Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Tora Selvåg Braadland

Alginate Biosynthesis in *Pseudomonas fluorescens* SBW25: Investigating Regulatory Aspects Involving Levansucrase and c-di-GMP

Master's thesis in Biotechnology
Supervisor: Helga Ertesvåg
May 2023

NTNU
Norwegian University of
Science and Technology

Tora Selvåg Braadland

Alginate Biosynthesis in *Pseudomonas fluorescens* SBW25: Investigating Regulatory Aspects Involving Levansucrase and c-di-GMP

Master's thesis in Biotechnology
Supervisor: Helga Ertesvåg
May 2023

Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

NTNU
Norwegian University of
Science and Technology

Abstract

Alginate is a widely utilised polysaccharide in the food and medicine industries, which is composed of the covalently linked monomers mannuronic acid (M) and its epimer guluronic acid (G). The composition of the monomers make alginate versatile, which contribute to commercial value. Alginate is primarily synthesised by brown algae but can also be synthesised by bacterial genera such as *Pseudomonas* and *Aztobacter.* All commercial available alginate today derives from algae, but bacterial alginate has great potential for the industry to gain purer and more predictable alginate composition.

Pseudomonas fluorescens can be genetically modified to synthesise alginate which is interesting to the industry, and the genes controlling the synthesis can be investigated. This study aimed to gain insight into genes and metabolites believed to influence alginate synthesis. It was tested through a system measuring levels of c-di-GMP, a second messenger positively regulating the alginate synthesis, and the importance of the levan-synthesising enzyme levansucrase on alginate synthesis was evaluated.

c-di-GMP positively regulates alginate synthesis by binding and activating Alg44, an essential constituent in the alginate synthesising complex. Measuring the cells' c-di-GMP values gives valuable information regarding alginate synthesis and growth. An altered version of the existing dual-fluorescence Bc3-5 c-di-GMP reporter system was constructed by replacing the reporter protein TurboRFP with mCherry_V1, exhibiting a fluorescens not interfering with the autofluorescence of *P. fluorescens*. The system was tested with alginate-producing mutants and mucR overexpressing mutants. Results implicated that the system effectively measured c-di-GMP levels in the cell, with levels being lowest in the alginate-producing strain, and that overexpression of mucR could negatively regulate alginate synthesis.

As of today, a negative selection marker for homologous recombination in *P. fluorescens,* without giving resistance towards antibiotics, is lacking. I-SceI is a homing endonuclease controlled by the TetR promoter, which is induced by anhydrotetracycline (ATc). In the presence of ATc, I-SceI is transcribed and cleaves the *I-sceI* recognition site, causing lethal double-stranded cuts. This study aimed to test the ability to use this I-SceI endonuclease system for negative counter-selection to select cells having undergone the second homologous recombination event. It was tested when selecting for *lsc* negative strains. However, results indicated that this system was not functioning.

The gene *lsc* encodes for levansucrase, an enzyme catalysing the synthesis of levan from sucrose. Levan is a polysaccharide, located extracellularly in the biofilm as a nutrient storage. Levan and alginate synthesis competes for carbon and energy in the cells. Therefore, the hypothesis was: removing lsc and the subsequent levan synthesis, will result in a higher alginate yield. This study aimed to investigate the effect of removing *lsc* from the chromosomal DNA of *P. fluorescens* SBW25 and the alginate-producing mutant *P. fluorescens* SBW25 *mucA* have on alginate synthesis and growth when cultivating the cells on different carbon sources. *lsc* negative strains were made. Results suggested that *lsc* is needed for growth and alginate production when cultivating in sucrose, and that the presence or absence of *lsc* does not affect alginate synthesis when cultivating the cells in fructose as the sole carbon source.

Sammendrag

Alginat er en mye brukt polysakkarid i mat- og legemiddelindustrien. Den består av de kovalent bundne monomerene mannuronsyre (M) og, dens epimer, guluronsyre (G). Sammensetningen av monomerene gjør alginat allsidig, hvilket bidrar til den kommersielle verdien. Alginat blir hovedsakelig syntetisert av brunalger, men kan også bli produsert av bakterieslekter som *Pseudomonas* og *Azotobacter.* Kommersielt alginat kommer i dag fra brunalger, men bakterielt alginat gir mulighet for industrien å oppnå renere og mer forutsigbar alginatsammensetning.

Pseudomonas fluorescens er genetisk modifisert til å syntetisere industrielt interessant alginat, og genene som kontrollerer syntesen kan undersøkes. Denne oppgavens mål var studere gener og metabolitter som antas å påvirke alginatsyntesen. Et system ble testet for å måle nivåene av c-di-GMP, en sekundær budbringer som positivt regulerer alginatsyntesen. Betydningen av levansucrase, enzymet som katalyserer syntesen av polysakkaridet levan, for alginatsyntesen og vekst ble evaluert.

c-di-GMP regulerer alginatsyntesen positivt ved å binde seg til og aktivere Alg44, en viktig komponent i alginatsyntese-proteinkomplekset. Måling av cellenes c-di-GMP-verdier kan gi verdifull informasjon, sammen med målinger av alginat og vekst, av hvordan c-di-GMP nivåene påvirker alginatsyntesen. En endret versjon av det eksisterende dobbelt-fluorescerende Bc3-5 c-di-GMP-rapporterings-systemet ble konstruert ved å erstatte det fluorescerende proteinet TurboRFP med mCherry_V1, som viser en fluorescens som ikke overlapper fluorescensen til *P. fluorescens*. Systemet ble testet med mutanter som produserer alginat og mutanter som over-uttrykker *mucR*. Resultatene viser at systemet effektivt målte c-di-GMP-nivåene i cellene, c-di-GMP-nivåene var lavest i alginat-produserende *P. fluorescens*, og overuttrykk av *mucR* kan påvirke alginatsyntesen negativt.

I dag mangler det en negativ seleksjonsmarkør for å kunne gjøre homolog rekombinering i *P. fluorescens* uten å gi den antibiotikaresistens. I-SceI er en endonuklease og kontrolleres av TetR-promotoren, som blir indusert av anhydrotetracyklin (ATc). Ved induksjon vil I-SceI bli transkribert og kutte I-SceI-gjenkjennings setet, som fører til et letalt dobbeltrådig kutt. Denne oppgaven testet muligheten for å bruke dette systemet som negativ seleksjon, for å selektere for celler som har gjennomgått det andre homologe rekombinasjonssteget. Systemet ble testet under konstruksjon av *lsc*-negative stammer. Resultatene indikerte imidlertid at det ikke fungerte.

lsc-genet koder for levansukrase, et enzym som katalyserer syntesen av levan fra sukrose. Levan er et polysakkarid som finnes ekstracellulært i biofilmen hvor det fungerer som et næringslager. Levan- og alginatsyntese konkurrerer om karbon og energi i cellene. Hypotesen var derfor: Fjerning av *lsc*, og dermed også syntesen av levan, vil føre til økt alginatmengde. Denne oppgaven undersøkte effekten av å fjerne *lsc* fra det kromosomale DNA-et til *P. fluorescens* SBW25 og den alginatproduserende mutanten *P. fluorescens* SBW25 *mucA.* Effekten av alginatsyntese og vekst når cellene dyrkes på ulike karbonkilder ble også undersøkt. *lsc*-negative stammer ble konstruert, og alginat-mengde ble målt. Resultatene antydet at lsc er nødvendig for vekst og alginatproduksjon når man dyrker cellene på sukrose som eneste karbonkilde, og at tilstedeværelsen eller fraværet av *lsc* ikke påvirker alginatsyntesen når fruktose er eneste karbonkilde.

Preface

The work described in this master thesis in Biotechnology was carried out between August 2022 and May 2023, at the Department of Biotechnology and Food Science at the Norwegian University of Science and Technology (NTNU) in Trondheim. It was written in collaboration with my supervisor Helga Ertesvåg.

Through this thesis, I have learned a lot about laboratory work, planning, patience and academic writing. Including, of course, the regulatory principles of *Pseudomonas fluorescens* regarding alginate synthesis.

First, I would like to thank my supervisor Helga Ertesvåg for being available and having open doors whenever I had a question or a problem. The fast responses and wise discussions helped me a lot.

I would also thank my classmates and friends for the lunches, coffee breaks, and discussions about everything and nothing. Additionally, I would like to thank my family for the support throughout the year. Especially my sister, Anna Selvåg Braadland, for guidance and help.

Lastly, I would like to thank my partner, Lars Isachsen Vassbø, for supporting me with food and love.

List of Contents

Abbreviations

P. fluorescens	*Pseudomonas fluorescens*
P. aeruginosa	*Pseudomonas aeruginosa*
P. syringae	*Pseudomonas syringae*
P. putida	*Pseudomonas putida*
M	β-D-mannuronic acid
G	α-L-guluronic acid
F6P	Fructose-6-phosphate
Man6P	Mannose-6-phosphate
Man1P	Mannose-1-phosphate
GDP-Man	GDP-mannose
GTP	Guanosine-5'-triphosphate
c-di-GMP	Bis-(3'-5')-cyclic-dimeric-GMP
DGC	Diguanylate cyclases
PDE	phosphoesterdiases
RBS	Ribosomal Binding Site
RFI	Relative Fluorescent Intensity
GMO	Genetically Modified Organism
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
m-toluate	3-methyl benzoate
RO	Reverse Osmosis
LB	Luria Broth
LA	Luria Agar
PIA	Pseudomonas Isolation Agar
SOC	Super Optimal Broth
TFB1	Transformation Buffer 1
TFB2	Transformation Buffer 2
TAE	Tris-acetate-EDTA
Amp	Ampicillin
Apr	Apramycin
Gen	Gentamycin
Kan	Kanamycin
ATc	Anhydro tetracycline
Abs	Absorbance
OD	Optical Density
UV	Ultraviolet
CIP	Calf Intestinal Phosphatase
PCR	Polymerase Chain Reaction
dNTP	Deoxynucleotide triphosphate
BLAST	The Basic Local Alignment Search
bp	base pair
Kbp	kilo base pair
FPLC	fast protein liquid chromatography

1 Introduction

This thesis assesses two aspects that can give insights into the biosynthesis of alginate. A counter-selection system for obtaining correct mutants was also tested. The effect of the expression of levansucrase, encoded by *lsc*, which is believed to influence the alginate synthesis, was evaluated. The counter-selection system I-sceI was tested for the potential of shortening time when selecting mutants undergone the second homologous recombination event. A system for testing c-di-GMP values was tested and c-di-GMP values were measured regarding growth, alginate synthesis and the overexpression of *mucR*.

This summarises the background information needed to understand the principles of the thesis. This chapter gives an introduction to *Pseudomonas fluorescens*. It also covers the bio-synthesis and regulation of alginate, in addition to an introduction to levan. Furthermore, it encompasses some genetic engineering approaches used in the thesis.

1.1 *Pseudomonas fluorescens*

P. fluorescens is a gram-negative, non-pathogenic rhizobacterium commonly found in terrestrial habitats, such as in soil and on the surface of plants (2, 3, 4). *P. fluorescens* strains display great heterogeneity (5, 6), and *P. fluorescens* SBW25 is one strain. *P. fluorescens* SBW25 is a commonly used model organism in biotechnology because it is non-pathogenic, easy to cultivate, and well characterized (2, 3, 7, 8). The strain entails plant-colonizing properties (2, 9).

The pathogenic *Pseudomonas aeruginosa* is another well caracterised *Pseudomonas,* as it can lead to infection in humans, and also produce alginate (9). *Pseudomonas syringae* is a well characterised phytopathogen, with similar biofilm production as in *P. fluorescens* (9). *Pseudomonas putida*, as *P. fluorescens*, is a microorganism beneficial for plants, with alginate performing similar roles (9).

A characteristic of *P. fluorescens* is that it synthesises pyoverdine, a fluorescent siderophore with a fluorescence that causes the cells to emit yellow-green colour (10). The *Pseudomonas* genus produces biofilm, a multicellular entity that involves the formation of an extracellular matrix that surrounds and envelops the bacterial cells (9, 11). The biofilm of wild-type *P. fluorescens* is mainly composed of polysaccharides, proteins and lipids (9). The polysaccharides consist mainly of cellulose, Psl and alginate, playing different roles in the biofilm, with levan also being a constitute (9, 12). However, under laboratory conditions, *P. fluorescens* wild-type strains do not produce alginate, because the promotor controlling alginate synthesis is tightly regulated, and production of the proteins responsible for alginate synthesis imposes a metabolic burden on the cells (1, 13).

The *mucA* mutant strain *P. fluorescens* SBW25 *mucA* has a point mutation in *mucA,* resulting in a truncated version of mucA, and the subsequent activated alginate synthesis (14, 15, 16). In this mutant, alginate is produced in late exponential phase and stationary phase (13).

1.2 Alginate

Alginate is a linear polysaccharide comprised of the two covalently 1-4 linked monomers, β-d-mannuronic acid (M) and α-L-guluronic acid (G)(1, 9, 17). It is synthesised by brown algae, Phaeophyceae, and some bacterial species. It behaves as a gel intracellularly and in the cell wall of brown algae, acting as the main skeletal compound giving it mechanical strength and flexibility (17). It is also found extracellularly on the gram-negative bacterial genera *Pseudomonas* and *Aztobacter*, acting as a bacterial biofilm constitute (12, 17). Alginate has important properties such as water and nutrient retention in the biofilm (15).

Alginate is a versatile polysaccharide of industrial value (1). It is used in foods, medicine, and cosmetics. Alginate has several applications, as it can function as a thickener stabiliser, encapsulating and gelling agent, synthetic fibre, and in drug delivery systems (17).

The presence of O-acetyl groups at O2 and/or O3 in bacterial alginates is the primary distinction from algal alginate (12, 17, 18). Algal alginate can be harvested in large quantities but has the disadvantage of not always displaying the desired composition (1). Bacterial alginate production is more costly, but have in return the opportunity to produce desired alginate composition by choosing mutants and bacterial strains, cultivating in controlled conditions, and epimerize subsequently *in vitro* (1).

1.2.1 Chemical Structure of Alginate

The copolymer alginate constitutes of O-acetylated M and G residues (9). Alginate can be described in the form of blocks, defining the composition of the polymer. Different compositions of bound monomers are described as different blocks (Figure 1.2-1) (9, 17).

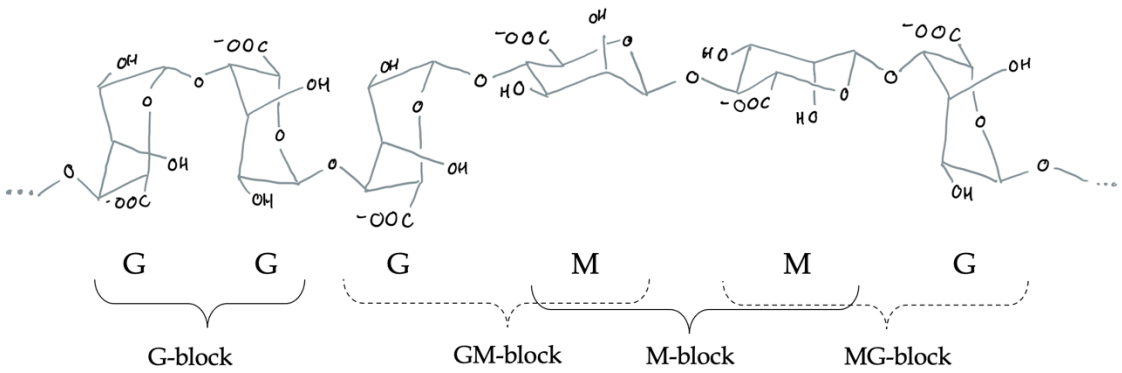


Figure 1.2-1: **Schematic drawing of alginate.** Two Ms bound together is referred to as an M block, two Gs bound together is referred to as G blocks, whereas an M and G bound together are referred to as an MG, and GM blocks are thus G and M bound together.

The blocks' composition and the polymer's length vary between different organisms, and which part of the algae it is harvested from. The physical properties of alginate are determined by the M and G content, the length of the polymer and to which degree it is acetylated (12). This variety of composition gives alginate versatile properties which can be exploited for commercial value (17).

M-blocks have an equatorial conformation, forming a ribbon-like structure (18), G-blocks forms have an axial formation and a buckled formation (18), and MG blocks alternate these formations. These different structures reflect in their physical properties. In the presence

of cations, such as Ca^{2+}, two facing G-block sequences will bind the Ca^{2+} and form an "egg box" model (18), which forms a gel. G-blocks are favourable in the industry because of this crosslinking ability to form a gel (1). Acetyl groups prevent the interaction between the polymer and cations, therefore preventing gel formation (19).

So far, alginates isolated from *Pseudomonas* spp. have not been found to have G-blocks. As alginate in *Pseudomonas* spp (12) does not form a gel, there may not be the same need for G-blocks as for algae (12).

1.2.2 Synthesis of Alginate

In *Pseudomonas* spp. (and *Azotobacter vinelandii*) the synthesis of alginate is similar, whereas the resulting alginate differs in character (20). Synthesizing alginate involves five steps – precursor synthesis (Figure 1.2-3), polymerization, periplasmic transit and modification and secretion (20).

The genes involved in alginate biosynthesis are all located in the same operon, tightly controlled by the algD promoter (1, 21). AlgC is an exception, located outside the operon (21). MucR regulates the c-di-GMP levels, and c-di-GMP binds Alg44 (1, 20).

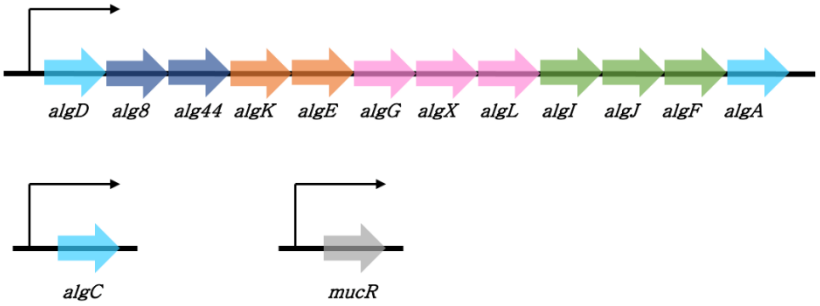


Figure 1.2-2: **The genes involved in alginate biosynthesis**. The figure is adapted from Maleki *et al.* (1). Promoters are indicated with the black arrow.

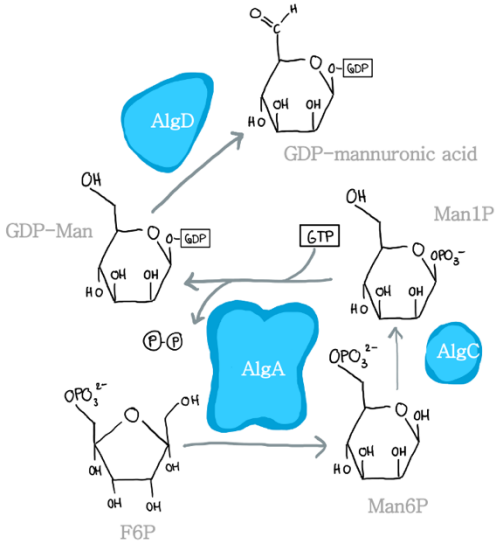


Figure 1.2-3: **The four steps in alginate precursor synthesis**. The first and third reaction are catalyzed by the same enzyme, AlgA.

