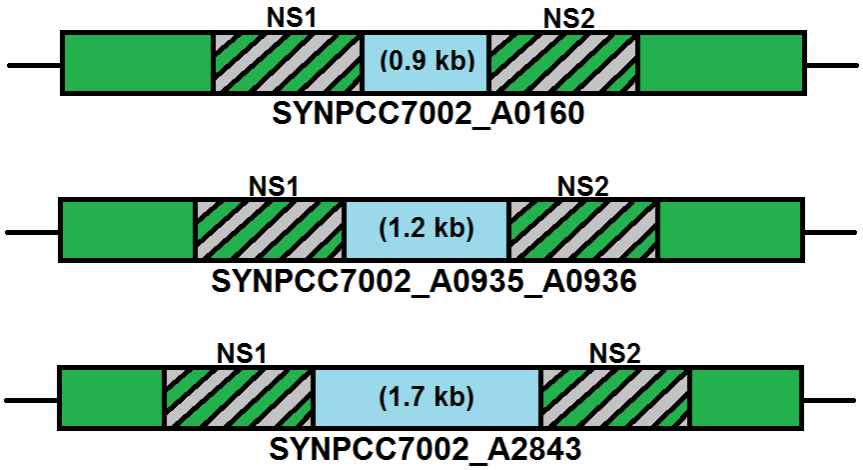

Figure A-1. Visualization of the three neutral integration sites within the *Synechococcus* sp. PCC 7002 genome, i.e. A0160, A0935 and A2843. DNA sequences homologous to two flanking regions of the neutral sites (NS1 and NS2) have been synthesized. The sequences are incorporated in the constructed counterselection plasmids as sequences flanking the constructed counterselection cassettes, and are utilized to facilitate homologous recombination and incorporation of cassettes within the *Synechococcus* genome. Incorporation leads to deletion of the sequences naturally occurring between NS1 and NS2 (blue).

APPENDIX B – DNA Ladders Utilized in this Project

DNA ladders are utilized during agarose gel electrophoresis as a means to investigate the size of DNA fragments present in samples from PCR or digestion reactions. By running the samples alongside a DNA ladder with DNA fragments of familiar sizes, the size of the DNA under study can be determined in comparison to the ladder. Two ladders were utilized in this project, i.e. GeneRuler™ 1 kb DNA Ladder and GeneRuler™ 1 kb Plus DNA Ladder. The ladders are presented in Figure B-1.