Alginate precursor synthesis

In the first step, called the alginate precursor synthesis, fructose-6-phosphate (F6P) is converted to GDP mannuronic acid catalysed by the enzymes AlgA, AlgC and AlgD in four subsequent steps (Figure 1.2-3)(21, 22).

F6P is a crucial intermediate metabolite that plays a significant role in the central carbon metabolism in *P. fluorescens*, utilised by the Entner-Doudoroff pathway (via glucose-6-phosphate) to produce cell mass and energy (1, 5). Consequently, the alginate precursor synthesis competes with the central carbon metabolism, imposing a burden to the cell (1, 5).

AlgA, a bifunctional enzyme with phosphomannose isomerase activity, catalyses the isomerization of F6P to mannose-6-phosphate (Man6P). Mannose-6-phosphate is then converted to Mannose-1-phosphate (Man1P) catalysed by the phosphomannomutase AlgC. Subsequently, Mannose-1-phosphate is converted to GDP-mannose (GDP-Man) through the GDP-mannose pyrophosphorylase activity of AlgA, jointly with the hydrolysis of GTP. Finally, GDP-mannose is oxidized to GDP-mannuronic acid catalysed by the dehydrogenase AlgD. GDP-mannuronic acid is the substrate for the polymerisation of alginate (21).

Polymerization, periplasmic transit, modification, and secretion

Polymerization of GDP-mannuroic acid to a polymeric chain consisting of β-d-mannuroic acid (M) and its C5 epimer α-L-guluronic acid (G) happens through a membrane-spanning complex consisting of six different proteins, illustrated in Figure 1.2-4 (1, 21).

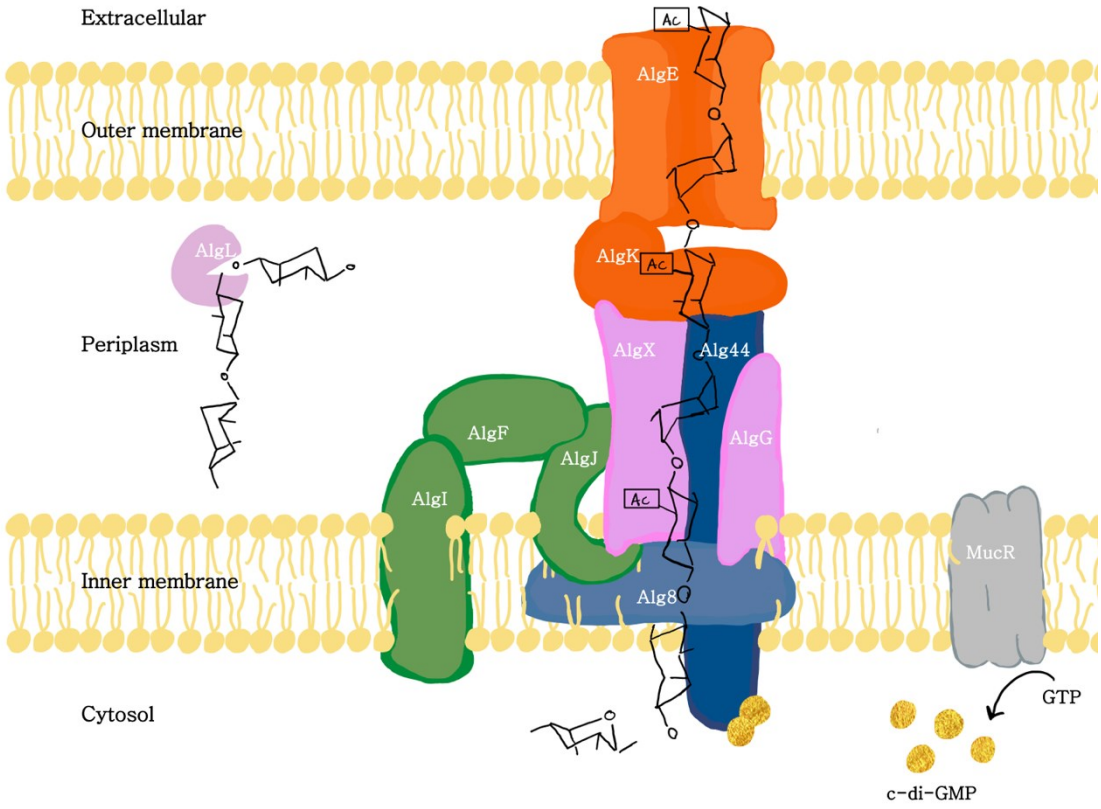


Figure 1.2-4: **Illustration of the membrane spanning complex.** The complex is involved in periplasmic transit, alginate modification and secretion. AlgL is a lyase that cleaves alginate misplaced in the periplasm. MucR regulates the synthesis of the second messenger c-di-GMP. c-di-GMP binds as a dimer to Alg44. The figure is adapted from Maleki *et al.* (1).

First, GDP mannuroic acid is polymerized by the polymerase Alg8, which is aided by Alg44, both important constituents in the complex (Figure 1.2-4) (1, 21, 23). The function of Alg44 is unclear, but deletion of alg44 results in no alginate production, and overexpressing it leads to overproduction (21), suggesting that it has an important function. It is suggested that Alg44 can be the connector that connects Alg8 to other membrane components, facilitating transit, modification, and secretion of alginate (21). Alg44 is activated by the second messenger bis-(3'-5')-cyclic-dimeric-GMP (c-di-GMP), which synthesis is regulated by the nearby MucR protein (24). MucR is discussed more in detail later in 1.2.5.

Then, the polymer of the mannuronic acid is modified while transported through the periplasm. AlgX, in conjunction with AlgI, AlgJ and AlgF, O-acetylates a various number of M-residues (1). AlgG, a mannuronan C-5-epimerase, can epimerize the not-acetylated M-residues to G-residues (12). Alginate erroneously located in the periplasm is broken down by the lyase AlgL. The presence of alginate in the periplasm is lethal for the cells because alginate attracts cations which, in turn, leads to high osmotic pressure and cell-lysis (12).

Alginate protected by acetylation, epimerization and the protein complex, will not be exposed for the lyase, but further secreted to the cell wall (12). The alginate export porin AlgE, located in the outer membrane (Figure 1.2-4), secretes the modified alginate chain facilitated by the lipoprotein AlgK (21).

1.2.3 Degradation of Alginate

Alginate is degraded and broken down by lyases in organisms that utilize alginate as carbon source, or organisms such as *Pseudomonas* with alginate in the periplasm. The polysaccharide is, cleaved at the O-glycosidic bond, creating the non-reducing end 4-deoxy-L-*erythro*-hex-4-enepyranosyluronate (12). The double bond created between C-4 and C-5 absorbs at 235 nm (25). This is used to monitor lyase activity and calculate alginate quantity in a sample. Because most lyases don't cleave acetylated residues, the samples need to be deacetylated prior to measuring (12).

Alginate consists of different blocks which also influence the reaction rates in which the different lyases cuts (12). The lyases are classified by their ability to cut G and M blocks, and are called G-lyases and M-lyases, respectively (12). This classification, however, does not include the ability to cut MG or GM blocks (12).

In this thesis, the M-lyase AlxM and the G-lyase AlyA are harvested and utilised to monitor the lyase activity and calculate the alginate quantity. AlxM is a D-mannuronan specific lyase, isolated and cloned from a marine bacterium (SFFB080483 Alg-A), initially isolated from the brown algae *Sargassum fluitans* (26). The G-lyase AlyA, deriving from *Klebsiella pneumoniae*, cleaves both G-G and G-M linkages (27).

1.2.4 Transcriptional Regulation of Alginate Synthesis

The synthesis of alginate is tightly regulated under natural conditions because it drains the cell's energy and carbon sources (22). Therefore, alginate is not synthesised under natural conditions unless it is needed, as cell wall stress (12).

The regulation of the gene *algU* encoding the σ factor AlgU is an important regulator in alginate biosynthesis because AlgU is required for the transcription of the alginate operon (Figure 1.2-2).

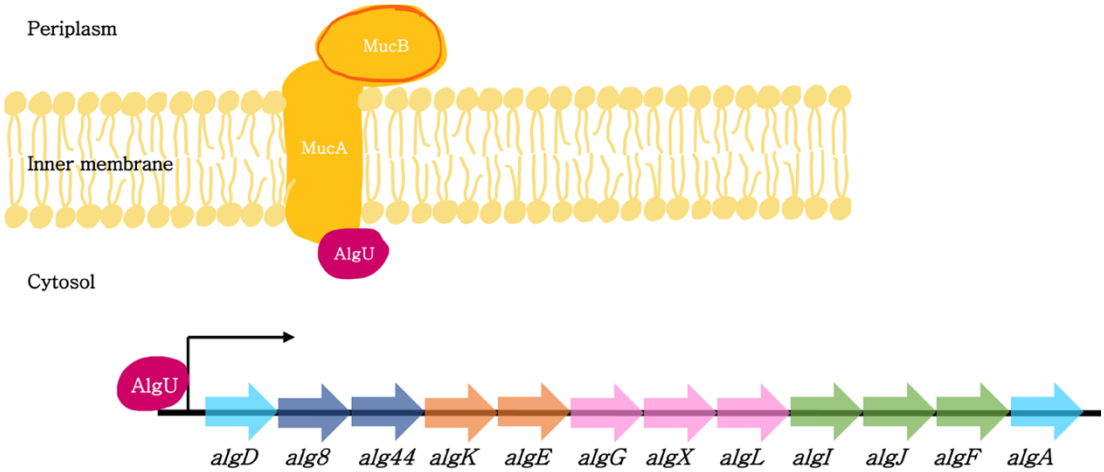


Figure 1.2-5: **Illustration of the function of MucA regulating the alginate biosynthesis.** The anti-σ factor MucA binds the σ-factor AlgU, preventing it from initiating expression of the AlgD promotor, controlling the operon.

The anti σ factor MucA binds MucB and AlgU, preventing it from initiating *algU* transcription (22). Alginate biosynthesis is induced by cleaving MucA, initiating the transcription of *algU* (22). Under laboratory conditions, the expression of the σ factor AlgU is usually turned off by binding to MucA, leading to no alginate synthesis (1, 13). The mucoid *P.fluorescens* SBW25 *mucA* mutant has a truncated version of *mucA*, which results in a defective MucA anti σ factor (15).

1.2.5 Post-Translational Regulation, MucR and c-di-GMP

The second messenger c-di-GMP, binds and activates Alg44, as mentioned. c-di-GMP is located in nearby pools, synthesised by MucR (Figure 1.2-4). The quantity of c-di-GMP is regulated by diguanylate cyclases (DGCs), synthesising c-di-GMP, and by phosphoester-diases (PDEs), degrading c-di-GMP. MucR is a bifunctional enzyme, exhibiting both DGC and PDE activity, thus regulating the levels of c-di-GMP in pools near Alg44, and thereby alginate production (1, 20). MucR is localised in the inner membrane, consisting of seven transmembrane regions, with the C-terminal residing in the cytosol. The C-terminal entails a GGDEF active site domain and an EAL active site domain exhibiting DGC and PDE activity, respectively. The EAL domain is suggested to reside at the end of the C-terminus (24).

Hay *et al.* found that deletion of *mucR* in an alginate over-producing strain of *P. aeruginosa* resulted in a reduced alginate production (24). The same study also tested the overexpression of mucR, and found that it led to a seven-fold increase in alginate production (24). This suggested that even though MucR exhibits PDE activity, the enzyme positively regulates the biosynthesis of alginate (24).

1.2.6 Measuring c-di-GMP Levels *in vivo*.

c-di-GMP is involved in many cellular processes, including that of binding and activating Alg44 (Figure 1.2-4)(28). Measuring c-di-GMP levels *in vivo* in alginate-producing strains, and also in *mucR* overexpressing strands, could give information about the effect different levels of c-di-GMP have on alginate production, in the different stages of growth.

A riboswitch is a regulatory RNA element that is usually in the 5'-untranslated region of mRNAs that regulates the translation of downstream genes by binding a ligand (29, 30). The riboswitch consists of two domains, one is an aptamer that binds the ligand, and the other, called expression platform, regulates the translation through a conformational switch controlled by ligand-binding (30, 31). Riboswitches exist both in simple and in tandem, whereas a tandem riboswitch is a stacking of homologous riboswitches, resulting in the need for less change in the concentration of the ligand for gene expression (31).

A fluorescent reporter system, based on a triple-tandem riboswitch, Bc3-5 RNA, to detect c-di-GMP levels was constructed by Zhou et al (30) in *Bacillus thuringiensis* subsp. *Chinensis* CT-43. Figure 1.2-6 presents this system, with mCherry_V1 as a fluorescent protein instead of the original TuroRFP.

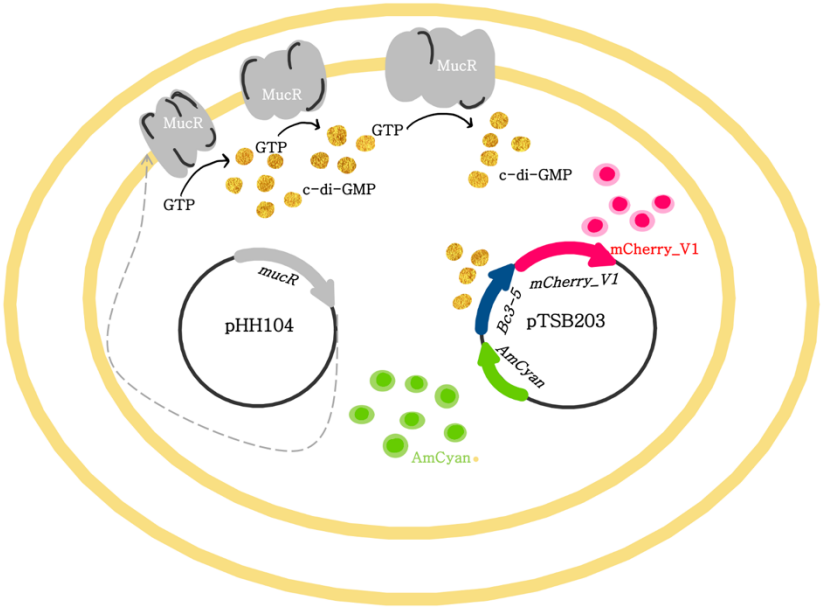


Figure 1.2-6: **An illustration of the triple-tandem c-di-GMP detecting riboswitch Bc3-5** coupled with *AmCyan* and *mCherry_V1*.The illustration includes the mucR overexpressing gene in pHH104, as this is utilised to test the system. pTSB203 is a derivative of pFY4535, with *mCherry_V1* instead of *TurboRFP*.

The plasmid pFY4535 contains the triple-tandem riboswitch Bc3-5 fused between the fluorescent proteins AmCyan and TurboRFP (32). *AmCyan* is located upstream of Bc3-5 and expressed constitutively under the control of the strong constitutive promoter PFP2. AmCyan exhibits the emission/excitation wavelengths 458/489 nm (30). Its transcription is not dependent on binding c-di-GMP, and is utilised to consider the promoter activity of PFP2, and thus differences between transcription and translation of TurboRFP (30) caused by the riboswitch Bc3-5.

TurboRFP is a fluorescent protein emitting a red/orange colour. It is located downstream Bc3-5 and translated upon activation of the ligand c-di-GMP. TurboRFP exhibits the excitation/emission wavelengths 553/574 nm. However, Haaland found that this excitation and emission spectre interferes with the autofluorescence of *P. fluorescens* SBW25 (33). Therefore, TurboRFP was changed to the alternative fluorescent protein *mCherry_V1* in this thesis.

mCherry_V1 emits a pink/red colour with emission/excitation wavelengths 576/610 nm. The re-engineered mCherry fluorescent protein mCherry_V1, constructed by Fages-Lartaud *et al.*, lacks the background expression found in other mCherry fluorescent proteins (34). Fages-Lartaud *et al.* engineered this protein by removing an alternative ribosomal binding site (RBS) which translated a shorter version of mCherry (34).

The vector to be constructed will have *mCherry_V1* under transcriptional control of the promoters PFP2 and rrnB P2, and under translational control of the levels of c-di-GMP (30). To evaluate the intracellular c-di-GMP levels, the relative fluorescence intensity (RFI) is calculated by dividing fluorescence of mCherry_V1 by the fluorescence of AmCyan. This gives a ratio between the translation of *AmCyan* and the c-di-GMP activated translation of *mCherry_V1*.

1.3 Levan

Levan is a branched polyfructan comprised of β-(2,6) linked D-fructofuranosyl residues with β-(2,1) branching points (35, 36). It is synthesised in plants, called phelins, and as an exopolysaccharide in bacteria. The branched, high molecular weight and low intrinsic viscosity make levan suitable for commercial applications in the food and pharmaceutical industries (36).

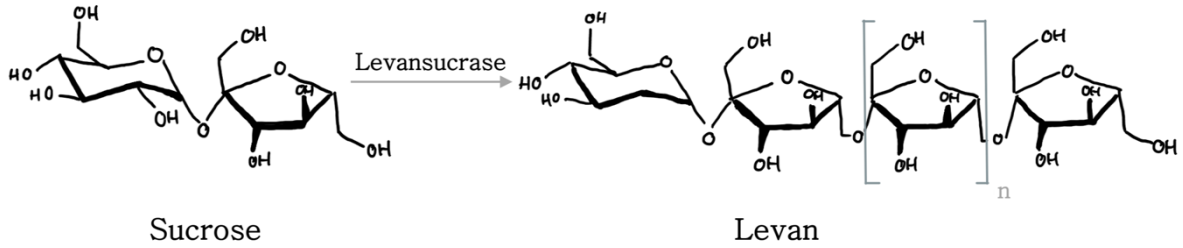


Figure 1.3-1: **The structure of levan**, and the substrate to levansucrase; sucrose.

Levan is synthesised exclusively from sucrose. The reaction is catalysed by the extracellular enzyme levansucrase, encoded by the *lsc* gene (15, 35, 36). Levansucrase cleaves the sucrose in the glycosidic bond between glucose and fructose, and polymerises the fructoses linked β(2-6) (37). The exact reaction in *Pseudomonas* is poorly understood (38, 39).

It is suggested that in *P. syringae*, levan is not fundamental for the maintenance or structure of the biofilm, but rather located in voids in the biofilm acting as a nutrient storage (35, 40). This implies that levan is used by the bacteria when the nutrient sources are depleted. In *P. syringae,* levansucrase catalyses the synthesis of levan from sucrose, and it is suggested that it also catalyses the hydrolysis of levan to fructose (41).

Levan and alginate, both exopolysaccharides in *P. fluorescens*, use carbon sources the cell uses for metabolic function in the cell. It is beneficial for the industry not to chemically remove levan when purifying alginate. Additionally, it was hypostisited that it could be beneficial for the alginate production in the cell to remove *lsc*, as it would make more energy and carbon sources available to produce alginate.

1.4 Genetic Engineering

Genetic engineering is modification of characteristics of an organism by manipulating the genome (42). Throughout the years, different methods to genetically modify organisms have been developed, tested, and improved for its specific purpose. In this thesis, genetically modified organisms (GMOs) is constructed by inserting non-replicating DNA plasmids into the cells for transcription of wanted proteins, and by removal of a gene in the cells' genomic DNA.

1.4.1 Promoters and Selection Systems

Plasmids are closed-circular DNA outside the bacterial chromosomal DNA, that are able to self-replicate and be transferred horizontally to other bacteria (42). The genes encoded by plasmids are not essential to the bacteria but encode proteins that could be necessary, as resistant towards antibiotics. Vectors are manipulated closed-circular DNA with the same characteristics, exploited to introduce desired DNA into host cells (42).

For the plasmid to be able to conjugate into other strains of bacteria, such as *P. fluorescens*, the origin of transfer gene, ***oriT***, is needed. This is the sequence at which conjugal transfer is initiated by acting as a recognition site for a nick in the DNA (43).

Selection markers

A requirement for a vector is that it must be able to transform. The transformation efficiency is generally low (44). Therefore, there is a need for a selection system that gives information about the vectors' presence in the cells. By imparting **antibiotic resistance** properties to the vector, and supplying the media with that antibiotic, only transformed cells will survive.

The gene selection marker ***lacZ*** encodes for the glycoside hydrolase β-D-galactosidase, which hydrolyzes 5-bromo-4-chloro-3-indolyl- β-d-galactopyranoside (X-Gal) to galactose and indoxyl, which are in turn oxidized (45). The oxidized form emits a blue colour. Therefore, the expression of *lacZ* on a media containing X-Gal will make the transformants blue and reveal if they contain the plasmid of interest. Cells lacking *lacZ* will emit their usual colour.

When selecting for homologue recombined cells, it can be challenging to select for the second homologue recombination event (1.4.3). A vector designed for homologous recombination can contain the negative-selection gene ***I-sceI***, derived from *Saccharomyces cerevisiae* (46) for time- and effort-saving purposes. *I-sceI* encodes for the homing endonuclease I-SceI, which makes double-stranded cuts at I-SceI recognition sequences (47). This is lethal for the cell when the recognition site is present in the genome, and lethal for a plasmid when present in a plasmid. *I-sceI* is under regularly control of the TetR regulator, which is induced by anhydro-tetracycline (ATc) (47). TetR is a repressor, tightly bound to the operator and represses the transcription. When ATc binds, TetR dissociates from the operator, and the transcription is initiated (48). When cultivating

the cells on a medium containing the inducer ATc, the cells undergone the second recombination event will survive and the cells at the first recombination event will die from lethal cutting. Another commonly used negative selection marker is *sacB*, encoding levansucrase. However, because *P. fluorescens* SBW25 have *lsc* encoding levansucrase, SacB is not usable as a counter-selection marker.

Promoters

To control the expression of the inserted gene(s), different promoter systems are used. One system is the **XylS/P_m** promoter system. This system derives from *P. putida* TOL pWW0 (49) and consists of the P_m promoter and the regulator gene *xylS*. The *xylS* gene encodes the transcriptional regulator protein XylS, which transcription is being activated by an effector. XylS binds as a dimer to the operator of P_m, inducing transcription from P_m (22). Transcription of *xylS* can be induced by 3-methyl benzoate, referred to as m-toluate (50).

The ***P_m-G5* promoter** (22) is a mutant of the P_m promoter. This promoter is induced by m-toluate and found to provide lower background expression, that is the expression in the absence of the inducer, then the P_m wild-type promoter (51). The *P_m-G5* promoter was selected in *P. fluorescens* as a gene with lower background expression levels and lower induced expression levels as opposed to P_m (22, 50). In this thesis, *P_m-G5* controls the overexpression of *mucR* in the pHH104 plasmid, constructed by Haaland (33).

1.4.2 Using *Escherichia coli* S17-1 *λpir* as a Plasmid Host.

Escherichia Coli is a model organism commonly used in genetic engineering. *E. coli* has a short generation time, it is well characterised, the strains used for modification purposes are non-pathogenic, and it is compliant to exchange genes with other bacterial species (42).

Various types of vectors and *E. coli* strains are used for different modification purposes. *E. coli* S17-1 was developed by Pühler *et al.* (52) in 1983 This strain has the ability to transfer *oriT*-containing vector to a recipient through conjugation. The conjugation is dependent on the RP4 transfer system, which is responsible for DNA transfer and mating pair formation, which is in the chromosome of the strain (53, 54). *E. coli* S17-1 *λpir* encodes a *λ* protein is necessary for the replication of the R6K plasmid (55). The RK6 plasmid is replicated in the presence of *pir*, whereas in an acceptor strain without *pir,* the plasmid will not be replicated, and therefore selecting for conjugants with exogenous DNA homologous replicated into the chromosome (55, 56).

1.4.3 Homologous Recombination

Homologous recombination is the rearrangement of DNA between two DNA sequences that share a sequence of homology. This homologous region functions as a target for the recombination and is where the recombination occurs. The process of homologous recombination happens spontaneously *in vivo* and can also be exploited for research purposes (42, 57).

Homologous recombination occurs in two events. In the first event, the entire vector is integrated into the chromosome, by aligning the homologous sequences and recombinate into the chromosomal DNA (Figure 1.4-1). This can result in two different outcomes, depending on which sequence that was aligned. In the second event, the now homologous sequences in the chromosomal DNA (purple and blue in Figure 1.4-1) align and loops-out the vector DNA. This results in a cell that is returned to wild type or the correct mutant is produced (58), depending on which sequences that aligned (illustrated in blue and purple lines in Figure 1.4-1).

In this thesis, the I-sceI negactive selection system, described in 1.4.1, was used to select for the transformants undergone the second homologous recombination event, by killing the cells with integrated vector DNA.

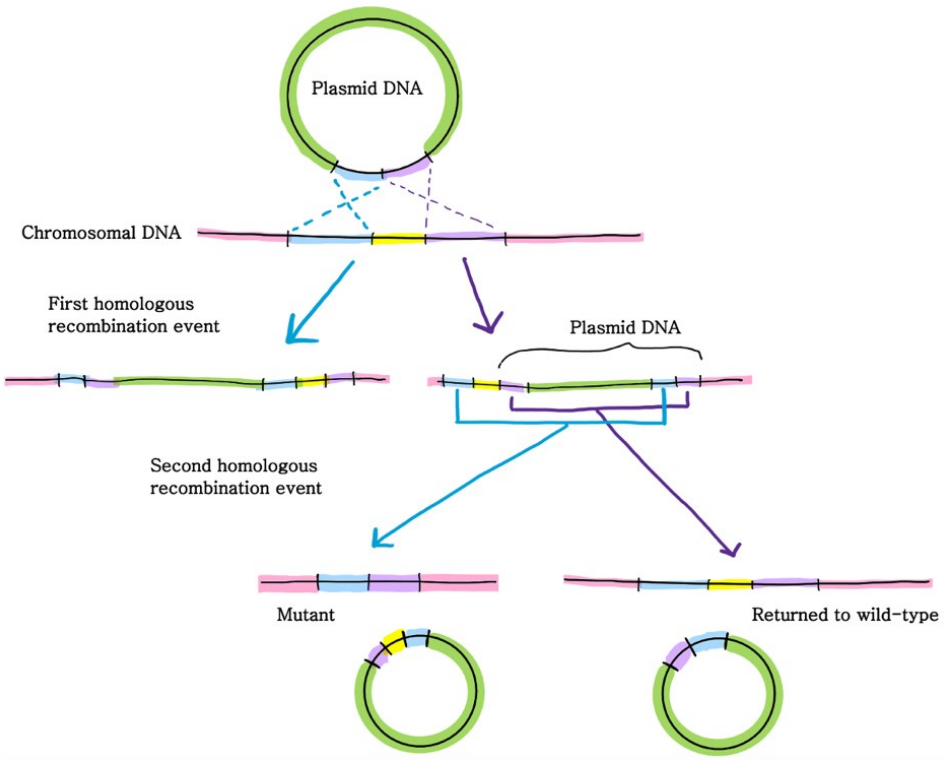


Figure 1.4-1: **Illustration of how homologous recombination can occur when removing a DNA fragment in chromosomal DNA.** Blue and purple represent homologous sequences, and the yellow sequence illustrate *lsc*, the gene that was to be removed in this thesis. Pink sequence represent the rest of the chromosomal DNA, and green sequence represent the vector DNA.

2 Aim of the Study

Alginate is a polysaccharide widely used in the food- and medicine industry. It is synthesised by brown algae, in larger quantities exploited by the industry, and by the bacterial genera *Pseudomonas* and *Aztobacter*. *P. fluorescens* is a non-pathogenic bacteria that has been genetically manipulated to produce alginate with higher consistency than algal alginate and in quantities relevant to the industry. There are many known genes influencing the amount of alginate (16, 22). Still, questions remain about what controls the initiation of alginate synthesis. In this thesis, the expression of genes believed to influence alginate synthesis is evaluated to gain a greater understanding of bacterial alginate synthesis. This study is divided into three parts.

Altering a reporter system for c-di-GMP and testing its functionality.

c-di-GMP positively regulates the alginate synthesis (24). Testing the c-di-GMP levels can give valuable information about the function in regard to growth and alginate production. An altered version of the dual-fluorescence c-di-GMP reporter system of Zhou *et al*. (30) was tested to monitor the c-di-GMP levels in the cell in relation to alginate synthesis and MucR overexpression. This system was tested for function in *P. fluorescens* by Vold and Haaland (33, 59). However, the autofluorescence of *P. fluorescens* interfered with the fluorescence of the reporter protein TurboRFP. This thesis aimed to change TurboRFP with another fluorescent protein, mCherry_V1, and test and evaluate the system's functionality.

If this was functional, the c-di-GMP levels in *P. fluorescens* SBW25 and the alginate producing *mucA* mutant, including in strains overexpressing MucR, were to be assessed in relation to growth and alginate production.

Constructing *lsc* negative mutants and evaluating the effect.

lsc is a gene encoding levansucrase, an enzyme that catalyses the synthesis of levan from sucrose (15). By removing *lsc* and consequently the synthesis of levan, it is hypothesised that alginate synthesis might increase, as levansucrase competes with alginate synthesis and the central metabolism on carbon sources. *lsc* negative strains are made to evaluate the influence *lsc* has on alginate synthesis and growth when different carbon sources are available.

This study aimed to investigate the effect of removing *lsc* from the chromosomal DNA of *P. fluorescens* SBW25 and the alginate-producing mutant *P. fluorescens* SBW25 *mucA* have on alginate synthesis and growth when cultivating the cells on different carbon sources.

Testing the I-SceI counter-selection system

Selecting for cells that have undergone the second homologous recombination event can be challenging. Today, there is no known negative selection marker for homolog recombination in *P. fluorescens,* without giving resistance toward antibiotics. Introducing

antibiotic resistance in a mutant is not favourable, and using the commonly used *sacB* as counter-selection is not possible in *P. fluorescens* SBW25 as it produces levansucrase itself. The I-SceI counter selection system has potential, as it has proven to function in *P. putida* (60) and exhibits promising potential as Haaland (33) found the restriction site to be absent in P. fluorescens SBW25. This study aimed to test the I-SceI counter-selection system.

The I-sceI counter-selection system was tested when selecting for mutants that have undergone the second homologous recombination event. If functional, the I-sceI counter selection system could reduce time in obtaining the correct mutants and reduce the need for antibiotic resistance bacteria for industrial purposes.

3 Materials and Methods

In this chapter, the methods and procedures utilised are described, and the recipes of media and solutions are presented.

3.1 Medias and Solutions

3.1.1 Luria Broth (LB)

10 g/L	Tryptone
5 g/L	Yeast extract
5 g/L	NaCl
	RO water

The media was stirred, autoclaved at 121 °C for 20 minutes and stored at room temperature.

3.1.2 Luria Agar (LA)

10 g/L	Tryptone
5 g/L	Yeast extract
5 g/L	NaCl
15 g/L	Bacteriological agar
	RO water

The media was stirred and autoclaved at 121 °C for 20 minutes. The preferable antibiotic solution was added after autoclavation when the media was approximately 50 °C. 25 mL of the agar was allocated into Petri dishes, set to solidify overnight, and stored at 4 °C.

For the LA no salt, 20% sucrose agar media, 20g/L sucrose was dissolved in 50% of the total reverse osmosis (RO) water volume and autoclaved separately. NaCl was not added. The sucrose and agar solution were mixed, and 0.05 mg ATc was added when the media was approximately 50 °C. ATc is light-sensitive, so the plating was done as dark as possible. 25 mL of the agar was allocated into Petri dishes, set to solidify overnight, and stored at 4 °C in the dark.

3.1.3 3X LB Medium

30 g/L	Tryptone
15 g/L	Yeast extract
5 g/L	NaCl
	RO water

The media was stirred and autoclaved at 121 °C for 20 minutes. The media was stored at room temperature.

3.1.4 Pseudomonas Isolation Agar (PIA)

45 g/L	Difco Pseudomonas Isolation Agar (PIA)
20 ml/L	Glycerol
	RO water

The media was stirred while heating and autoclaved at 121 °C for 20 minutes. A preferable antibiotic solution was added after autoclavation when the media was approximately 50 °C. 25 mL of the agar was allocated into Petri dishes, set to solidify overnight, and stored at 4 °C.

3.1.5 Super Optimal Broth (SOC)

20 g/L	Tryptone
5 g/L	Yeast extract
0.5 g/L	NaCl
2.5 mM	KCl
3.6 g/L	Glucose
5.08 g/L	$MgCl_2$
	RO water

The media was sterile filtered, allocated 1 mL into 1.5 mL Eppendorf tubes and stored at -20 °C. Readily made media was used.

3.1.6 Psi Medium

20 g/L	Tryptone
5 g/L	Yeast extract
5 g/L	$MgSO_4$
	RO water

The media was pH adjusted to 7.6 using 1M KOH and autoclaved at 121 °C for 20 minutes. The media was stored at room temperature.

3.1.7 DEF3 Minimal Medium

0.39 g/L	Yeast extract
0.65 g/L	KH_2PO_4
2.734 g/L	$(NH_4)_2HPO_4$
0.9 g/L	Citric acid $*H_2O$
3.413 mL/L	6 g/L Fe (III) citrate hydrate stock solution
0.034 mL/L	30 g/L H_3BO_3 stock solution
0.513 mL/L	10 g/L $MnCl_2*4H_2O$ stock solution
0.047 mL/L	84 g/L EDTA $*2H_2O$ Stock solution
0.034 mL/L	15 g/L $CuCl_2$ * $2H_2O$ stock solution
0.034 mL/L	25 g/L Na_2 Mo_4O_4 $*2H_2O$ stock solution
0.034 mL/L	25 g/L $CoCl_2O_4$ $*6H_2O$ stock solution
0.683 mL/L	4 g/L Zn $(CH_3COO)_2$ $*2H_2O$ Stock solution
1.561 g/L	NaCl
5 g/L	MOPS
	RO water

The ingredients were added in this order to 900 mL/L RO-water. The medium is pH adjusted to 7.0 using 10M NaOH and autoclaved at 121 °C for 20 minutes.

20g fructose is dissolved in 100 mL of RO water and autoclaved at 121 °C for 20 minutes separately. After autoclavation, the autoclaved fructose and sterile filtered 2.34 mL/L 246.47 g/L $MgSO_4$ $*7H_2O$ stock solution was added.

The media was stored at room temperature.

3.1.8 Transformation Buffer 1 (TFB1)

0.588 g	KH_2PO_4
2.42 g	RbCl
0.294 g	$CaCl_2$ * $2H_2O$
2.0 g	$MnCl_2$ * $4H_2O$
30 mL	Glycerol
	RO water

The ingredients were added to 180 mL/L RO-water. The buffer was pH adjusted to 5.8 using 10% acetic acid. RO water was added to the total of 200 mL, sterile filtered and stored at 4 °C.

3.1.9 Transformation Buffer 2 (TFB2)

0.21 g	MOPS
1.1 g	$CaCl_2 * 2H_2O$
0.121 g	RbCl
15 mL	Glycerol
	RO water

RO water was added to the total of 100 mL. The buffer was pH adjusted to 6.5 using diluted NaOH, sterile filtered and stored at 4 °C. Readily made buffer was used.

3.1.10 Phosphate Buffer Solution

18.52 mL	1M Na_2HPO_4
21.48 mL	1M NaH_2PO_4
	RO water

RO water was added to the total of 400 mL. The buffer was pH adjusted to pH 6.8 using 1M NaOH, stirred and stored at 4 °C.

3.1.11 Phosphate Buffer A and B

200 mL	Phosphate buffer solution
1 mL	1M $CaCl_2$
	RO water

RO water was added to the total of 1 L. The buffer was stirred, sterile filtered and stored at 4 °C. For Phosphate Buffer B, 1M NaCl was added.

3.1.12 0.05M TRIS Buffer A and B

100mL	0.5M Tris-HCl, pH 7.5
	RO water

RO water was added to the total of 1000 mL. The buffer was stirred, sterile filtered and stored at 4 °C. For TRIS Buffer B, 1M NaCl was added.

3.1.13 Lyaseassay Buffer

10 mL	0.5M Tris-HCl, pH 7.5
0.1 mL	1M $CaCl_2$
20 mL	1M NaCl
	RO water

RO water was added to the total of 100 mL. The buffer was stirred, sterile filtered and stored at 4 °C. For usage, 10 mL of the Lyaseassay Buffer was mixed with 2.5 mL 1% LF 10/60 alginate.

3.1.14 1X Tris-acetate-EDTA Buffer (TAE)

242 g/L	Tris-base
57.1 mL/L	100% acetic acid
100 mL/L	0.5 M EDTA (pH 8)
	RO water

RO water was added to the total of 1L. The buffer was autoclaved. 400mL of the buffer was diluted with RO water to a total volume of 20L.

3.1.15 Agarose Solution

3.2 g	LE Agarose
400 mL	1X TAE Buffer

The agarose was melted in the microwave until all the agarose was dissolved, then 20 µL of GelGreen or GelRed was added. The agar was stored at 60 °C.