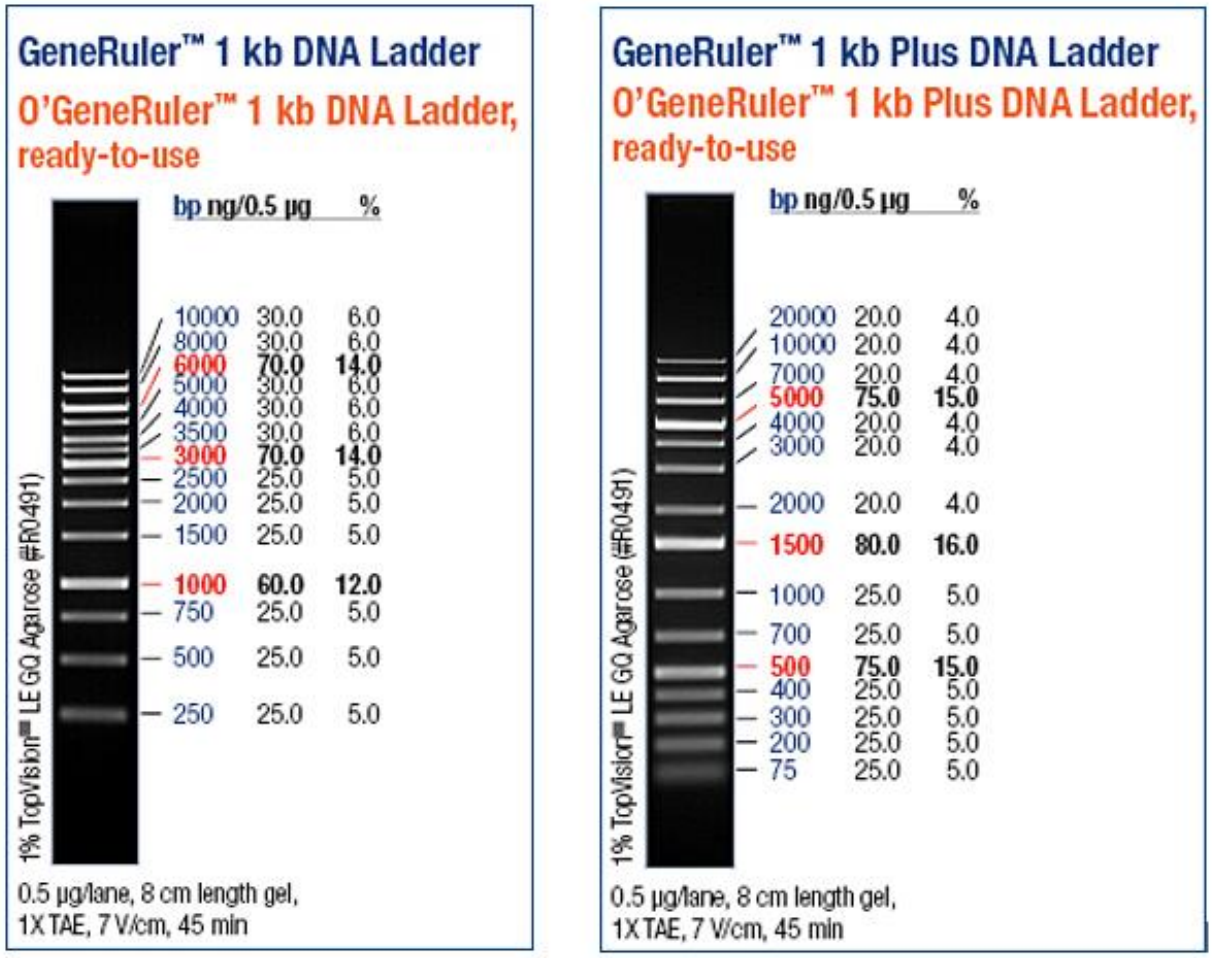

Figure B-1. DNA ladders utilized in this project.

APPENDIX C – Cloning Vectors Utilized in This Project

A cloning vector is a small piece of DNA that can self-replicate within a cell and be used for carrying cloned genes or segments of DNA. A variety of DNA molecules can be utilized as vectors. The DNA should be small and manageable, be easy to move from cell to cell, and it should be straightforward to generate and purify large amounts of vector DNA. Bacterial plasmids are by far the most popular choice, as well as small viral genomes (Clark and Pazdernik, 2013).

The cloning vectors utilized in this project are presented in Figure C-1 and Figure C-2. pK19mobsacB was used to amplify the *sacB* counterselection gene, coding for an enzyme called levansucrase. Furthermore, pLitmus28i-ChlorR was used to amplify the *Tn9cat* gene, conferring chloramphenicol resistance. Together with the *kan*^{r} gene from pA0160-KanR, *sacB* and *Tn9cat* were utilized to create counterselection cassettes. The cassettes were thereafter incorporated into vectors backbones of pA0160, pA0935 and pA2843, which together constitute the complete counterselection plasmids created in this project.

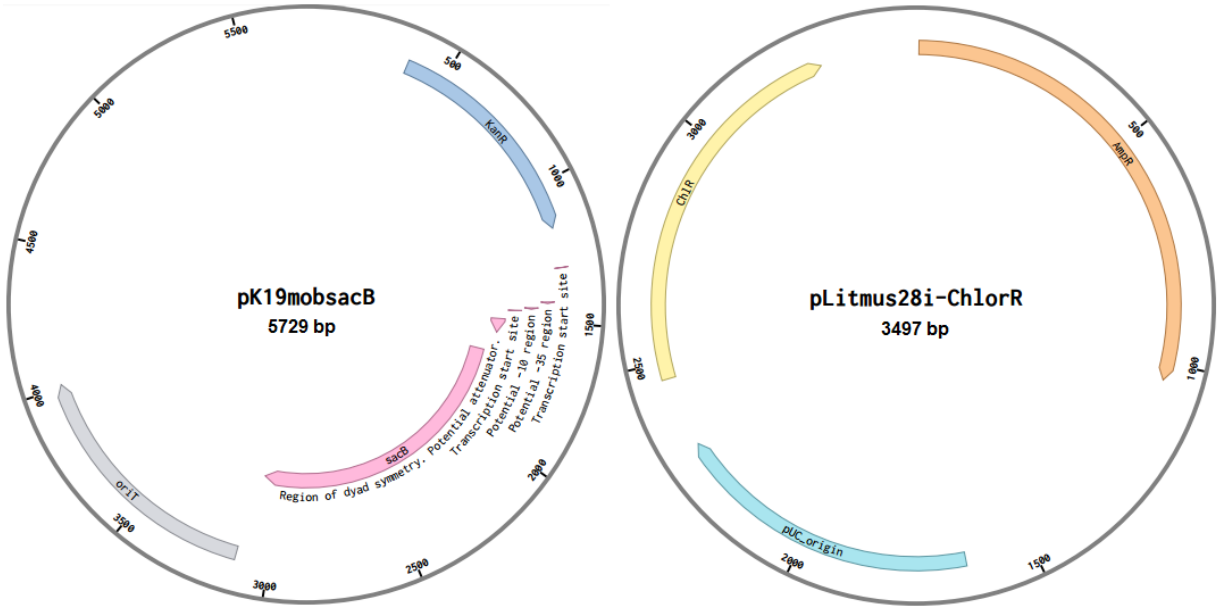

Figure C-1. Visualization of the cloning vectors pK19mobsacB and pLitmus28i-ChlorR. The plasmids are partly annotated, with emphasis on sequences of interest. pK19mobsacB was utilized to amplify the *sacB* counterselection gene (pink), coding for an enzyme called levansucrase. The plasmid also contains a kanamycin resistance gene, used for positive selection of transformants. pLitmus28i-ChlorR was utilized to amplify the *Tn9cat* gene (yellow), which confers chloramphenicol resistance. The plasmid maps are collected from Benchling.