3.1.16 0.5 g/L LF Alginate Standard Solution

25 mg	10/60 LF Alginate
	RO water

RO water was added to the total of 50 mL. The solution was stirred and shaken at 225 rpm at 37 °C overnight. 0.1 g/L, 0.2 g/L, 0.3 g/L and 0.4 g/L solutions was made by diluting the 0.5 g/L LF alginate solution. The solutions were stored at 4 °C.

3.1.17 Antibiotic Stock Solutions

Table 3.1-1: The antibiotics stock solutions and concentrations utilised in this thesis.

Antibiotic	**Concentration used (µg/mL)**
Ampicillin (Amp)	200 and 100
Apramycin (Apr)	200
Gentamycin (Gen)	20
Kanamycin (Kan)	50
Anhydro tetracycline (ATc)	0.5 and 1

For antibiotic stock solutions, the antibiotic was dissolved in RO water. For ATc, 80% were dissolved in ethanol. The stock solutions were sterile filtered, allocated 1 mL to 1,5 mL Eppendorf tubes and stored at -20 °C.

3.2 Cultivation and Storage

The bacterial cells used in this thesis were inoculated as described in methods for the particular purpose. All *E. coli* strains were incubated at 37 °C, and all *P. fluorescens* strains were incubated at 30°C. When antibiotics were utilised, the concentrations were as stated in Table 3.1-1.

The cells were stored differently depending on their purpose. Cells grown on agar plates were stored at 4 °C for up to three weeks usage. For longer storage the strain was stored at -80 °C. This was done by first inoculating the strain in 3 mL LB and the appropriate antibiotics and incubating overnight at 225 rpm and the preferred °C for the strain. Then, 1 mL of this culture was mixed with 300 µL 60% Glycerol in a cryotube, and frozen to -80 °C.

3.3 Strains and Plasmids

In this section, an overview of the bacterial strains and plasmids utilised in this thesis is presented in Table 3.3-1 and Table 3.3-2.

Table 3.3-1: **Overview of the bacterial strains utilised in this thesis.**

Bacterial strain	**Description**	**Source**
E. coli S17-1 *λpir*	*λpir* (replication of R6K-plasmids) recA, thi pro hsdR- M^+ RP4::2-Tc::Mu::Kan $Tn7Tp^r$ Sm^r	(16)
E. coli RV308	A derivative of the wild-type *E. coli* K-12. su^-, Δ*lacX74*, *gal IS II*::OP*308*, *strA*	(61)
P. fluorescens SBW25	Non-mucoid wild type of *P. fluorescens*.	(7)
P. fluorescens SBW25 *mucA*	Derivative of *P. fluorescens* SBW25. A stop-codon introduced in *mucA*.	(14)
P. fluorescens SBW25 *lsc*- A and B	Derivative of *P. fluorescens* SBW25. Deletion of *lsc.*	This thesis
P. fluorescens SBW25 *mucA lsc*- A and B	Derivative of *P. fluorescens* SBW25 *mucA.* Deletion of *lsc.*	This thesis

Table 3.3-2: **Overview of the plasmids utilised in this thesis.**

Plasmid	**Description of the relevant characteristics**	**Source**
pFY4535	The triple-tandem riboswitch dual-fluorescence c-di-GMP biosensor, coupled with *AmCyan* and *TurboRFP.* Gm^r	(32)
pLitmus28Tc	Derivative of pLitmus28 (ColE1 cloning vector) and pUC7Tc. Amp^r, Tet^r.	(62)
pTSB200	Derivative of pCR™-Blunt II-TOPO® containing *mCherry_V1*, Kan^r	This thesis
pTSB201	Derivative of pFY4535 and pLitmus28Tc. The triple-tandem riboswitch dual-fluorescence c-di-GMP biosensor, coupled with *AmCyan* and *TurboRFP.* Amp^r	This thesis
pTSB202	Derivative of pTSB200 and pTSB201.The triple-tandem riboswitch dual-fluorescence c-di-GMP biosensor, coupled with *AmCyan* and *mCherry_V1.* Amp^r	This thesis
pTSB203	Derivative of pTSB202 and pFY4535. The triple-tandem riboswitch dual-fluorescence c-di-GMP biosensor. Gm^r	This thesis
pHH104	Derivative of pHH103 and pSV6 (RK2 based expression vector). Contains P_m-*G5* controlling *mucR*. Kan^r	H. Haaland (33)
pHH108	Derivative of pFOK (R6K suicide vector) and pIO100. Contains *I-sceI* and I-sceI restriction site. Apr^r. Kan^r.	H. Haaland (33)
pSV11	Derivative of pSV10 and pHNB4. Contains *LacZ.* Tet^r, Amp^r.	S. Vold (59)
pHE510	Derivative of pCR™-Blunt II-TOPO® containing *oriT.* Kan^r	H. Ertesvåg, *unpublished*
pTSB401	Derivative of pHH108 and pSV11. *LacZ*, *I-sceI* and I-sceI restriction site. Kan^r. Apr^r.	This thesis
pTSB402a	Derivative of pCR™-Blunt II-TOPO® containing fragment upstream of *lsc* (O). Kan^r	This thesis
pTSB402b	pCR™-Blunt II-TOPO®, fragment downstream of *lsc* (N). Kan^r	This thesis
pTSB402	Derivative of pCR™-Blunt II-TOPO® containing fragments up- and downstream of *lsc* (*lsc*-). Kan^r	This thesis
pTSB403	Derivative of pTSB401 and pTSB402. *lsc-. LacZ. I-sceI* and I-sceI restriction site. Kan^r	This thesis

pTSB404	Derivative of pTSB403 and pHE510. *oriT*. *lsc-. LacZ I-sceI* and I-sceI restriction site. Kan^r	This thesis
pLE3	Contains *alxM* controlled by XylS/P_m. Amp^r.	L. Ellevog *unpublished*
pAT77	Contains *alyA* (from Klebsilla pneumoniae) controlled by XylS/P_m. Amp^r.	(27)

3.4 Isolation of Total Genomic DNA

When working with DNA for cloning, sequencing or quantifying purposes, isolation and purification of the Total Genomic DNA of an organism is a principal procedure. In this thesis, the MasterPure^{TM} Complete DNA and RNA Purification Kit by EPICENTRE was utilised to isolate genomic DNA (63). This method uses an enzymatic and desalting process to lyse and purify the total genomic DNA.

Procedure

All centrifugations were performed at 16,000 g at 4 °C using Eppendorf Centrifuge 5424.

The first step of the process is to lyse the cells containing the DNA of interest. The bacterial cells containing the DNA of interest were plated from a frozen culture and inoculated in a 30 °C cabinet overnight. A cell lump from this plate was scraped and resuspended into a mixture of 2.5 µL *Proteinase K* and 300 µL *Tissue and Cell Lysis Solution* by flicking and pipetting. Proteinase K denatures aliphatic and aromatic amino acids and increases the DNA yield by inactivating DNases and RNases (63). The solution was incubated at 65 °C, the optimised temperature for Proteinase K, for 15 minutes and flicked every five minutes. The sample was placed on ice for 4 minutes before 1 µL of *RNAse A* was added and mixed to cleave DNA and retrieve a purer DNA sample. Next, the mixture was incubated at 37 °C for 30 minutes and put on ice for 4 minutes.

In the second step, the proteins are removed. 150 µL of *MPC Protein Precipitation Reagent* was added to the sample to precipitate proteins and vortexed for 10 seconds. The tube was centrifuged for 10 minutes, and the supernatant was transferred to a new 1.5 mL sterile Eppendorf tube.

In the third step, DNA is precipitated. 500 µL of *isopropanol* was added to the recovered supernatant, and the tube was inverted 30 to 40 times before centrifugation for 10 minutes. The supernatant was carefully removed, and the pellet was rinsed twice with 70% ethanol without dislodging the pellet. Isopropanol can precipitate protein residues and wash off the salt (63). Ethanol also washes salt residues which purifies the sample. The DNA was resuspended in 100 µL 10mM Tris, pH 7.5 (ready buffer EB, TE Buffer) to help maintain the pH in storage and shaken overnight until the DNA was dissolved (63).

The total genomic DNA was stored at -20 °C.

3.5 Isolation of Plasmid DNA

To manipulate genes *in vitro*, it is necessary to isolate plasmid DNA from chromosomal DNA and the cell itself. One standard method is the alkaline lysis procedure, first described by Birnboim and Doly (1979), where linear DNA denatures at alkali pH while closed-circular DNA remains intact (42, 64). In this study, the kit ZR Plasmid Miniprep™-*Classic* was utilised to isolate plasmid DNA from different strains of *E. coli*. The kit provides reagents for lysing cells, denaturation of chromosomal DNA and protein and the subsequent plasmid DNA isolation using a modified alkaline lysis approach (65).

Procedure

All centrifugations were performed at 16,000g using Eppendorf Centrifuge 5424. Prior to the isolation of plasmid DNA, an overnight culture was made.

1.5 mL of culture was transferred to a 1.5 mL Eppendorf tube and centrifuged for 2 minutes for the culture to form a pellet. After discarding the supernatant, the pellet was resuspended in 200 µL P1 Buffer by pipetting. Next, 200 µL of alkaline Buffer P2 was added, mixed by inverting the tube three times and incubated for less than two minutes. Buffer P2 lysate the cells and denature chromosomal DNA (42, 64).

Thereafter, the mixture was neutralised by adding 400 µL Buffer P3, mixed by inverting the tube five times and incubated at room temperature (23 °C) for 5 minutes. This causes chromosomal DNA, cell remains, proteins, and buffer material, to aggregate. The solution was then centrifuged for 5 minutes, which pellets the aggregation, leaving the plasmid DNA in the supernatant (42, 64).

To bind the plasmid DNA to a column, the supernatant was transferred to a "Zymo-Spin™IIN" column assembled on top of a collection tube, centrifuged for one minute, and the flow through was disposed of. Next, 200 µL of "Endo-wash Buffer" was added and centrifuged for one minute. Then, 400 µL of "Plasmid-wash Buffer" was added and centrifuged for additionally two more minutes. This elutes impurities and purifies the sample (42, 64).

The column containing plasmid DNA was transferred to a sterile 1,5 mL Eppendorf tube. Then, 50 µL of Tris-EDTA buffer was added at the centre of the column matrix and incubated at room temperature (23 °C) for at least one minute before 1.5-minute centrifugation to elute the plasmid. This stabilises the plasmid for storage and further use (42, 64).

The eluted plasmid DNA was stored at -20 °C.

3.6 DNA Concentration Measurement

When working with DNA, knowing the concentration of dsDNA in a sample is of interest. In this thesis, NanoDrop One by ThermoFisher was used to determine the sample's concentration and purity. Measuring the concentration of DNA was done in conjunction with applications such as digesting DNA using restriction enzymes, sequencing plasmid DNA and PCR.

NanoDrop One uses a UV absorbance method to measure the concentration of DNA. Both purine and pyrimidine rings in DNA contain conjugated double bonds, which have an absorption peak at 260 nm. This is proportional to the concentration of nucleic acids (66, 67). Therefore, NanoDrop One measures UV at 260 nm and calculates the amount of DNA in the sample. Beer-Lamberts law is used to correlate the absorbance value with the concentration in the sample.

Equation 1: Beer-Lamberts Law $c = \frac{A}{\varepsilon * b}$

A =	UV absorbance in absorbance units (AU)	b =	pathlength in cm
ε =	Extinction coefficient in L/mol-cm	c =	concentration in M

For dsDNA, ε * b = 50 ng-cm/µL. NanoDrop One gives the concentration in ng/µL.

NanoDrop One also measures the sample's purity by measuring the absorbance ratio of 260/230 and 260/280. A260/A280 measures how much DNA and RNA there is in a sample, whereas a number around 1.8 is preferable for pure DNA. A lower number indicates the presence of proteins, phenol or other contaminants that absorb near 280nm, and a higher number may indicate more RNA in the sample. This number may vary depending on the pH of the sample and the base composition in DNA. A260/A230 is also a measure of contaminants which absorb at 230 nm, such as carbohydrates and phenols. This number should be between 2 and 2.2 (68).

Procedure

Before measuring, the NanoDrop One Spectrophotometer pedestal was cleansed by pipetting 2 µL of Milli-Q water, let sit for 30 seconds and wiped off using a lab wipe.

Before measuring, a blank was established. The buffer for blanking was the buffer used to elute the plasmid DNA or the buffer in which the DNA was resuspended. 1 µL of buffer was used for blanking. After the preparations, 1 µL of the sample was loaded on the pedestal and used to measure the DNA amount. After measuring, the pedestal was cleansed by pipetting 2 µL of Milli-Q water, let sit for 30 seconds and wiped off using lens paper.

3.7 Restriction Cutting of Plasmid DNA

In genetic engineering, cutting DNA using restriction endonucleases was an enormous progress step when it was first discovered in 1970 by Hamilton Smith and Daniel Nathans (69, 70). Naturally, in bacteria, endonucleases protect bacteria from foreign DNA. Type II restriction enzymes are site-specific and bind to and cleave DNA at specific restriction sites. Many restriction enzymes have since then been discovered and improved to be used for research purposes, as in this thesis.

In this thesis, digesting of DNA using restriction enzymes was used in the construction of plasmids and for validating plasmids. The restriction enzymes used in this thesis, including the restriction sites and the optimal buffer, are listed in Appendix A.

When using one restriction enzyme to digest a vector, there is a high probability of religation later. Calf Intestinal Phosphatase, CIP, is therefore used to inactivate the religation. The phosphatase removes the 5' phosphate group, preventing internal ligases to function (71). CIP was added to the cutting mix 30 minutes before loading the samples on an agarose gel.

The software Benchling was used to find restriction sites for different restriction enzymes in the respective plasmid. The most convenient enzymes were used based on the location of the restriction site and the compatibility with other restriction enzymes.

Procedure

A sterile 1,5 mL Eppendorf tube was loaded with 100 ng to 200 ng plasmid DNA, 2 µL of the buffer with the highest buffer activity for the restriction enzyme, 0.5 µL of the enzyme(s) needed, and finally autoclaved RO water up to a total volume of 20 µL. If a small size DNA fragment was going to be isolated, a greater amount of DNA was used.

The contents were mixed and spun down, and incubated in a water bath at 37 °C. The duration of incubation varied from one hour to overnight. The sample was used directly for gel electrophoresis or stored at -20 °C.

3.8 Separation of DNA Fragments by Agarose Gel Electrophoresis

Separating DNA fragments based on size and charge in a semi-porous material is essential in genetic engineering. In this thesis, agarose gels were used to isolate fragments of DNA and for the validation of PCR products and digested transformed plasmids. The DNA ladders used for size reference, are listed in Appendix C.

The separation of DNA fragments takes advantage of the negative charge of nucleotides and the migration speed of the fragments through a semi-porous gel. The negatively charged dyed DNA fragments are loaded in wells on one edge of the gel and placed in an electrophoresis chamber containing negative and positive electrodes. The negatively charged DNA migrates toward the positive electrode through the gel. The smallest fragments have less resistance in the gel – and therefore migrate longer. The bigger pieces have greater resistance and migrate less (69).

ChemiDocTM XRS+ Gel Imaging System by BioRad Laboratories Inc. was used to visualise the DNA fragments. This instrument utilises UV light to visualise the bands and creates a photograph visible through the Image Lab™ Software.

Procedure

A GelGreen or GelRed containing agarose gel (3.1.15) was poured into a casting tray containing a comb to set wells in the gel. The gel was set to solidify for 15 to 30 minutes. GelGreen binds and stains DNA and can be used when it is preferable not to damage DNA with UV light and is therefore compatible with blue light instead (72). It is utilised when the purpose is to isolate DNA fragments from the gel. GelRed also binds and stains DNA, and it was used when the aim was to visualise the sample´s fragment size(s).

The gel was then submerged in a gel electrophoresis tray containing TAE Buffer. The wells in the gel were loaded with samples containing DNA, autoclaved RO water and Purple Gel Loading Dye 6X.

The amount of DNA sample loaded on the gel depended on the DNA concentration in the sample and the purpose of the gel. For λ PstI and λ HindIII standards, 2.5 µL of sample DNA was added. 1.5 µL of Purple Gel Loading Dye (6X) was added to each sample, and autoclaved RO-water was added to the total volume of 11.5ul. The loading dye is added to ease the loading and tracking of the sample in the wells by colouring and densifying it, which keeps the sample in the well. The RO water is autoclaved and as sterile as possible to not contaminate the samples with other DNA.

After loading the gel with a sample, the voltage was set to 100V and 400mA. The gel ran for 30 to 50 minutes. ChemiDoc™ XRS+ Gel Imaging System and Image Lab™ Software were then used to visualise the DNA fragments.

When extracting DNA from a GelGreen agarose gel, XcitaBlue™ Conversion Screen was used to convert UV light to blue light. The fragment was cut out using X-tracta™ Gel Extractor Tool from Promega.

The gel piece was quickly isolated using the Monarch Gel DNA isolation kit (3.9) or frozen at -20 °C.

3.9 DNA Extraction from Agarose Gel

Isolating DNA fragments based on their size is essential when making recombinant plasmids and purifying DNA fragments from a PCR. This thesis utilised Monarch DNA Gel Extraction Kit from New England BioLabs to isolate DNA fragments from an agarose gel. The kit purifies dsDNA from agarose gels, using reagents that dissolve the agarose gel and remove trace amounts of TAE buffer salts, DNA binding dyes and gel loading buffer (73).

Procedure

All centrifugations were done at 13,000 rcf using Eppendorf Centrifuge 5424.

When extracting DNA from an agarose gel, a GelGreen agarose gel (3.1.15) was loaded with a DNA sample containing approximately 100 ng, depending on the size of the fragment to cut out.

The excised gel piece from an agarose gel was transferred to a clean 1.5 mL Eppendorf tube and weighed. Next, four volumes of Monarch Gel dissolving Buffer were added, and the solution was incubated at 50 °C for approximately 5 minutes until the gel was dissolved, flicking in between. If the DNA fragment to be isolated was larger than 8 kbp, 1.5 volumes of autoclaved RO water were added after incubating the tube at 50 °C.

The dissolved sample was then loaded on a column on a collection tube. This was centrifuged for 1 minute for the DNA to bind to the column. The flow-through was discarded. 200 µL of DNA Wash Buffer was added to the column and centrifuged for one minute. This step was repeated once.

Then, the column was transferred to a clean 1.5 mL Eppendorf tube, and 17 µL of DNA elution buffer was added. When eluting a DNA size larger than 10 kbp, the DNA elution buffer was heated to 50 °C prior to addition. The column was soaked in the buffer for at least one minute before a 1.5-minute centrifugation step to elute the DNA. The eluted DNA was stored at -20 °C.

3.10 Ligation of DNA Fragments

To construct a recombinant plasmid, the DNA fragments purified from the gel must be joined. Ligation of DNA fragments using DNA ligases is a commonly used tool in biotechnology. In this thesis, ligation was used to construct recombinant plasmids.