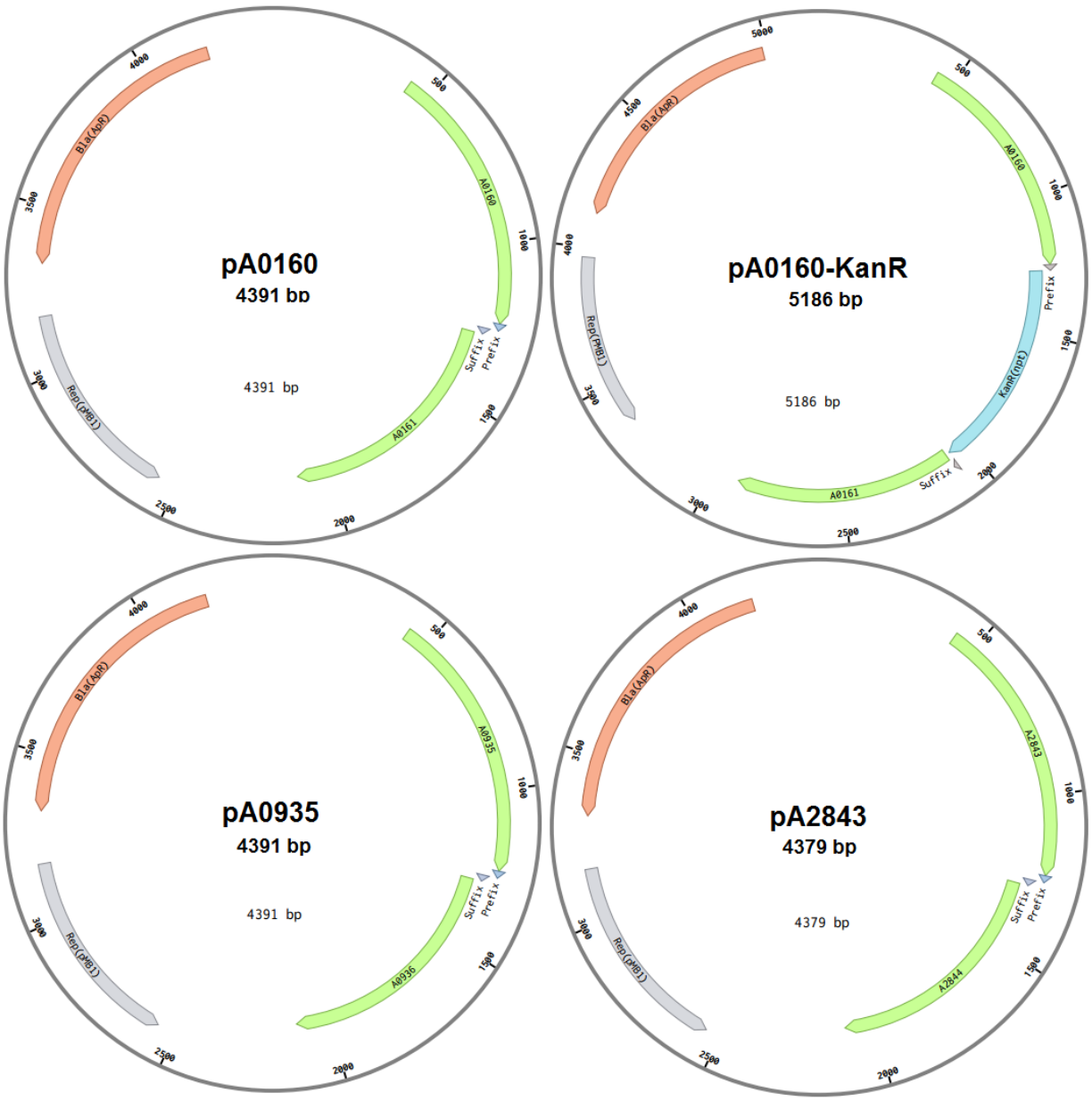

Figure C-2. Visualization of the cloning vectors pA0160, pA0160-KanR, pA0935 and pA2843. The plasmids are partly annotated, with emphasis on sequences of interest. A0160, A0935 and A2843 (green) refers to DNA fragments identical to three neutral integration sites within the genome of *Synechococcus* sp. PCC 7002. The neutral sites are incorporated into a vector backbone of pUC57-Simple, and contains BioBrick cloning sites, i.e. a prefix and a suffix, to facilitate molecular cloning. Two of the structures in the backbones are annotated, i.e. the *bla* gene (red) providing ampicillin resistance, and the origin of replication from the pMB1 plasmid (grey). KanR (blue) refers to the *kan*^{r} gene, providing kanamycin resistance. The plasmid maps are collected from Benchling.

APPENDIX D – PCR Primers and Expected Sizes of Amplicons

Polymerase Chain Reaction (PCR) is a powerful method utilized in molecular biology to amplify a specific sequence of DNA *in vitro*. PCR requires two oligonucleotide primers, designed to bind flanking regions at each side of the DNA sequence to be amplified (Reece, 2004). The primers utilized in this project are presented in Table D-1, and the annealing temperatures utilized for thermocycling in Table D-2. Expected sizes of selected amplicons are presented in Table D-3.

Table D-1. PCR primers utilized in this project. Important primer characteristics are presented, including sequence, target sequence, length and GC-content. The melting temperature (Tm) of each primer is calculated for both Q5 High-Fidelity DNA Polymerase and *Taq* DNA Polymerase, utilizing the Tm calculator from New England Biolabs Inc (NEB(c)). Forward primers are denoted FP and reverse primers RP. All primers are ordered from Sigma-Aldrich. The annealing temperatures utilized for thermocycling are denoted in Table D-2.

Primers	Sequence (5'-3')	Target	Length	GC-content	Tm (Q5)	Tm (Taq)
A0160_800_FP	CCGATTAGACCCTAAATTTC	A0160 *Synecho.* Neutral Site	20 nt	40%	55°C	47
A0160_800_RP	TTTCTGCAAGGCGTTGTTGG		20 nt	50%	65°C	57
A0160_400_FP	TACCGCCAGGATCGGAAAGC		20 nt	60%	68	61
A0160_400_RP	CACCAGGGCTAACACTTCAG		20 nt	55%	63	56
A0160_200_FP	ATCGCCAGGGCCAAACAATG		20 nt	55%	67	59
A0160_200_RP	TCTCGATCATAGGCGATCTC		20 nt	50%	61	53
A0160_100_FP	ATGCAAAGTACAGTCCGCTC		20 nt	50%	63	55
A0160_100_RP	CGGCTGGTAATACAAGTGAG		20 nt	50%	61	53
A0935_800_FP	AGTTTCCGGATCGATGAACC	A0935 *Synecho.* Neutral Site	20 nt	50%	63	55
A0935_800_RP	AATTGAGACTTCAATTTATC		20 nt	25%	49	41
A0935_400_FP	CGCCCTTCCTTTGTGCTCAG		20 nt	60%	67	60
A0935_400_RP	CTGTTTACAGCAGCCCTTAG		20 nt	50%	61	53
A0935_200_FP	CCAATCTTCAGCGGAAACAG		20 nt	50%	62	54
A0935_200_RP	ACCACGCCAATGTCTACACC		20 nt	55%	66	58
A0935_100_FP	ACGGGCTTCTAGCACAAATG		20 nt	50%	63	56
A0935_100_RP	CGGTACAGATGTCTTGATCG		20 nt	50%	60	53
A2843_800_FP	CGATAATCCAGCAACCCAAC	A2843 *Synecho.* Neutral Site	20 nt	50%	62	54
A2843_800_RP	TAGTAATAATTCAGCAGCAC		20 nt	35%	54	46
A2843_400_FP	TTATGGAGGATGGCCACGAG		20 nt	55%	65	57
A2843_400_RP	AATCTGGCACTGTGGCAAGG		20 nt	55%	66	59
A2843_200_FP	CCAGCAGCGGCTTGTGTTAG		20 nt	60%	67	60
A2843_200_RP	CGTTGGTGAGGACAAAGAAG		20 nt	50%	61	54
A2843_100_FP	CGATCGCCACACCGACTTTG		20 nt	60%	67	60
A2843_100_RP	TCTAGATCTTGAATAATGAG		20 nt	30%	50	42
BioBrick_FP	GAATTCGCGGCCGCTTCTAGAG	Prefix	22 nt	59%	69	62
BioBrick_RP	CTGCAGCGGCCGCTACTAGTA	Suffix	21 nt	62%	70	63
Chlo_FP	GAATTCGCGGCCGCTTCTAGAG ATGAGACGTTGATCGGCACG	Tn9 *cat* (pLitmus 28i)	42 nt	57%	81	73
Chlo_RP	CTGCAGCGGCCGCTACTAGTA ATTCAGGCGTAGCACCAGGC		41 nt	61%	83	75
sacB_FP	GAATTCGCGGCCGCTTCTAGAG CACATATACCTGCCGTTCAC	*sacB* (pK19 mobsacB)	42 nt	55%	80	72
sacB_RP	CTGCAGCGGCCGCTACTAGTA ATTTCGCTCGGTACCCATCG		41 nt	59%	81	74

Table D-2. Annealing temperatures utilized in this project. The melting temperatures (Tm) in Table D-1 are used to calculate the recommended annealing temperatures (Ta) of each primer pair. Ta is calculated for both Q5 High-Fidelity DNA Polymerase ($Ta=Tm_{lower}+3°C$) and Taq DNA Polymerase ($Ta=Tm_{lower}-5°C$), utilizing the Tm-calculator from New England Biolabs Inc (NEB(c)). All primers are ordered from Sigma-Aldrich.

Primer pairs	Target sequence	Ta (Q5 Pol.)	Ta (Taq Pol.)
A0160_800_FP A0160_800_RP	A0160 *Synecho.* Neutral Site	58°C	42°C[2]
A0160_400_FP A0160_400_RP	A0160 *Synecho.* Neutral Site	66°C	51°C
A0160_200_FP A0160_200_RP	A0160 *Synecho.* Neutral Site	64°C	48°C
A0160_100_FP A0160_100_RP	A0160 *Synecho.* Neutral Site	64°C	48°C
A0935_800_FP A0935_800_RP	A0935 *Synecho.* Neutral Site	52°C	36°C[2]
A0935_400_FP A0935_400_RP	A0935 *Synecho.* Neutral Site	64°C	48°C
A0935_200_FP A0935_200_RP	A0935 *Synecho.* Neutral Site	65°C	49°C
A0935_100_FP A0935_100_RP	A0935 *Synecho.* Neutral Site	63°C	48°C
A2843_800_FP A2843_800_RP	A2843 *Synecho.* Neutral Site	57°C	41°C[2]
A2843_400_FP A2843_400_RP	A2843 *Synecho.* Neutral Site	68°C	52°C
A2843_200_FP A2843_200_RP	A2843 *Synecho.* Neutral Site	64°C	49°C
A2843_100_FP A2843_100_RP	A2843 *Synecho.* Neutral Site	53°C	37°C[2]
BioBrick_FP BioBrick_RP	Prefix Suffix	72°C	57°C
Chlo_FP Chlo_RP	Tn9 *cat* (pLitmus28i)	72°C[1]	68°C
sacB_FP sacB_RP	*sacB* (pK19mobsacB)	72°C[1]	67°C

[1]According to the formula for Q5® High-Fidelity DNA polymerase, the chloramphenicol and sacB primer pairs have a Ta of 84°C and 83°C. As this value is outside the recommended annealing range (50-72°C), the temperatures are set to the highest value within range.