The process of ligation utilises a ligase enzyme to join two nucleosides. In the presence of nicked dsDNA, the ligase, in this case, the T4 ligase deriving from the bacteriophage T4, binds to and catalyses the synthesis of the phosphodiester bonds between adjacent 5’ phosphoryl and 3’-hydroxyl groups (74). First, the 5’ phosphorylated donor strand is adenylated by ATP, and then a phosphodiester bond is formed between the adenylated donor and the acceptor 3’ hydroxyl groups (75).

T4 ligase can ligate both sticky and blunt ends. Utilising restriction enzymes with complementary sticky ends that result in different ends on each side of the fragment, the ligation of the fragments is predictable. When ligating blunt-end fragments, the insert can be inserted in two ways. In this thesis, T4 DNA ligase was utilised when ligating DNA fragments with sticky ends.

Procedure

A solution of 14 µL insert DNA, 3 µL of vector DNA, 2 µL of T4 DNA ligase buffer and 1 µL of T4 ligase is added to a 1.5 mL clean Eppendorf tube, mixed and incubated overnight at 14 °C or in the refrigerator at 4 °C for two nights. The ligation mix was then used for transformation or frozen at -20 °C.

3.11 Preparation of Supercompetent Cells

Competent cells are cells that are cells that can take up DNA from the environment. Some bacteria have genes encoding proteins that mediate this process, and others can be made competent by e.g. chemical treatment (69). In this thesis, *E. coli* strain *E. coli* S17-1 *λpir* was made competent by chemical treatment followed by a heat shock.

The chemical treatment consists of treating the cells with the divalent cation $CaCl_2$ in combination with the monovalent ion RbCl. $CaCl_2$ is believed to help DNA to adhere to the cell surface, while RbCl is used to increase the transformation efficiency (76, 77).

Procedure

All centrifugations were performed at 4000 rpm at 4 °C using Sorvall Lynx 6000 Centrifuge by Thermo Scientific.

E. coli S17-1 *λpir* cells were inoculated in a 125 mL Erlenmeyer flask with 10 mL Psi medium at 37 °C and 225 rpm overnight. 2 mL of the pre-night culture was transferred to a 1L baffled Erlenmeyer flask with 200 mL Psi medium and incubated at 37 °C and 225 rpm for 1 hour and 40 minutes.

SpectraMax Plus 384-Microplate Reader was used to measure OD_{600}. When OD_{600} was approximately 0.4, the flask was put on ice for 15 minutes. The cold culture was transferred to two 250 mL sterile Nalgene Centrifuge Flasks and centrifuged for 5 minutes. The supernatant was discarded, and each pellet was carefully resuspended in 40 mL TFB1 Buffer. The solute was put on ice for 5 minutes before a 5-minute centrifugation. The supernatant was discarded, and the pellets were each carefully resuspended in 6 mL TFB2 Buffer and put on ice.

100 µL of the resuspended cells were allocated into sterile 1.5 mL Eppendorf tubes on ice. The tubes were snap-frozen using liquid nitrogen for 5 seconds and then transferred to a -80 °C freezer, where they were stored.

3.12 Transformation of Plasmid DNA to *E. coli* S17-1 *λpir*

Horizontal gene transfer in bacteria can happen in three ways, conjugation, transduction, and transformation. Transformation is when bacteria take exogenous DNA from the environment. Different strains of bacteria have different potentials to transform, and *E. coli* has a low potential.

However, *E. coli* can be transformed through electroporation or rendered competent through chemical transformation and then transform through heat-shock treatment (42).

In this thesis, the chemically competent *E. coli* S17-1 *λpir* strain was transformed through heat-shock treatment. A selection marker, for example, antibiotic resistance in this thesis, was present in the plasmid to be transformed to select the transformed bacteria.

Procedure

The super-competent *E. coli* S17-1 *λpir* cells (from -80 °C freezer, 100 µL) are thawed on ice for 5 to 10 minutes. Next, 10 µL of plasmid DNA is added to the super-competent cells and incubated on ice for 30 minutes. The tube is then heat-shocked at 37 °C in a water bath to allow plasmid DNA to be introduced into the cell and cooled on ice for 2 minutes. Next, 900 µL of SOC medium, heated to 37 °C, is added, and the tube is incubated at 37 °C at 225 rpm for between 1 and two hours. This allows the cells to grow and the plasmids to express desired properties, such as antibiotic resistance.

The transformed cells are selected on LA plates containing the preferred antibiotics and incubated at 37 °C overnight.

3.13 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction, PCR, is an in vitro method to amplify distinct fragments of DNA. The procedure utilises DNA polymerase to synthesise DNA fragments using a template DNA and dNTPs as substrates. The DNA polymerase adds nucleotides to the 3' end of a primer complementary to the annealed DNA template strand. It is a 3-step cycle repeated 35 times, including an initiation step and a final step Table **3.13-1**.

Table 3.13-1: **Thermocycler settings in a PCR reaction using Q5 polymerase.** Annealing temperature and extension time vary between samples.

Step		**Temperature**	**Duration time**
Initial denaturation		98 °C	3 minutes
Denaturation	35 cycles	98 °C	10 seconds
Annealing		5 °C lower than primer T_m	30 seconds
Extension		72 °C	30 seconds per kbp
Final extension		72 °C	2 minutes
Hold		4 °C	∞

The initial denaturation step is the heating of the system, which causes the double-stranded DNA to denature to single-stranded DNA. The initial denaturation step lasts 3 minutes to denature the template DNA. Then, the 3-step cycle is initiated (Table 3.13-1) The system is cooled so that the single-stranded oligonucleotide, used as a primer, anneals to its complementary template. The system is then heated to the optimised temperature for the DNA polymerase to elongate the dsDNA strands. After elongation, the system is heated to denature the newly synthesised dsDNA strand and the cycle is repeated. During the PCR cycles, the DNA segment of interest amplifies exponentially (78).

This thesis utilised the Q5 high-fidelity DNA polymerase from New England Biolabs, a thermostable DNA polymerase with a low error rate. For amplifying *mCherry_V1* from RK2-mCherry-V1 plasmid, the CloneAmp HiFi PCR Premix by Takara was used. For CloneAmp HiFi Polymerase, 5 seconds per kbp is needed for extension.

Colony PCR is a screening method used in this thesis to detect a desired genetic sequence, the lsc-absence, in a bacterial colony. The PCR reaction is equivalent, but the template DNA is exchanged with colony DNA.

Procedure

Table 3.13-2 specifies which solutes are added to a 0.2 mL PCR tube when utilising Q5 high-fidelity DNA polymerase. The solutes are mixed and spun down.

Table 3.13-2: **Solutes mixed in a 0.2 mL PCR Tube**

Volume	**Solute**
10 µL	5X Q5 Reaction Buffer
10 µL	5X Q5 High GC Enhancer
1 µL	10 mM dNTP
2.5 µL	10 µM Forward primer
2.5 µL	10 µM Reverse primer
0.5 µL	100 µg Template DNA
To 50 µL	Autoclaved RO water
0.5 µL	Q5 High-Fidelity DNA polymerase

When utilising CloneAmp HiFi Polymerase, 12.5 µL CloneAmp HiFi PCR Premix, primers to the final concentration of 0.2-0.3 µM, 100 ng Template DNA and autoclaved RO water up to 25 µL is added to a 0.2 mL PCR tube and mixed and spun down (79).

In colony PCR, template DNA is exchanged with treated colony DNA. Colony DNA is retrieved by scraping a lump of cells from a cell colony on a plate and resuspending it in 25 µL of water. The dissolved cells are heated at 98°C for 15 minutes and cooled on ice before using 1 µL as template DNA in the PCR reaction.

This thesis used the Eppendorf thermocycler instruments Mastercycler Gradient and Mastercycler Personal. The settings are set as in Table **3.13-1**. The elongation time and annealing temperature varied between samples. The PCR samples were frozen at -20 °C or used directly. When loading PCR samples on an agarose gel, 2-2.5 µL PCR sample was added.

3.14 Purification of PCR Products

PCR solutions consist of polymerases, primer sequences, detergents and dNTPs, which are favourable to remove when using the PCR products, for example, in a ligation reaction (80). For this purpose, the Monarch^{R} PCR & DNA Cleanup Kit from New England BioLabs was utilised.

Procedure

All centrifugations were performed at 16,000 rcf using Eppendorf Centrifuge 5424.

The dsDNA was diluted in the 2:1 ratio with DNA Cleanup Binding Buffer and mixed by pipetting. The sample was put on a column and centrifuged for one minute. 200 µL of DNA Wash Buffer was added to the column and centrifuged at for one minute. This step was repeated, with the exception being that the tube was centrifuged for 2 minutes. The column was transferred to a clean 1.5 mL Eppendorf tube, and 10 µL of DNA Elution Buffer was added, marinated for one minute and centrifuged for one minute to elute the PCR products. The sample was stored at -20 °C.

3.15 Zero Blunt® TOPO® PCR Cloning

When inserting a blunt-end DNA fragment from a PCR reaction, Zero Blunt® TOPO® PCR Cloning can be utilised. In this thesis, Zero Blunt® TOPO® PCR Cloning was used in association with transformation.

Zero Blunt® TOPO® PCR Cloning utilises the function of topoisomerase I from the Vaccinia virus. Topoisomerase I is covalently bound to the 3' end of the plasmid vector, called pCR^{TM}-Blunt II-TOPO®. The enzyme binds to DNA at a specific site, cleaves, and covalently binds to the phosphodiester backbone in one strand. The covalent bond can then be attacked by the 5' hydroxyl end of the original cleaved strand or by another strand's 5' hydroxyl end.

To select recombinants containing the segment of interest in its vector, not the realigned vector, the vector comprises the lethal ccdB gene. The recombinant with disrupted ccdB gene, with insert, will survive upon plating, whilst the cells containing realigned plasmid die (81).

Procedure:

2 µL of PCR product, 0.5 µL of salt solution (200 mM NaCl, 10mM $MgCl_2$) and pCR^{TM}II-Blunt-TOPO^{R} was added to a sterile 1.5 mL Eppendorf tube, mixed by flicking the tube and incubated at room temperature (23 °C) for 30 minutes. Next, the solution was transformed into super-competent *E. coli* S17-1 *λpir* cells according to 1.7.

3.16 Sequencing

DNA sequencing has been the most important technological advance in the history of genetics (69).

DNA sequencing is the determination of the nucleotide sequence in a DNA molecule and is one of the most important technological advances in the history of genetics (69). Different techniques have been developed to further enhance the speed, lowering the cost and improving the accuracy of the read. In this thesis, a Sanger sequencing method was used by the LightRun Tube sequencing service of Eurofins Genomics.

This sequencing method is a chain-terminating method that utilises 2′,3′-Dideoxyribonucleoside triphosphates to generate DNA sequences of different sizes to determine the sequence of a fragment. The dsDNA fragment to be sequenced is denatured to ssDNA by heat. A primer DNA, complementary to a segment, is aligned, and a polymerase elongates the strands by adding dNTPs. dNTPs without oxygen at C3 at its base called ddXTP, or chain terminators, are also added to the mix. When a polymerase adds a ddXTP to the strand, The elongation reaction stops, and a DNA fragment of a specific size is synthesised. The ddXTP are detected, and the length of the strand is measured by fluorescence. From this data, a sequence is modelled (69).

Procedure

3ul of the plasmid DNA (ca. 150 ng/µL), 0.75 µL DMSO, 1.75 µL of 20 pmol/µL primer and 9,5 µL autoclaved RO water was added to a 1,5 mL Eppendorf tube and put in the sending box for not longer than 2 hours before predicted pick up.

The software Benchling was used to process the sequence results.

3.17 Conjugation of Plasmid DNA From *E. coli* S17-1 *λpir* to *P. fluorescens* SBW25 Strains

Conjugation is another possible horizontal gene transfer mechanism in bacteria. It is the transfer of genetic material through direct cell-cell contact via pili or adhesins (82). The donor cell expresses a pilus, which latches on the recipient, and the genetic material is transferred from the donor cell to the recipient via the pilus (83). To transfer plasmids from a donor to a recipient, the plasmids need the gene *oriT*, an origin of transfer. Prior to conjugation, DNA is unwinded and separated to ssDNA, and a nick is introduced in *oriT* (83). Then, the strand is transferred to the recipient cell and is synthesised to dsDNA by complementary base pairing (84).

In this thesis, the donor cells were *E. coli* S17-1 *λpir*. *E. coli* S17-1 *λpir* have integrated conjugal RP4 transfer functions, mediating conjugation to various organisms (53, 55). Furthermore, the strain is a *λpir* lysogen of S17-1, which entails that by irradiation with UV light, phage genes that lyse the cells to death are switched on(85). The pir-encoded λ protein is necessary for the replication of the R6K plasmid. In the recipient strain absence of *pir*, the R6K plasmid will not be replicated and behaves like a suicide vector, thus allowing exogenous DNA to homologous replicate into the chromosome (55, 56).

Procedure

Preculture of the donor, *E. coli* S17-1 *λpir*, containing the desired plasmid, was made by inoculating the cells in 10 mL LB (125 mL Erlenmeyer flask) and its preferred antibiotics and incubated at 37 °C at 225 rpm overnight. Preculture of the recipient *P. fluorescens* SBW25 strain was made by inoculating in 10 mL LB (125 mL Erlenmeyer flask) and incubated at 30°C at 225 rpm overnight.

The following day, 200 µL of the *P. fluorescens* SBW25 preculture was inoculated in 10 mL LB (125 mL Erlenmeyer flask) and incubated at 30°C at 225 rpm for 3.5 hours. Next, 200 µL of the *E. coli* S17-1 *λpir* preculture was inoculated in 10 mL LB (125 mL Erlenmeyer flask) and incubated at 37°C at 225 rpm for 1.5 hours because *E. coli* S17-1 *λpir* has a steeper growth rate than *P. fluorescens* SBW25 (86). After incubation, the OD_{600} of the cells was measured and compared. Desired OD_{600} is 0.4.

3 mL of each donor *E. coli* S17-1 *λpir* and the recipient P. fluorescens SBW25 strain were mixed in a sterile 13 mL tube. If the OD_{600} differed, the volume was adjusted appropriately to regain as similar density as possible.

The tube was centrifuged at 7000 rcf using Eppendorf Centrifuge 5430 R at room temperature. Most of the supernatant was removed, leaving approximately 100 µL of supernatant to resuspend the pellet. The resuspended pellet was placed as a droplet on LA without antibiotics and incubated at 30 °C overnight.

The next day, the cell growth was scraped off by a platinum inoculation loop and resuspended in 1 mL LB. A dilution series was made, and 100 µL of undiluted, 10^{-2}, up to 10^{-5}, was selected on PIA containing the desired antibiotics and incubated at 30 °C for 2 to 4 days until growth.

3.18 Homologous Recombination

Homologous recombination is the rearrangement of DNA between two DNA molecules that share a sequence of homology. This region functions as a target for the recombination and is where the recombination occurs (42, 57, 87). The process of homologous recombination happens spontaneously *in vivo* and can also be exploited for research purposes. In this thesis, homologous recombination was utilised to knock out the gene *lsc* in *P. fluorescens* SBW25 and in *P. fluorescens* SBW25 *mucA*.

Homologous recombination occurs in two events. In the first event, the entire vector is integrated into the chromosome. In this thesis, the plasmid to be integrated contained the *lacZ* gene, therefore the lacZ-selection system (1.4.1) was used (5). In the second event, the bacteria are returned to wild type, or the correct mutant is produced (58), as described in 1.4.3. For the second recombination event, the negative-selection gene *I-sceI* and its recognition site, as described in 1.4.1, were tested.

Procedure

To mediate the first recombination event, the plasmid containing the homologue sequence must first be conjugated into the bacteria, as described in 3.17. Then, 60 µL X-Gal (20mg/mL in DMSO) was plated on PIA Kan, and the plate rested for one hour in the dark at room temperature. A dilution series up to 10^{-4} of conjugated *P. fluorescens* containing the plasmid of interest was made. 100 µL of undiluted, 10^{-2}, 10^{-3} and 10^{-4} were plated on

PIA Kan X-Gal and incubated in the absence of light at 30 °C for 48 hours until colonies were visible.

A series of subcultures of the blue colonies from these plates were made to mediate the second homologous recombination event. Three blue colonies were picked and inoculated in 3 mL LB in 13 mL tubes and incubated at 30 °C overnight. This culture was denoted K1. Further, 3 µL of K1 is inoculated in 3 mL LB in 13 mL tubes, incubated at 30 µL overnight, and denoted K2. Dilution series of subcultures K3 to K6 were plated on LA Kan X-Gal and incubated at 30 °C for two nights.

When utilising the negative selection system using *I-sceI*, precultures from the blue colonies were made by inoculating them in 10 mL LB in a 125 mL Erlenmeyer flask and incubating at 30 °C and 225 rpm overnight. To select for the second recombination event, the precultures were plated on LA – NaCl + 20% sucrose and 0.5 µg/mL ATc and incubated at 30 °C in the dark overnight.

Next, approximately 50 white colonies from these plates were transferred to LA and LA Kan and inoculated at 30 °C overnight. The colonies that did not grow on LA Kan were tested using colony PCR as described in 0. The mutants containing the gene of interest were frozen at -80 °C.

3.19 Cultivating Cells for, and Conducting the Fluorescens Measurement

When measuring the cells' fluorescence and concentration throughout the growth, the cells must be cultivated and sampled. In this thesis, the fluorescence of the fluorescent proteins mCherry_V1 and AmCyan were measured in *P. fluorescens* SBW25 *mucA* (pTSB203), *P. fluorescens* SBW25 *mucA* (pTSB203) (pHH104), *P. fluorescens* SBW25 (pTSB203), *P. fluorescens* SBW25 (pTSB203) (pHH104) and *P. fluorescens* SBW25 *mucA. The* fluorescence of mCherry_V1 was measured in *P. fluorescens* SBW25 as a control.

In addition to the fluorescence measurements, the OD_{600} of the strains at the same time points were also measured.

Procedure

The strains to be measured were inoculated in 3 mL LB in a sterile 13 mL tube and incubated at 30 °C and 225 rpm overnight, adding the appropriate antibiotics. The next day, 200 µL from the 3 mL cultures was inoculated in 10 mL DEF3 medium and the appropriate antibiotics in a 125 mL Erlenmeyer flask and incubated at 30 °C and 225 rpm for 12-14 hours. Three replicates from each strain were made from the 3 mL cultures. Then, OD_{600} was measured, and a calculated amount of culture was added to two new 125 mL Erlenmeyer flasks with 10 mL DEF3 medium to obtain OD_{600} 0.05. To one of the replicates, 20 µL 0.5 M m-toluate was added. For the control strain, only one, uninduced replica was made.