[2]The Ta are below the recommended annealing range of *Taq* DNA polymerase (45-68°C).

Table D-3. Expected sizes of selected amplicons.

	BioBrick F+R[1]	800 F+R[2]	400 F+R[2]
Counterselection plasmids			
Without insert	0.0 kb	1.6 kb	0.8 kb
With KanR	0.8 kb	2.4 kb	1.6 kb
With ChlR	0.9 kb	2.5 kb	1.7 kb
With *sacB*	1.9 kb	3.5 kb	2.7 kb
With KanR-sacB	2.9 kb	4.5 kb	3.7 kb
With ChlR-KanR-sacB	3.7 kb	5.3 kb	4.5 kb
Wild-Type Synechococcus			
A0160 without insert	-	2.5 kb	1.7 kb
A0935 without insert	-	2.8 kb	2.0 kb
A2843 without insert	-	3.3 kb	2.5 kb
A0160 with KanR-sacB	2.9 kb	4.5 kb	3.7 kb
A0935 with KanR-sacB	2.9 kb	4.5 kb	3.7 kb

[1]F refers to forward primers and R refers to reverse primers.

[2]A0160-800 primers used to amplify A0160-sequences and so on. The length might vary with a few nucleotides depending on which neutral site is under study.

APPENDIX E – Clustal Omega Multiple Sequence Alignment

Sequencing was utilized to examine the counterselection cassette within pA0160-KanR-sacB, to verify that both the kanamycin resistance gene and the *sacB* gene was incorporated into the vector backbone.

The sequencing was conducted by GATC Biotech, and the resulting sequences analyzed by multiple sequence alignment utilizing the Clustal Omega tool (version 1.2.1) from EMBL-EBI. The following alignment was downloaded from the Clustal Omega internet page, and the sequence was annotated in Microsoft Word.

P-KAN-sacB-S refers to the DNA sequence collected from Benchling, used as a reference. Forward and Reverse refers to the two sequencing results: one utilizing prefix as a forward primer, and the other suffix as a reverse primer. The fragment was thereby sequenced from both sides, in two separate reactions.

The blue and yellow markings within the P-KAN-sacB-S reference sequence correspond to the kanamycin resistance gene coding sequence (blue) and the *sacB* coding sequence (yellow), respectively. The green markings are sequences were the sequencing results are identical to the aforementioned coding sequences within the reference DNA.

The alignment verifies that both the kanamycin resistance gene and the *sacB* gene are present in the purified counterselection plasmids utilized as a template for sequencing.

```
Forward          ------------------------------------------------------------ 0
P-KAN-sacB-S     tgaattcgcggccgcttctagaggcgaaacgatcctcatcctgtctcttgatcagatctt 60
Reverse          ------------------------------------------------------------ 0

Forward          ---------------------GTAAACTGGATGGCTTTCTTGCCGCAGGATGGCGCAGGG 39
P-KAN-sacB-S     gatcccctgcgccatcagatccttggcggcaagaaagccatccagtttactttgcagggc 120
Reverse          ------------------------------------------------------------ 0

Forward          GATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGAT 99
P-KAN-sacB-S     ttcccaaccttaccagagggcgccccagctggcaattccggatgattgaacaagatggat 180
Reverse          ------------------------------------------------------------ 0

Forward          TGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAAC 159
P-KAN-sacB-S     tgcacgcaggttctccggccgcttgggtggagaggctattcggctatgactgggcacaac 240
Reverse          ------------------------------------------------------------ 0

Forward          AGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTC 219
P-KAN-sacB-S     agacaatcggctgctctgatgccgccgtgttccggctgtcagcgcaggggcgcccggttc 300
Reverse          ------------------------------------------------------------ 0
```

```
Forward          TTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAAGACGAGGCAGCGCGGC 279
P-KAN-sacB-S     ttttgtcaagaccgacctgtccggtgccctgaatgaactgcaagacgaggcagcgcggc 360
Reverse          ------------------------------------------------------------ 0

Forward          TATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAG 339
P-KAN-sacB-S     tatcgtggctggccacgacgggcgttccttgcgcagctgtgctcgacgttgtcactgaag 420
Reverse          ------------------------------------------------------------ 0

Forward          CGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACC 399
P-KAN-sacB-S     cgggaagggactggctgctattgggcgaagtgccggggcaggatctcctgtcatctcacc 480
Reverse          ------------------------------------------------------------ 0

Forward          TTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTG 459
P-KAN-sacB-S     ttgctcctgccgagaaagtatccatcatggctgatgcaatgcggcggctgcatacgcttg 540
Reverse          ------------------------------------------------------------ 0

Forward          ATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTC 519
P-KAN-sacB-S     atccggctacctgcccattcgaccaccaagcgaaacatcgcatcgagcgagcacgtactc 600
Reverse          ------------------------------------------------------------ 0

Forward          GGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGC 579
P-KAN-sacB-S     ggatggaagccggtcttgtcgatcaggatgatctggacgaagagcatcaggggctcgcgc 660
Reverse          ------------------------------------------------------------ 0

Forward          CAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGATGATCTCGTCGTGA 639
P-KAN-sacB-S     cagccgaactgttcgccaggctcaaggcgcgcatgcccgacggcgatgatctcgtcgtga 720
Reverse          ------------------------------------------------------------ 0

Forward          CCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGA-TCA 698
P-KAN-sacB-S     cccatggcgatgcctgcttgccgaatatcatggtggaaaatggccgcttttctggattca 780
Reverse          ------------------------------------------------------------ 0

Forward          TCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTG 758
P-KAN-sacB-S     tcgactgtggccggctgggtgtggcggaccgctatcaggacatagcgttggctacccgtg 840
Reverse          ------------------------------------------------------------ 0

Forward          ATATG-CTGAAGAGCTTGGCGGCGAATGGGCTGACGCTTCCTCGTGCTTTTACGGTATCG 817
P-KAN-sacB-S     atattgctgaagagcttggcggcgaatgggctgaccgcttcctcgtgctttacggtatcg 900
Reverse          ------------------------------------------------------------ 0

Forward          CCGCTCCCGATTCGCACGCATCGCCT--TCATCGCCTTCTTGACGAGTTTCTCTGATAAT 875
P-KAN-sacB-S     ccgctcccgattcgcagcgcatcgccttctatcgccttcttgacgagttcttctgataCT 960
Reverse          ------------------------------------------------------------ 0

Forward          AATACTAGACACATATACCTGCCGTTCACTATATTTAGTGAAATGGAGATATTGAT--AT 933
P-KAN-sacB-S     AGA-----GCACATATACCTGCCGTTCACTATTATTTAGTGAAATGAGATATTATGATAT 1015
Reverse          ------------------------------------------------------------ 0

Forward          TTTCTGAA---------------------------------------------------- 941
P-KAN-sacB-S     TTTCTGAATTGTGATTAAAAAGGCAACTTTATGCCCATGCAACAGAAACTATAAAAAATA 1075
Reverse          ------------------------------------------------------------ 0
```

Appendix

```
Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     CAGAGAATGAAAAGAAACAGATAGATTTTTTAGTTCTTTAGGCCCGTAGTCTGCAAATCC 1135
Reverse          ------------------------------------------------------------ 0

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     TTTTATGATTTTCTATCAAACAAAAGAGGAAAATAGACCAGTTGCAATCCAAACGAGAGT 1195
Reverse          ------------------------------------------------------------ 0

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     CTAATAGAATGAGGTCGAAAAGTAAATCGCGCGGGTTTGTTACTGATAAAGCAGGCAAGA 1255
Reverse          ------------------------------------------------------------ 0

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     CCTAAAATGTGTAAAGGGCAAAGTGTATACTTTGGCGTCACCCCTTACATATTTTAGGTC 1315
Reverse          ------------------------------------------------------------ 0

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     TTTTTTTATTGTGCGTAACTAACTTGCCATCTTCAAACAGGAGGGCTGGAAGAAGCAGAC 1375
Reverse          ------------------------------------------------------------ 0

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     CGCTAACACAGTACATAAAAAAGGAGACATGAACGATGAACATCAAAAAGTTTGCAAAAC 1435
Reverse          ------------------------------------------------------------ 0

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     AAGCAACAGTATTAACCTTTACTACCGCACTGCTGGCAGGAGGCGCAACTCAAGCGTTTG 1495
Reverse          ------------------------------------------------------------ 0

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     CGAAAGAAACGAACCAAAAGCCATATAAGGAAACATACGGCATTTCCCATATTACACGCC 1555
Reverse          ------------------------------------------------------------ 0

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     ATGATATGCTGCAAATCCCTGAACAGCAAAAAAATGAAAAATATCAAGTTTCTGAATTTG 1615
Reverse          ------------------------------------------------------------ 0

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     ATTCGTCCACAATTAAAAATATCTCTTCTGCAAAAGGCCTGGACGTTTGGGACAGCTGGC 1675
Reverse          ------------------------------------------------------------ 0

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     CATTACAAAACGCTGACGGCACTGTCGCAAACTATCACGGCTACCACATCGTCTTTGCAT 1735
Reverse          GGCCATTAAACGCTGAC------GGCACTGCAAACTTCACGGCTACCATCGTCTTTGCAT 54

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     TAGCCGGAGATCCTAAAAATGCGGATGACACATCGATTTACATGTTCTATCAAAAAGTCG 1795
Reverse          TAGCCGGAGATCCTAAAAATGCGGATGACACATCGATTTACATGTTCTATCAAAAAGTCG 114

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     GCGAAACTTCTATTGACAGCTGGAAAAACGCTGGCCGCGTCTTTAAAGACAGCGACAAAT 1855
Reverse          GCGAAACTTCTATTGACAGCTGGAAAAACGCTGGCCGCGTCTTTAAAGACAGCGACAAAT 174
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Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     TCGATGCAAATGATTCTATCCTAAAAGACCAAACACAAGAATGGTCAGGTTCAGCCACAT 1915
Reverse          TCGATGCAAATGATTCTATCCTAAAAGACCAAACACAAGAATGGTCAGGTTCAGCCACAT 234

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     TTACATCTGACGGAAAAATCCGTTTATTCTACACTGATTTCTCCGGTAAACATTACGGCA 1975
Reverse          TTACATCTGACGGAAAAATCCG-TTATTCTACACTGATTTCTCCGGTAAACATTACGGCA 293

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     AACAAACACTGACAACTGCACAAGTTAACGTATCAGCATCAGACAGCTCTTTGAACATCA 2035
Reverse          AACAAACACTGACAACTGCACAAGTTAACGTATCAGCATCAGACAGCTCTTTGAACATCA 353

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     ACGGTGTAGAGGATTATAAATCAATCTTTGACGGTGACGGAAAAACGTATCAAAATGTAC 2095
Reverse          ACGGTGTAGAGGATTATAAATCAATCTTTGACGGTGACGGAAAAACGTATCAAAATGTAC 413

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     AGCAGTTCATCGATGAAGGCAACTACAGCTCAGGCGACAACCATACGCTGAGAGATCCTC 2155
Reverse          AGCAGTTCATCGATGAAGGCAACTACAGCTCAGGCGACAACCATACGCTGAGAGATCCTC 473

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     ACTACGTAGAAGATAAAGGCCACAAATACTTAGTATTTGAAGCAAACACTGGAACTGAAG 2215
Reverse          ACTACGTAGAAGATAAAGGCCACAAATACTTAGTATTTGAAGCAAACACTGGAACTGAAG 533