8 hours after incubation, OD_{600} and fluorescence were measured every two hours for 10 hours, and two additional time points. 200 µL of culture was used for measuring fluorescence, and 100 µL of culture was used for measuring OD_{600}. TECAN-Infinite M200pro-Microplate reader was used for the measurements, using the excitation and emission wavelengths, and gain, described in Table 3.1-1. The Nuclon 96 Flat Bottom

Transparent Polystyrene by Thermo Fisher Scientific was used for the OD_{600} measurements. The Nuclon 96 Flat Bottom Black Polystyrene by Thermo Fisher Scientific was used for the fluorescent measurements.

Table 3.19-1: **Emission and excitation wavelengths** of the fluorescent proteins mCherry_V1 and AmCyan. Gain is also given.

	mCherry_V1	**AmCyan**
Excitation wavelength (nm)	576	458
Emission wavelength (nm)	610	489
Gain	50	50

3.20 Production of M-Lyase and G-Lyase

To harvest recombinant enzymes, the cells expressing the enzymes have to be cultivated, the protein expression needs to be induced, the cells need to be lysed to access the enzymes, and then the enzymes need to be eluted, purified, and tested for activity. In this thesis, this process was utilised to harvest M- and G-lyases for usage in alginate assay. M-lyase was harvested from *E. coli* RV308 (pLE3), and G-lyase was harvested from *E. coli* RV308 (pAT77).

The XylS/Pm promotor system was utilised to express the recombinant M- and G-lyases. The cells were lysed to access the proteins. Cell lysis by sonication is a procedure to lyse the cells by using ultrasonic vibrations(88). The cells are suspended in a cold buffer solution to avoid deviations in pH, which can disrupt the functionality of the enzymes to be eluted.

The activity of the enzymes (U_{enzyme}) eluted in different mol NaCl, was tested using spectrophotometry. The lyases cut alginate, leaving a non-reducing end with a double bond that absorbs UV at 235 nm (25). The rate at which alginate is cleaved determines the amount and activity of the enzyme. The activity of an enzyme is given in U/mL, where U is defined as the amount of enzyme that increase the absorbance at 235 nm by 1 per minute (89). U/mL was calculated from the linear slope, using the using the Equation 2 in excel.

Equation 2: Enzyme activity given in U/mL

$$U_{enzyme} = \frac{\left(\frac{Abs_{235}}{minutes}\right)}{volume\ sample\ (uL)} * 1000$$

Procedure cultivating cells

Precultures of *E. coli* RV308 (pAT77) and *E. coli* RV308 (pLE3) were made by inoculating the strains in 10 mL LB and 10 µL 100 mg/mL ampicillin in 125 mL Erlenmeyer flasks and incubating them at 37°C and 225 rpm overnight.

3 mL preculture was transferred to 400 mL 3X LB medium and 400 µL 100 mg/mL ampicillin in a 2L Erlenmeyer baffled flask and incubating them at 37 °C and 225 rpm for 3 hours. To induce recombinant protein expression, 400 µL 0.5 mM m-toluate was added to each flask and incubated at 37 °C and 225 rpm for 4 more hours. Then, the cultures were allocated evenly to two 50 mL tubes and centrifuged at 5752 rcf for 20 minutes using the

Eppendorf Centrifuge 5430 R (V 2.3). The supernatant was removed, and the pellets were frozen at -20°C.

Procedure sonication of cells

Four of the pellets were gathered into one 50 mL tube and resuspended in 40 mL cold Phosphate Buffer A or 0.5M Tris-HCl Buffer A. To disrupt the cells, the diluted samples were sonicated using the Sonic Dismembrator, model 505, Sonicator by Fisher Scientific Fisherbrand. The pulses lasted for 10 seconds with 20-second intervals. The amplitude was set to 40%. During the sonication, the 50 mL tube was submerged in ice, and the microtip was placed 1 cm under the sample surface without touching the tube. The sonication process was paused during the procedure, to ensure the sample did not heat up. The sonication lasted for 3-4 minutes until the sample had big bubbles.

The crude extract was transferred to a 50 mL sterile Nalgene centrifuge tube and centrifuged at 16,000 rpm using Sorvall Lynx 6000 Centrifuge by Thermo Scientific for 30 minutes. The supernatant was sterile-filtered and cooled in ice prior to protein purification.

Procedure protein elution and purification through ion-exchange chromatography

A 5 mL anion-exchange chromatography column was equilibrated using Phosphate Buffer A or 0.5M Tris-HCl Buffer A. The sterile-filtered extract was pumped onto the column in a fast protein liquid chromatography (FPLC) system. The protein is eluted by a stepwise gradient of NaCl (0M to 1M) using Phosphate Buffer B or 0.5M Tris-HCl Buffer B. 5mL of fractions was collected during each elution stage.

Procedure enzyme-activity testing

A Costar 96-well UV plate was loaded with a 25 µL sample and 175 µL Lyaseassay Buffer containing LF 10/60 alginate. The substrate was added using an 8-Channel pipette right before measuring the activity. The absorbance at 235 nm was measured every 9 seconds for 10 minutes using SpectraMaxPlus 384 and the software SoftMaxPro 4.7.1. The lyase activity was estimated from the linear slope in the increase in OD_{235}. The samples were analysed for activity, and 50 µL was distributed evenly into 1.5 mL Eppendorf Tubes and stored at -20°C.

3.21 Cultivating Cells For, and Conducting Alginate Assay

An enzymatic alginate assay for quantifying alginate, utilising harvested M- and G-lyases that cleaves alginate, resulting in a double bond with absorption at 235 nm, was utilised in this thesis to determine the amount of alginate produced. At the 24- and 48-hour sampling time points, OD_{600} was measured for later determination of alginate per cell concentration. Alginate assays were conducted on *P. fluorescens* SBW25 strains and *P. fluorescens* SBW25 *mucA* strains.

To measure the consequences removing *lsc* have on alginate levels and growth at 24- and 48-hour timepoints, samples of *P. fluorescens* SBW25 *mucA* and *P. fluorescens* SBW25, and its corresponding *lsc*- mutants, cultivated in fructose and sucrose were taken. An alginate assay was conducted to determine the quantities of alginate in each sample.

The alginate concentration in g/L was calculated based on the delta absorbance at which the curve was maximized. In this study, the curves were maximised at 24 hours after adding lyases. The samples were corrected with lyases and blank values, and g/L were calculated by using a standard curve (Appendix E) with known alginate amount. To calculate the original g/L, the concentrations were multiplied with its dilution factor.

Procedure cultivation

Precultures of the strains to be measured was made by inoculating them in 3 mL LB and preferred antibiotics in a sterile 13 mL tube and incubate them at 30 °C and 225 rpm overnight. The next day, 500 µL preculture was inoculated in 25 mL DEF3 medium containing 0.15 mL/L alkalase 2.4L and 0.15 m/L neutrase 0.8L in a 250 mL baffled Erlenmeyer flask and incubated at 30 °C and 225 rpm for 24 hours and 48 hours. Three replicates were made for each strain.

At the sampling timepoints, OD_{600} was measured and 1g of culture was weighed into a 13 mL tube. The sample was diluted and mixed with 4 mL 0.2M NaCl, or 7 mL if viscous. The diluted sample was transferred to two 1.5 mL Eppendorf tubes and centrifuged at 16,000 rcf Eppendorf Centrifuge 5424 for 10 minutes. 1 mL of the supernatant was transferred to new 1.5 mL Eppendorf tubes, and 33 µL 3M NaOH was added to each tube. The tubes were mixed and frozen at -20 °C.

Procedure measurement

The samples to be tested were thawed and deacetylated by shaking at 30 °C and 225 rpm for one hour. Then, the samples were centrifuged at 16,000 rcf for 10 minutes. 100 µL of the supernatant was added to a new 1.5 mL Eppendorf tube containing 300 µL Tris-Buffer (0.05M Tris-HCl, 0.2M NaCl, pH 7.5).

To construct a standard curve for alginate concentration, six concentrations of LF 10/60 alginate were prepared (0.0 g/L, 0.1 g/L, 0.2 g/L, 0.3 g/L, 0.4 g/L and 0.5 g/L).

For the assay, a Costar 96-well UV plate was used. To each well, 150 µL Tris-Buffer (0.05M Tris-HCl, 0.2M NaCl, pH 7.5) and 75 µL prepared sample was added. Three parallels were used, except for the no-alginate standard, where six parallels were used. OD_{235} was measured using the SpectraMax Plus 384-Microplate Reader. Then, 8 µL M-lyase (1 U/mL) and 8 µL G-lyase (1 U/mL) was added to all wells, except three of the non-alginate standards, and mixed by pipetting. The plate was covered and incubated at room temperature. OD_{235} was measured after 3, 4 and 8 hours, including measurements the next day until the amount of alginate was stabilised.

3.22 Software

3.22.1 Benchling

Benchling is a software company, providing tools for visualising and constructing DNA plasmids, fragments, alignments, and clones. In this thesis, Benchling was utilised as a bioinformatic tool for said purposes.

3.22.2 Microsoft

Microsoft OneNote and Microsoft PowerPoint were used for the illustration of figures.

Microsoft Excel was used for data processing, calculations and visualising the data through graphs and plots.

The standard deviation (SD) was calculated using Equation 3 through Excel.

Equation 3: Standard deviation (SD)

$$\sqrt{\frac{\sum(x-\bar{x})^2}{n-1}}$$

x = the measured value n = total number of samples

x lined = the mean of the measured values

3.22.3 BLAST by NCBI

The Basic Local Alignment Search Tool (BLAST) is a bioinformatics tool that finds regions of similarity between a given sequence and sequences in a database, calculating the statistical significance of the alignment. In this thesis, BLAST was utilised to search for homologue sequences in a given strain, by using a DNA sequence of another bacterial strain as a query.

3.22.4 UniProt

UniProt is a database of protein sequences. In this thesis, UniProt was used to search for a given protein, and obtain the protein sequence of the given protein.

4 Results

In this chapter, the results of the thesis are presented. Section 4.1 describes the construction of a plasmid containing an altered triple-tandem c-di-GMP riboswitch Bc3-5 dual-fluorescence reporter system, and the subsequent c-di-GMP and alginate measurements. Section 4.2 describes the construction of the recombinant plasmid pTSB404, the testing the I-SceI counter-selection system under homologous recombination, the production of the lyases for use in the alginate assay, and the cultivation and subsequent alginate assays performed.

4.1 Inserting *mCherry_V1* in Bc3-5, and Measuring c-di-GMP Levels in Relation to Alginate Production and Overexpressance of *mucR*

This section presents the results of replacing the fluorescent protein *TurboRFP* with *mCherry_V1* in the triple-tandem c-di-GMP riboswitch (Bc3-5)-based dual-fluorescence reporter system (30). The replacement was done to minimize the background fluorescence by *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA*, which had an overlapping fluorescence spectrum with that of *TurboRFP* (33). By using *mCherry_V1* as the fluorescent reporter gene, the effect c-di-GMP levels have on alginate production throughout the growth can be measured. *mucR* overexpressing strains were also tested for their c-di-GMP levels and alginate production. In this chapter, these tests are presented and evaluated.

First, the fluorescent expression of *mCherry_V1* was tested in *P. fluorescent* SBW25. Then, a plasmid with *mCherry_V1* exchanged for *TurboRFP*, called pTSB203, is constructed. An overview of the steps in constructing pTSB203, containing *mCherry_V1*, is illustrated in Figure 4.1-2. Lastly, c-di-GMP measurements were conducted using alginate and non-alginate synthesising *P. fluorescens* SBW25, and *mucR* overexpressing alginate and non-alginate synthesising *P. fluorescens* SBW25. Alginate measurements were conducted on the alginate producing strains.

4.1.1 Testing the Expression of *mCherry_V1* in *P. fluorescens* SBW25

To use *mCherry_V1* as a fluorescent reporter gene, it is required for it to be expressible in *P. fluorescens* SBW25. Furthermore, the emission and excitation wavelength should also be distinguishable towards the fluorescence of *P. fluorescens* SBW25. This section presents the results of testing the expression of *mCherry_V1* in *P. fluorescens* SBW25 and the fluorescence of *P. fluorescens* SBW25 in the same fluorescence spectre.

The plasmid RK2-mCherry_V1 was conjugated into *P. fluorescens* SBW25. Three biological replicates of *P. fluorescens* SBW25 RK2-mCherry-V1 and one culture of *P. fluorescens* SBW25 as negative control were cultivated in DEF3 medium for 24 hours. OD_{600} and fluorescence of mCherry_V1 (Emission/Excitation576/610 nm, gain 100) were measured (Figure 4.1-1).

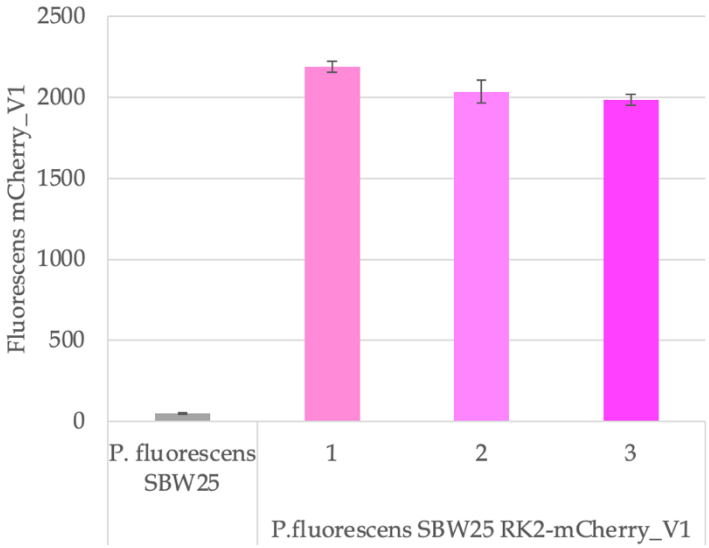


Figure 4.1-1: **Average of mCherry_V1 fluorescence** measured in quintuplicates. The bars represent each standard deviation.

Figure 4.1-1 indicate that *mCherry_V1* is expressible in *P. fluorescens* SBW25 RK2-mCherry-V1, while *P. fluorescens* SBW25 exhibits low autofluorescence in the fluorescent spectre. Based on these results, *mCherry_V1* could be a useful replacement for *TurboRFP*.

4.1.2 Constructing pTSB203

This section presents the results of constructing the plasmid pTSB203, which replaces the reporter gene *TurboRFP* with the reporter gene *mCherry_V1* in the Bc3-5 riboswitch. Figure 2.0-1 illustrates the steps in constructing the plasmid. This section is divided into construction of each plasmid.

The constructed plasmids are listed in Appendix B.

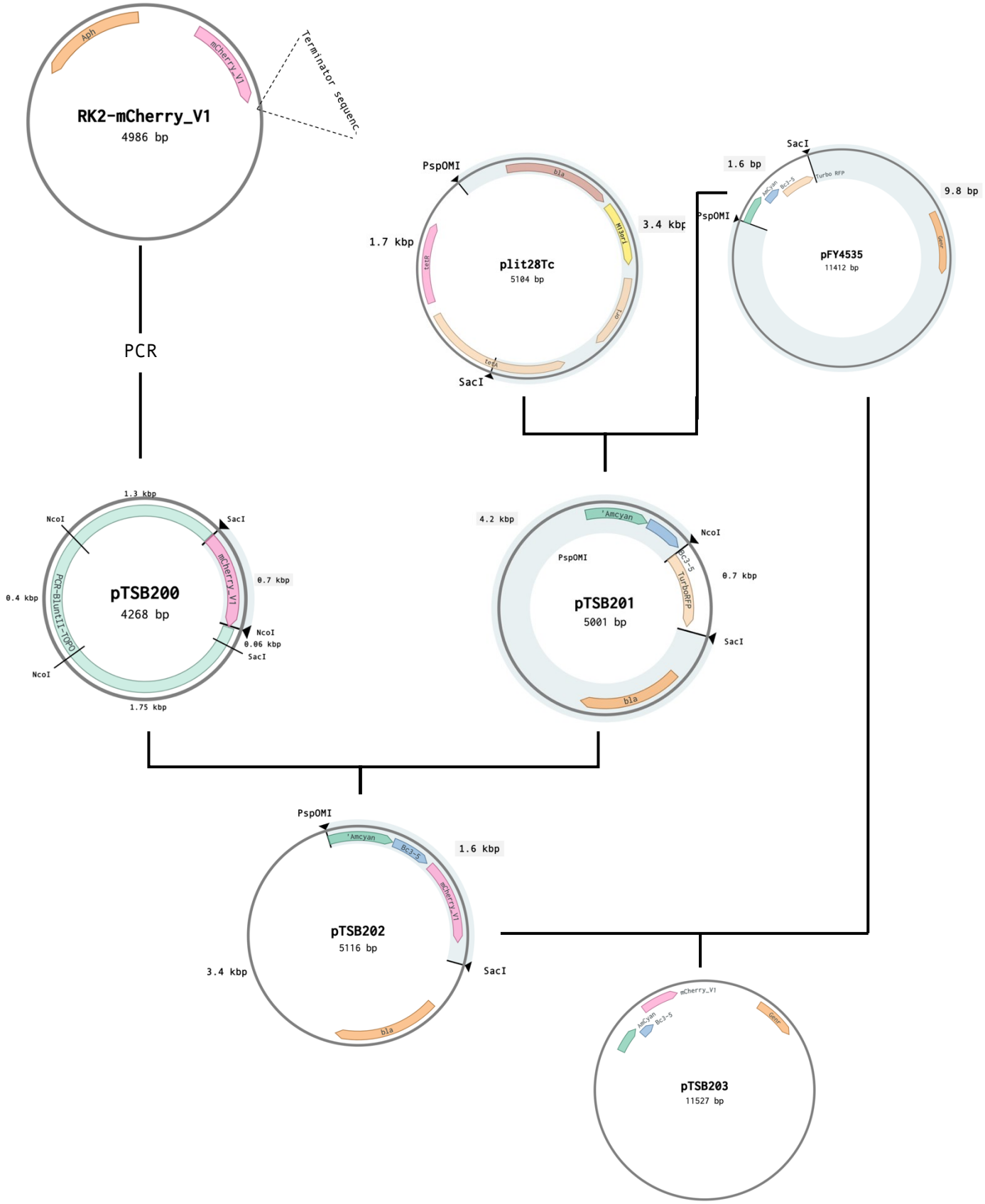


Figure 4.1-2: **Overview of the steps in constructing pTSB203.** The fragments to be ligated in the next step is marked in grey. A terminator sequence in "RK2-mVherry_V1" plasmid is illustrated as outside of the plasmid, because of results have shown that the terminator sequence could be absent.

4.1.2.1 Cloning mCherry_V1 from RK2-mCherry_V1 and Construction of pTSB200

This section presents PCR results of amplifying and isolating *mCherry_V1* from RK2-mCherry_V1, and the results of inserting *mCherry_V1* into the vector DNA pCR™II-Blunt-TOPO®.