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     ATGGCTACCAAGGCGAAGAATCTTTATTTAACAAAGCATACTATGGCAAAAGCACATCAT 2275
Reverse          ATGGCTACCAAGGCGAAGAATCTTTATTTAACAAAGCATACTATGGCAAAAGCACATCAT 593

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     TCTTCCGTCAAGAAAGTCAAAAACTTCTGCAAAGCGATAAAAAACGCACGGCTGAGTTAG 2335
Reverse          TCTTCCGTCAAGAAAGTCAAAAACTTCTGCAAAGCGATAAAAAACGCACGGCTGAGTTAG 653

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     CAAACGGCGCTCTCGGTATGATTGAGCTAAACGATGATTACACACTGAAAAAAGTGATGA 2395
Reverse          CAAACGGCGCTCTCGGTATGATTGAGCTAAACGATGATTACACACTGAAAAAAGTGATGA 713

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     AACCGCTGATTGCATCTAACACAGTAACAGATGAAATTGAACGCGCGAACGTCTTTAAAA 2455
Reverse          AACCGCTGATTGCATCTAACACAGTAACAGATGAAATTGAACGCGCGAACGTCTTTAAAA 773

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     TGAACGGCAAATGGTACCTGTTCACTGACTCCCGCGGATCAAAAATGACGATTGACGGCA 2515
Reverse          TGAACGGCAAATGGTACCTGTTCACTGACTCCCGCGGATCAAAAATGACGATTGACGGCA 833

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     TTACGTCTAACGATATTTACATGCTTGGTTATGTTTCTAATTCTTTAACTGGCCCATACA 2575
Reverse          TTACGTCTAACGATATTTACATGCTTGGTTATGTTTCTAATTCTTTAACTGGCCCATACA 893

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     AGCCGCTGAACAAAACTGGCCTTGTGTTAAAAATGGATCTTGATCCTAACGATGTAACCT 2635
Reverse          AGCCGCTGAACAAAACTGGCCTTGTGTTAAAAATGGATCTTGATCCTAACGATGTAACCT 953
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Appendix

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Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     TTACTTACTCACACTTCGCTGTACCTCAAGCGAAAGGAAACAATGTCGTGATTACAAGCT 2695
Reverse          TTACTTACTCACACTTCGCTGTACCTCAAGCGAAAGGAAACAATGTCGTGATTACAAGCT 1013

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     ATATGACAAACAGAGGATTCTACGCAGACAAACAATCAACGTTTGCGCCGAGCTTCCTGC 2755
Reverse          ATATGACAAACAGAGGATTCTACGCAGACAAACAATCAACGTTTGCGCCGAGCTTCCTGC 1073

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     TGAACATCAAAGGCAAGAAAACATCTGTTGTCAAAGACAGCATCCTTGAACAAGGACAAT 2815
Reverse          TGAACATCAAAGGCAAGAAAACATCTGTTGTCAAAGACAGCATCCTGAACAAGGACAATT 1133

Forward          ------------------------------------------------------------ 941
P-KAN-sacB-S     TAACAGTTAACAAATAAAAACGCAAAAGAAAATGCCGATGGGTACCGAGCGAAATTACTA 2875
Reverse          -AACAGTAACAATAAAACGC--------------------------------------- 1152

Forward          ----------- 941
P-KAN-sacB-S     GTAGCGGCCGC 2886
Reverse          ----------- 1152
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APPENDIX F – Calculations utilized for Preparation of Overnight Cultures of *Synechococcus* sp. PCC 7002

When starting the transformation procedure of *Synechococcus* sp. PCC 7002, the OD_{730} of the overnight culture need to be 0.4. If the OD_{730} exceeds this value, the culture cannot be used. It is therefore important to be precise with the setup of the overnight culture. The amount of wildtype cells to be added depends on the OD_{730} of the starter culture used, amount of fresh growth media, planned incubation time, doubling time of the cells, and the end OD_{730} that should be reached. The calculations are demonstrated in equation **(1) (2)** below, followed by an example.

Since the cells were not grown under optimal conditions, the doubling time needed to be estimated under the specified growth conditions. This was achieved by another member of the PhotoSynLab research group, by conducting several OD_{730} measurements at specific time intervals. The doubling time was estimated to be 6.84 hours, as compared to the doubling time of 2.6 hours reported under optimal conditions (Chen and Widger, 1993, Ludwig and Bryant, 2012).

FIND START OD:

$$Start\ OD\ (C2) = \frac{End\ OD}{10^{\frac{\left({}^{1}/_{Doubling\ time\ (hrs)}\right)*Incubation\ time\ (hrs)}{3.3}}} \quad \textbf{(1)}$$

FIND START AMOUNT:

$$Start\ amount\ (V1) = \frac{Start\ OD\ (C2) * Volume\ growth\ media\ (V2)}{OD\ overnightculture\ (C1)} \quad \textbf{(2)}$$

EXAMPLE:

$$C2 = \frac{0.4}{10^{\frac{\left({}^{1}/_{6.84}\right)*13}{3.3}}} = 0.106 \quad \textbf{(3)}$$

$$V1 = \frac{0.106 * 200\ mL}{1.06} = 20\ mL \quad \textbf{(4)}$$

In conclusion, when using a starter culture with OD_{730}=1.06 and a 13-hour incubation time, 20 mL wild-type cells should be added to 200 mL AA+ media to reach a final OD_{730} of 0.4 the following day.

APPENDIX G – Calculating Transformation Efficiency of Competent *Escherichia coli* DH5α

The process where free DNA molecules released from one bacterium is taken up by another is called transformation. Cells capable of taking up free DNA and be transformed are thereby called competent cells. Not all bacteria are naturally transformable, including *Escherichia coli*. However, cells can be made competent by treatment of high amounts of calcium ions. The procedure utilized in this project is adapted from a protocol by Julian Eaton-Rye (Madigan et al., 2012, Snustad and Simmons, 2012, Hanahan, 1983, Eaton-Rye, 2011).

The transformation efficiency was tested by transforming the cells with a plasmid of known concentration, grown on selective LA medium. An aliquot of competent cells was transformed with 1 µL of a plasmid solution of pTA16, with a concentration of 111.5 ng/µL. The plasmid contains a kanamycin resistance gene, which could be used to select for transformed cells harboring the plasmid. A dilution series was made, and 100 µL from each solution was plated on LA plates with 50 µg/mL kanamycin. In addition, a negative control was included with wild-type cells, to confer that only transformed cells are capable of growing in the presence of the antibiotic. By counting the number of colonies on each growth plate and taking into account the dilutions and the concentration of plasmid, the transformation efficiency could be estimated. Two of the plates had an appropriate amount of colonies to count, and these were utilized to calculate the transformation efficiency, as presented below. The plates are named D and E, in conjugation with the results presented in the thesis.

Plate D:

Colony forming units (cfu): 200
Dilutions: 1/6 x 1/10 x 1/10 = 1/600
Amount of plasmid DNA added: 1 µL / 111.5 ng/µL = 111.5 ng
Amount of plasmid DNA present in the sample: 111.5 ng x 1/600 = 1.86×10^{-4} µg DNA
Transformation efficiency: 200 cfu / 1.86×10^{-4} µg DNA = 1.1×10^{6} cfu/µg DNA

Plate E:

Colony forming units (cfu): 25
Dilutions: 1/6 x 1/10 x 1/10 x 1/10 = 1/6000
Amount of plasmid DNA added: 1 µL / 111.5 ng/µL = 111.5 ng
Amount of plasmid DNA present in the sample: 111.5 ng x 1/6000 = 1.86×10^{-5} µg DNA
Transformation efficiency: 25 cfu / 1.86×10^{-5} µg DNA = 1.3×10^{6} cfu/µg DNA

In conclusion, the competent cells have a transformation efficiency of 1.2×10^{6} cfu/µg DNA.

APPENDIX H – OD_{730} Measurements Obtained from NaCl Growth Experiment in *Synechococcus* sp. PCC 7002

A growth experiment was conducted with wild-type *Synechococcus* sp. PCC 7002 to investigate whether the cells were able to grow under NaCl limiting conditions. Media was prepared with 0, 25, 50 and 100 NaCl, compared to the amount usually present within AA+ media. The cells were grown with agitation for 3 days, with the cell growth measured at OD_{730} twice a day. The results obtained are presented in Table H-1.

Table H-1. OD_{730} measurements obtained in NaCl growth experiment. The experiment was conducted with wild-type Synechococcus sp. PCC 7002, grown in liquid AA+ media with different amounts of NaCl. The liquid cultures were incubated at 33°C and 90 $\mu Em^{-2}s^{-1}$, with 300 rpm shaking.

OD_{730} measured:	0% NaCl:	25% NaCl:	50% NaCl:	100% NaCl:
Start	0.013	0.012	0.010	0.012
18 hrs.	0.020	0.029	0.026	0.023
28 hrs.	0.058	0.102	0.118	0.121
43 hrs.	0.062	0.245	0.275	0.264
51 hrs.	0.117	0.338	0.389	0.354
65 hrs.	0.284	0.529	0.604	0.551
77 hrs.	0.471	0.717	0.792	0.676