The plasmid RK2-mCherry_V1 was isolated and purified. Several PCR reactions were conducted using 55 °C annealing temperature and 1 minute elongation time with the primer pairs mCherryV1F/R. The PCR products were separated on an agarose gel (Figure 4.1-3). The expected results were one fragment of 845bp. However, the fragments were not of the expected size.

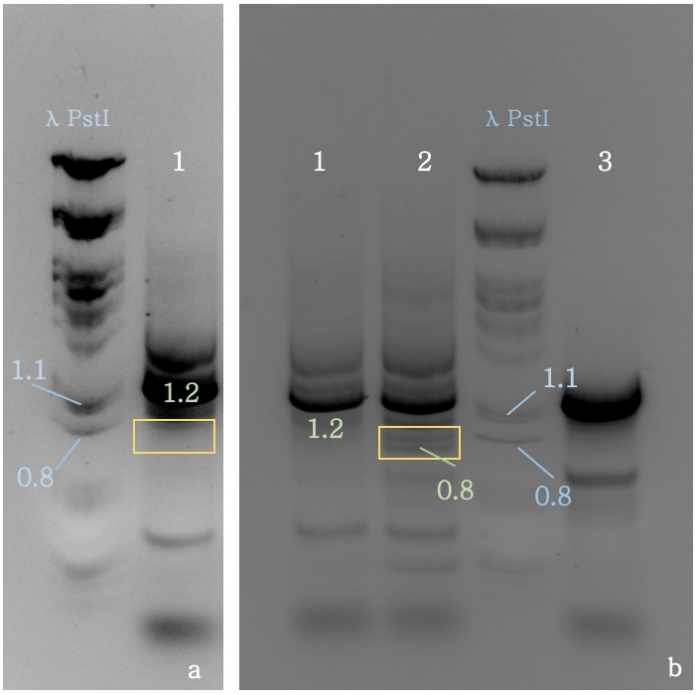


Figure 4.1-3: **Separated PCR products of expectedly amplified mCherry_V1 from the RK2-mCherry_V1 plasmid**. a) Lane 1 contains the PCR products of the first PRC reaction conducted. The marked fragment was cut out and purified to be used as DNA template in a third PCR reaction. b) Lane 1 contains the same PCR product as in gel a. Lane 2 contains the PCR products of a repeated PCR reaction. Lane 3 contains the PCR products of a third PCR reaction using isolated DNA from the first PCR reaction (a) as DNA template. λ PstI as DNA standards. Fragment sizes in kbp.

Even though the product was not pure, the seemingly weak 0.8 kbp fragment, marked in Figure 4.1-3 b, was cut out, purified, and used as the blunt end fragment DNA in the Zero Blunt® TOPO® PCR Cloning system and further cloned as the illustration shows (Figure 4.1-2). Errors followed tough out, therefore new primer pairs were ordered to repeat the initial PCR reactions. The forward primer, mCherryV1F (34), was identical to the former. Two reverse primers, one including the terminator sequence, mCherryV1R3, were different to the former. All primers utilised are listed in Appendix D.

PCR reactions were conducted using the primer pairs mCherryV1F2/R2 (without terminator sequence) and mCherryV1F2/R3 (Including terminator sequence), 51 °C annealing temp. and 1 minute elongation time.

The PCR products were separated on an agarose gel (Figure 4.1-4). The expected fragments were 1.3 kbp for mCherryV1F2/R3 primer pair and 0.7 kbp for mCherryV1F2/R2 primer pair. This resulted in no products.

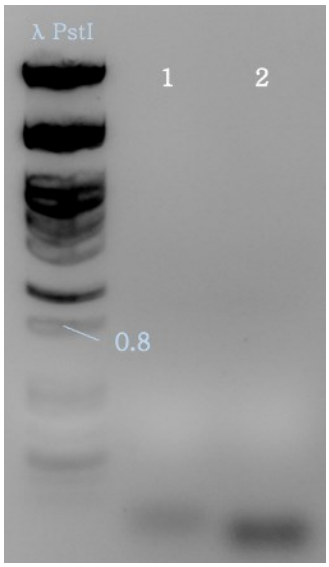


Figure 4.1-4: **Separated PCR products of expectedly amplified mCherry_V1 from the RK2-mCherry_V1 plasmid.** Lanes 1 and 2 represent the same PCR product. λ PstI as DNA standard. Fragment sizes in kbp.

An explanation for unsuccessful PCR reactions (Figure 4.1-4) could be sequential flaws in the RK2-mCherry_V1 plasmid. RK2-mCherry_V1 was therefore cut in three different reactions using the restriction enzymes HindIII, PstI and XmnI, and separated on an agarose gel (Figure 4.1-5). The expected fragment size when cut by HindIII is 3.2 kbp, 1.5 kbp and 0.9 kbp. The expected fragment size when cut by XmnI and PstI is 2.3 kbp, 1.4 kbp and 1.2 kbp. The fragments were not of the expected size (Figure 4.1-5).

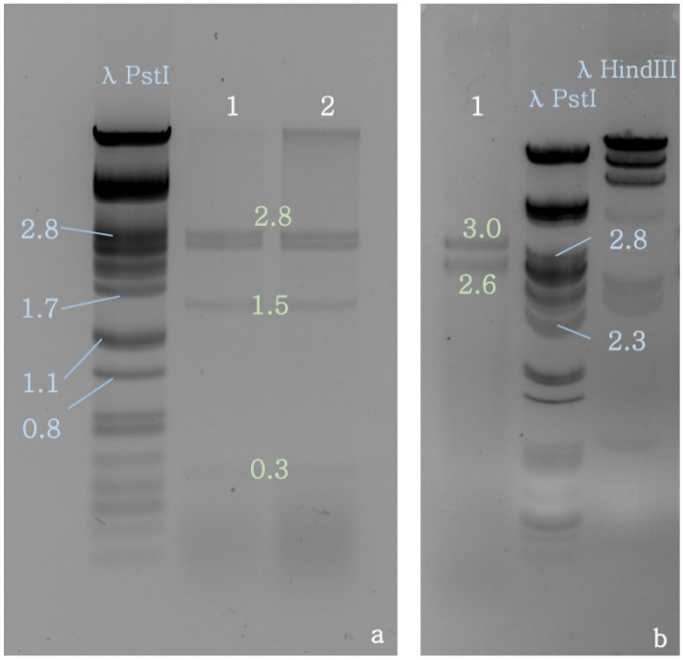


Figure 4.1-5: **Digested RK2-mCherry_V1 plasmid. λ** PstI and λ HindIII as DNA standards. a) Digested RK2-mCherry_V1 by HindIII. Two individual digestions. Only 1.5 kbp fragment is expected. b) Digested RK2-mCherry_V1 by XmnI and PstI. Fragment sizes in kbp.

The results in Figure 4.1-5 support the assumption that there could be sequential flaws in the RK2-mCherryV1 plasmid.

Simultaneously as the digestion of RK2-mCherry_V1, a new PCR reaction using the primer pair mCherryV1F2/R3 was conducted using CloneAmp HiFi PCR Premix. The PCR product was separated on an agarose gel (Figure 4.1-6)The expected fragment sizes were 1.3 kbp. (Figure 4.1-6) The fragment was not of the expected size.

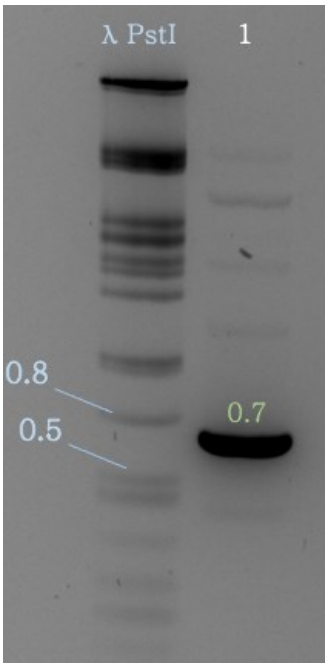


Figure 4.1-6: **Separated PCR (using CloneAmp HiFi PCR Premix) products of expectedly amplified mCherry_V1 from the RK2-mCherry_V1 plasmid.** λ PstI as DNA standard**.** Fragment sizes in kbp.

Because of the possibility that the amplified sequence still contained the *mCherry_V1* gene, the PCR product was used directly as the blunt end fragment in the Zero Blunt^{R} TOPO^{R} PCR Cloning system.

The PCR product possibly containing *mCherry_V1* was introduced as an insert in the vector DNA pCR^{TM}II-Blunt-TOPO^{R}. The plasmid was transformed and selected on LA Kan.

Preculture of the transformed cells was made, and the plasmid was isolated and tested by digesting with the restriction enzyme HindIII (Figure 4.1-7). The expected fragment size was 4.6 kbp. The expected fragment sizes of re-ligation of the parent vector DNA pCR^{TM}II-Blunt-TOPO^{R} are 3.5 kbp. The fragments were of unexpected sizes. The fragments of transformant number 1, 3, 5 and 6 display weak fragments of approximately 2.8 and 1.5 kbp, the fragment sizes similar to RK2-mCherry_V1 plasmid digested by HindIII (Figure 4.1-5), suggesting that it could be traces of RK2-mCherry_V1. The fragments of transformant numbers 2 and 4 display fragments of approximately 4.4 and 0.6. (Figure 4.1-7)

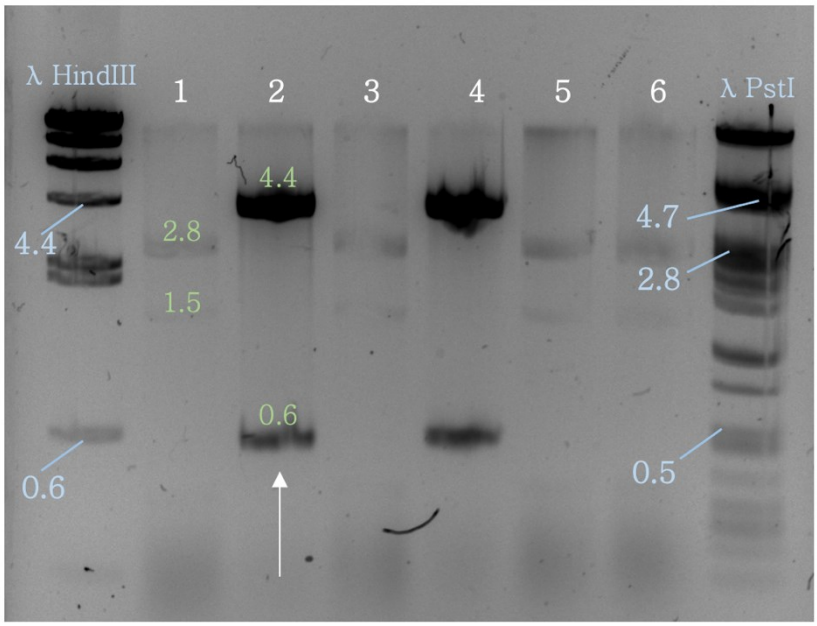


Figure 4.1-7: **HindIII digested plasmids isolated from 6 transformants annotated 1-6.** λ PstI and λ HindIII as DNA standards. Fragment sizes in kbp.

The colony colours of transformants 2 and 4 emitted a pink colour, while transformants 1,3,5 and 6 were white. Transformants 2 and 4 were sequenced because of an assumption that *mCherry_V1* was the reason for the pink colour.

The sequencing results (Appendix I) revealed that the given sequence of plasmid RK2-mCherryV1 near *mCherry_V1* was not correct. The terminator sequence was absent, and the terminator codon of *mCherry_V1* was TGA, not TAA. The results explain the fragments in Figure 4.1-7, in which the expected fragments from digestion with HindIII now were 3.8 kbp and 0.5 kbp, which is correct. It also explains the results of Figure 4.1-6, where the expected results regarding the sequencing results is a fragment of 0.7 kbp, which is displayed.

Transformant number two was chosen (Figure 4.1-7), and the plasmid was named pTSB200.

4.1.2.2 Constructing pTSB201

To replace *TurboRFP* in pFY4535 with *mCherry_V1* from RK2-mCherry_V1, some intermediate steps had to be done (Figure 4.1-2). The results of the process are presented in this section.

The plasmids pFY4535 and pLitmus28Tc were digested by the restriction enzymes SacI and PspOMI. The digested plasmids were separated on agarose gels. Also, uncut pFY4535 was separated on an agarose gel (Figure 4.1-8)

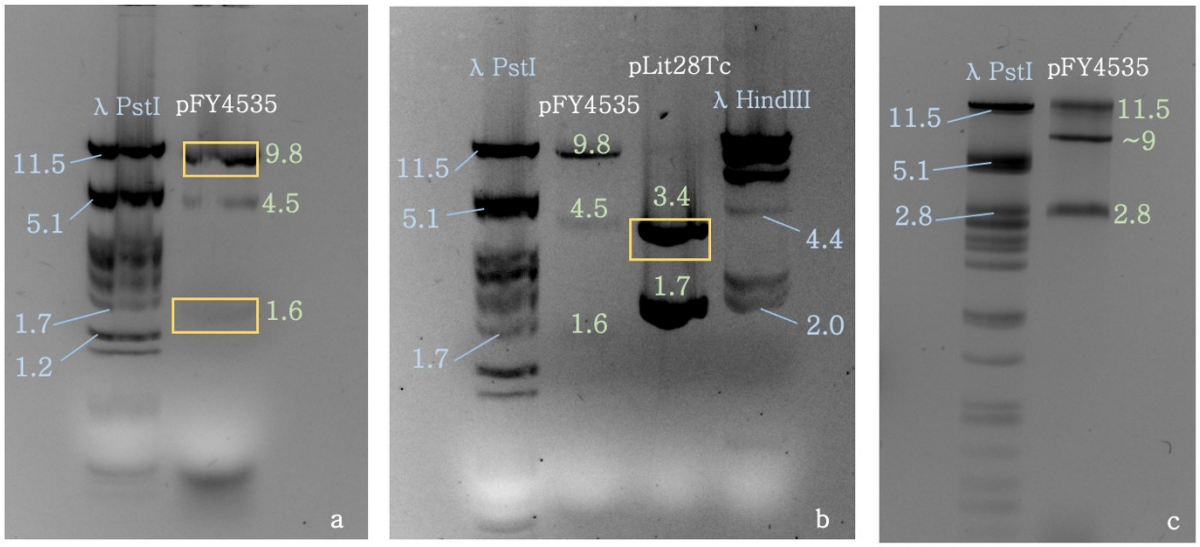


Figure 4.1-8: **a) and b) Separated pFY4535 and pLit28Tc digested by SacI and PspOMI c) Separated uncut pFY4535**. λ PstI and λ HindIII as DNA standards. Fragment sizes in kbp.

The marked 3.4 kbp fragment from pLitmus28Tc and the marked fragments of 9.8 kbp and 1.6 kbp from pFY4535 (Figure 4.1-8) were cut out and purified. pFY4535 displayed a weak, not expected, 4.5 kbp fragment (Figure 4.1-8). This could be an uncut pFY4535 plasmid, and therefore uncut pFY4535 plasmid was separated on an agarose gel. Uncut pFY4535 displays two blurry and one much clearer fragment in between. The fragment of approximately 2.8 kbp is possibly supercoiled pFY4535, the clear bond in the middle could be the closed circular pFY4535, while the 11.5 kbp fragment could be pFY4535 cut once since this is the expected size of linear pFY4535 (Figure 4.1-8).

The 3.4 kbp fragment of pLitmus28Tc (Figure 4.1-8) was ligated with the 1.6 kbp fragment of pFY4535 (Figure 4.1-8). The purified fragment of 9.8 kbp (Figure 4.1-8) was stored for later use at -20 °C.

The ligation mix was transformed to *E. coli* S17-1 *λpir* and selected on LA Amp. Plasmids from 8 transformants were isolated, digested with the restriction enzymes NcoI and SacI and separated on an agarose gel (Figure 4.1-9). NcoI and SacI cut on both sides of the reporter gene *TurboRFP*, making the restriction enzymes suitable for removing *TurboRFP* from pFY4535. The expected fragments were 0.7 and 4.3. The expected fragment sizes of the re-ligated parent plasmid are 5.1 and 0.04 kbp.

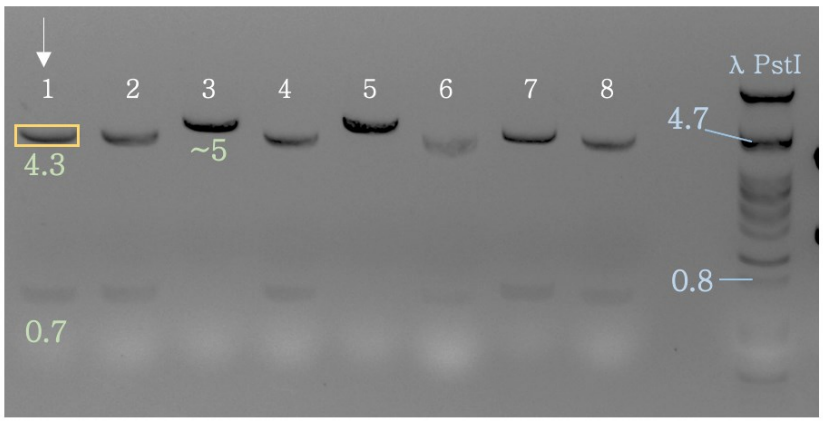


Figure 4.1-9: **Separated NcoI and SacI digested plasmids isolated from 8 transformants annotated 1-8.** λ PstI as DNA standard. Fragment sizes in kbp.

Transformants 1, 2, 4, 6, 7 and 8 were the expected size, and transformants number 3 and 5 display possible re-ligated pLitsmus28Tc fragments (Figure 4.1-9). Transformant number 1 was chosen and called pTSB201. The marked fragment of 4.3 kbp was cut out and purified (Figure 4.1-9).

4.1.2.3 Constructing pTSB202

mCherry_V1 from pTSB200 was inserted into the vector sequence of pTSB201 (Figure 4.1-2). It was inserted into this vector to be correctly oriented in relation to the riboswitch system. In this section, the result of this process is presented.

The sequenced plasmid of transformant 2 (pTSB200) and 4 from section 2.1 was digested using the restriction enzymes SacI and NcoI and separated on an agarose gel (Figure 4.1-10). The expected fragment sizes are 1.7, 1.3, 0.7, 0.4 and 0.06.

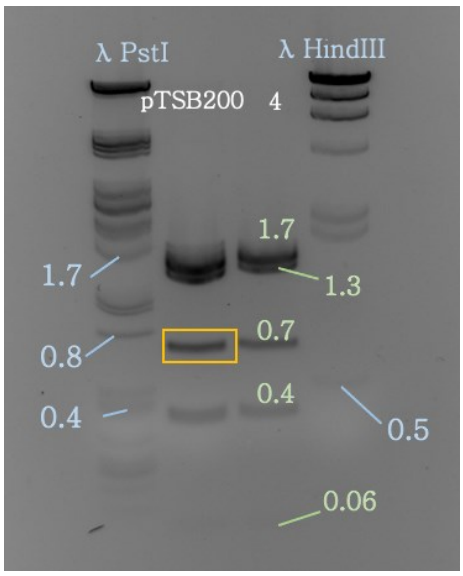


Figure 4.1-10: **pTSB200 and the sequenced plasmid from transformant number 4 (Figure 4.1-7) digested by SacI and NcoI and separated.** λ PstI and λ HindIII as DNA standards. Fragment sizes in kbp.

The marked 0.7 kbp fragment from pTSB200 (Figure 4.1-10) was cut out and purified. The purified 0.7 kbp fragment was ligated with the purified 4.3 kbp fragment of pTSB201 (Figure 4.1-9). The ligation mix was transformed into *E. coli* S17-1 *λpir* and selected on LA amp.

Plasmids from 6 transformants were isolated and digested using the restriction enzymes PspOMI and SacI and separated on an agarose gel (Figure 4.1-11). The expected fragment sizes of pTSB202 were 3.4 kbp and 1.6 kbp. The expected fragment sizes of re-ligation of the parent plasmid pTSB201 are 3.4 kbp and 0.9 kbp. The fragments were of the expected sizes.

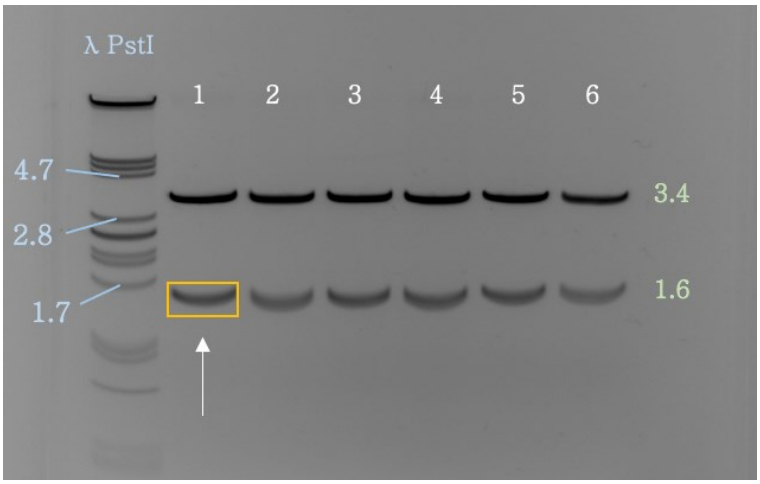


Figure 4.1-11: **Separated PspOMI and SacI digested plasmids isolated from 6 transformants annotated 1-6.** λ PstI as DNA standard. Fragment sizes in kbp.

The plasmid from transformant number 1 was chosen and named pTSB202. The marked 1.6 kbp fragment (Figure 4.1-11) was cut out and purified.

4.1.2.4 Constructing pTSB203

Finally, *mCherry_V1* and the rest of the triple-tandem riboswitch system (Bc3-5 and *AmCyan*) from pTSB202 (Figure 4.1-11) were inserted into the vector sequence of pFY4535, resulting in pTSB203 with the only difference from pFY4535 being the *TurboRFP -mCherry_V1* exchange. (Figure 4.1-2) In this section, the results of constructing pTSB203 are presented.

The marked 1.6 kbp fragment from pTSB202 (Figure 4.1-11) and the marked 9.8 kbp fragment from pFY4535 (Figure 4.1-8) was ligated. The ligation mix was transformed into *E. coli* S17-1 *λpir* and selected on LA gm.

Plasmids from 6 transformants were isolated and digested using the restriction enzyme HindIII and separated on an agarose gel (Figure 4.1-12). Expected fragment sizes are 9.4 kbp, 1.6 kbp and 0.4 kbp. The expected sizes of the religation of the parent plasmid pFY4535 are 9.4, 0.3 and 0.04. The fragments were not of the expected size but resembles the sequence pattern as for uncut pFY4535 (Figure 4.1-8).

Therefore, the isolated plasmids were also digested by SacI and PspOMI, and by PstI and EcoRV. The expected fragment sizes of the plasmid cut by SacI and PspOMI are 1.6 and 9.8 kbp. The expected size of the religation of the parent plasmid pFY4535 is 9.8. The expected fragment sizes of the plasmid cut by PstI and EcoRV are 5.3, 4.4 and 1.7. The plasmids were separated on an agarose gel (Figure 4.1-12). The expected sizes of the religation of the parent plasmid pFY4535 are 4.4 kbp, 3.7 kbp, 1.7 kbp and 0.03 kbp. The plasmid was of expected sizes in the two digestion reactions, plus extra unexpected fragment(s) (Figure 4.1-12).

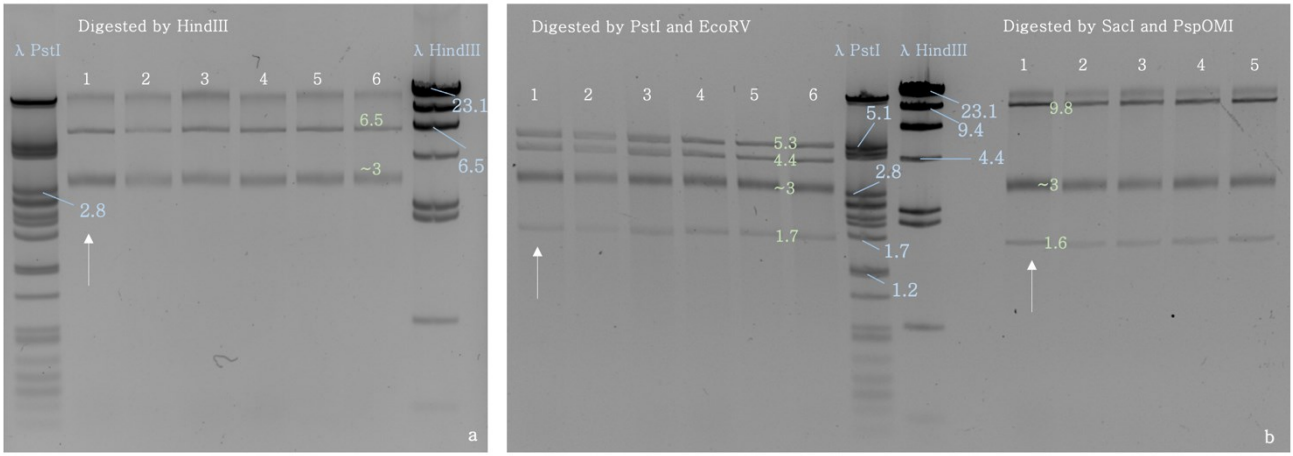


Figure 4.1-12: **Separated plasmids isolated from 6 transformants annotated 1-6, digested by different restriction enzymes.** λ PstI and λ HindIII as DNA standards. Fragment sizes in kbp. a) separated plasmids digested by HindIII. b) To the left of λ Pst: Separated plasmids digested by PstI and EcoRV. To the right of λ HindIII: Separated plasmids digested by PspOMI and SacI. The isolated plasmid of transformant number 6 was left out because of space issues.

Because of unexpected results, a new agarose gel was made, separating the digestion reactions with plasmid isolated from transformant number one, including an uncut plasmid (Figure 4.1-13). This was to examine the digestion results easier.

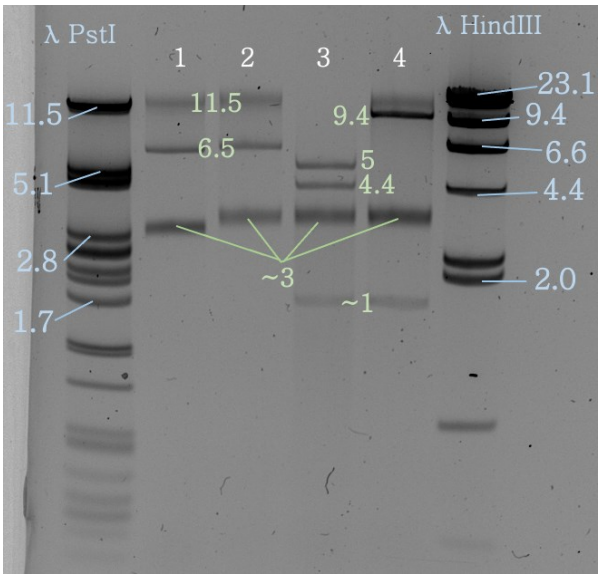


Figure 4.1-13: **Digested and separated plasmid isolated from transformant number one with different restriction enzymes.** Lane 1 contains uncut plasmid. Lane 2 contains HindIII digested plasmid. Lane 3 contains PstI and EcoRV digested plasmid. Lane 4 contains PspOMI and SacI digested plasmid. λ PstI and λ HindIII as DNA standards. Fragment sizes in kbp.

By analysing the digestion results and considering the uncut plasmids from the parent plasmid pFY5435 (Figure 4.1-8), the ca. 3k bp fragment and the largest fragment look like an uncut plasmid. The isolated plasmid digested by PstI and EcoRV, and the plasmid digested by SacI and PspOMI, display the expected fragment sizes, including the uncut plasmid. The isolated plasmid digested by HindIII resembles an uncut plasmid, which concludes that the digestion reaction did not occur. The plasmid of transformant number 1 was chosen and named pTSB203.

As a second verification test, *E. coli* S17-1 *λpir* (pFY4535) and *E. coli* S17-1 *λpir* (pTSB203) were streaked out on LA Gen and incubated at 30 °C for 2 nights (Figure 4.1-14).

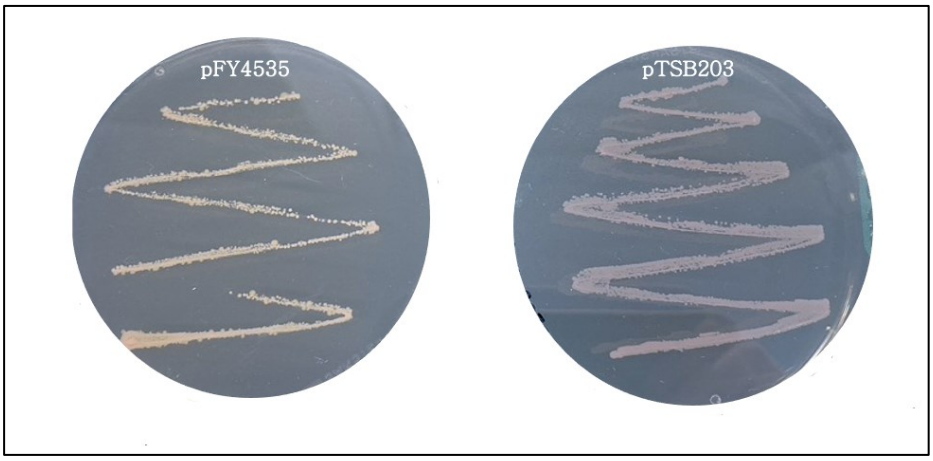


Figure 4.1-14: ***E. coli* S17-1 *λpir* (pFY4535) and *E. coli* S17-1 *λpir* (pTSB203) on LA gm.** The plates are next to each other with the same lightning. The phenotype of pTSB203 expressing mCherry_V1 and pFY4535 expressing TurboRFP are different.

Figure 4.1-14 shows that pFY4535, expressing *TurboRFP*, emits a bright orange colour, while pTSB203 emits a pink colour, with the only difference between the two plasmids being the expression of the fluorescent proteins TurboRFP and mCherry_V1. This result is also an indication that *mCherry_V1* is present in pTSB203.

4.1.3 Assessment of the *mCherry*_V1 Coupled Bc3-5 Riboswitch in *P. fluorescens* SBW25 and the Alginate Producing *P. fluorescens* SBW25 *mucA*

The tripe-tandem riboswitch Bc3-5 fused between the fluorescent reporter genes *AmCyan* and *mCherry_V1*, in pTSB203, should activate the translation of *mCherry_V1* upon binding of the ligand c-di-GMP. By measuring the levels of g-di-GMP dependent fluorescent mCherry_V1 and the constitutive fluorescent AmCyan, this system could measure the c-di-GMP levels *in vivo*.

If the system is reliable, it can be used to describe if, and if so- which, effect the c-di-GMP levels could have on the alginate producing *P. fluorescent* SBW25 *mucA* mutant. The system could also measure the effect overexpressing *mucR*, the protein synthesizing and degrading c-di-GMP, have on c-di-GMP levels in both *P. fluorescent* SBW25 and *P. fluorescent* SBW25 *mucA.*

pHH104, the plasmid containing *mucR* controlled by the P_{mG5} promoter, resulting in over-expressing of MucR, was conjugated into *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA.* pTSB203, the vector containing the *AmCyan*-Bc3-5-*mCherry_V1* riboswitch was conjugated into *P. fluorescens* SBW25 *mucA* and *P. fluorescens* SBW25, as a control, to measure if the *mucA* mutation affects the levels of c-di-GMP. pTSB203 was also conjugated into *P. fluorescens* SBW25 *mucA* (pHH104) and *P. fluorescens* SBW25 (pHH104), to measure which effect *mucA* mutation and overexpression of *mucR* have the levels of c-di-GMP, and on the alginate synthesis. To sum up: c-di-GMP levels were tested in *P. fluorescens* SBW25 *mucA* (pTSB203), *P. fluorescens* SBW25 *mucA* (pTSB203) (pHH104), *P. fluorescens* SBW25 (pTSB203), *P. fluorescens* SBW25 *mucA* (pTSB203) (pHH104) and *P. fluorescens* SBW25 *mucA* as a control.

The cultivation of the five strains was conducted as described in 3.19, using Kan to select for pHH104 and Gen to select for pTSB203. *P. fluorescens* SBW25 *mucA* was used as the control strain as it doesn't contain the overexpression of *mucR*, nor the expression of *mCherry_V1* or *AmCyan*, thus revealing the autofluorescence of *P. fluorescens* SBW25 *mucA* in the fluorescence spectre to be measured. *P. fluorescens* SBW25 were not used as a control strain, because it did not exhibit any autofluorescence in the given fluorescent spectre (Figure 4.1-1).

Six biological replicates were cultivated, three induced by 1 mM m-toluate. OD_{600} and the fluorescence of mCherry_V1 and AmCyan were measured at nine different time points. (Figure 4.1-15,Figure 4.1-16)

The measurement was conducted twice, the first time only measuring fluorescence 610 and OD_{600}, therefore the results from the first measurements are not shown.

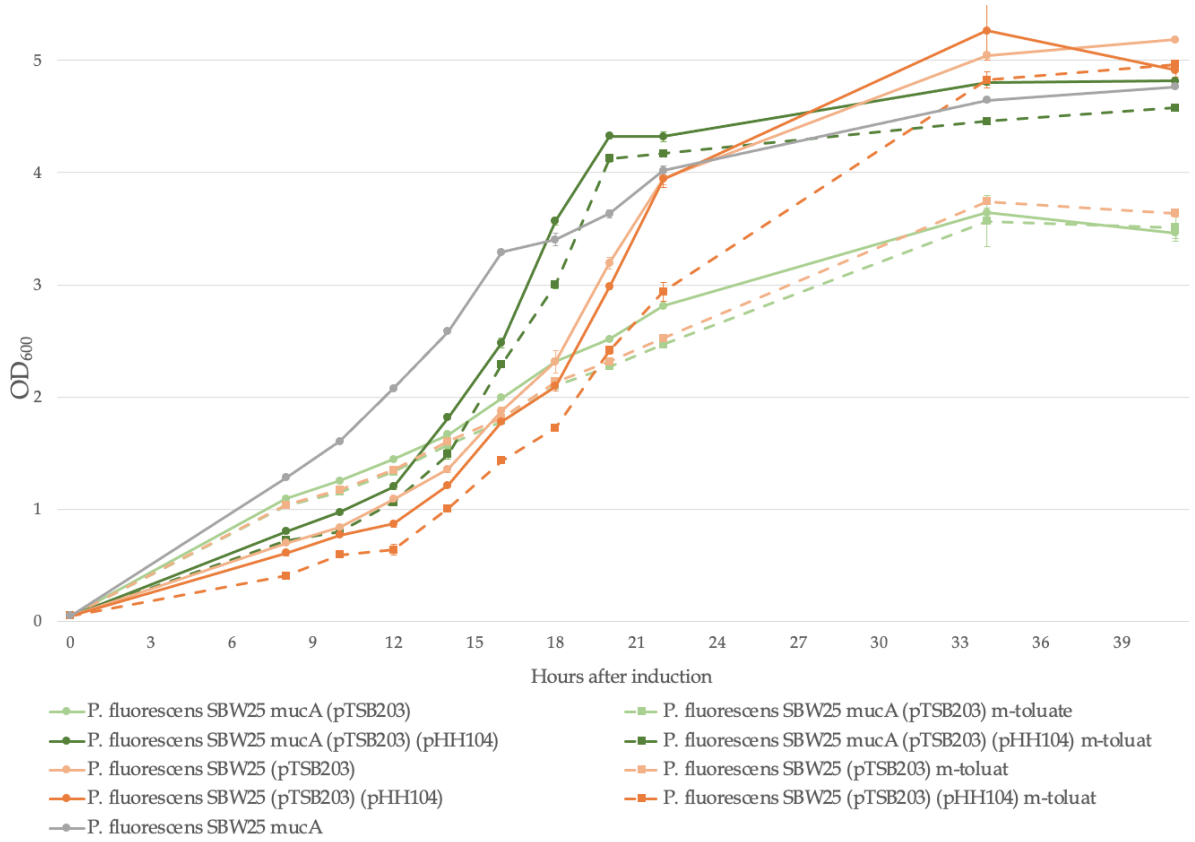


Figure 4.1-15: **OD_{600} measurements of induced (dotted line) and uninduced (line) *P. fluorescens* SBW25 *mucA* (pTSB203), *P. fluorescens* SBW25 *mucA* (pTSB203) (pHH104), *P. fluorescens* SBW25 (pTSB203) and *P. fluorescens* SBW25 (pTSB203) (pHH104).** Uninduced *P. fluorescens* SBW25 *mucA* was used as control strain. For each data point, the average OD_{600} of three biological replicates, measured in duplicate, corrected by blank and pathlength are given. The bars represent the standard deviations of each timepoint.

Figure 4.1-15 shows that the strains exhibit somewhat different growth rates. The control strain *P. fluorescens* SBW25 *mucA* grow faster than the mutants, and *P. fluorescens* SBW25 *mucA* (pTSB203) (pHH104) exhibit a steeper log-phase than the other mutants. The induced strains *P. fluorescens* SBW25 *mucA* (pTSB203) and *P. fluorescens* SBW25 (pTSB203) exhibit similar growth curves. *P. fluorescens* SBW25 *mucA* (pTSB203) exhibit less OD_{600} at the stationary phase. In general, the induced replicates exhibit a lower OD_{600} than those not induced by m-toluate (Figure 4.1-15).

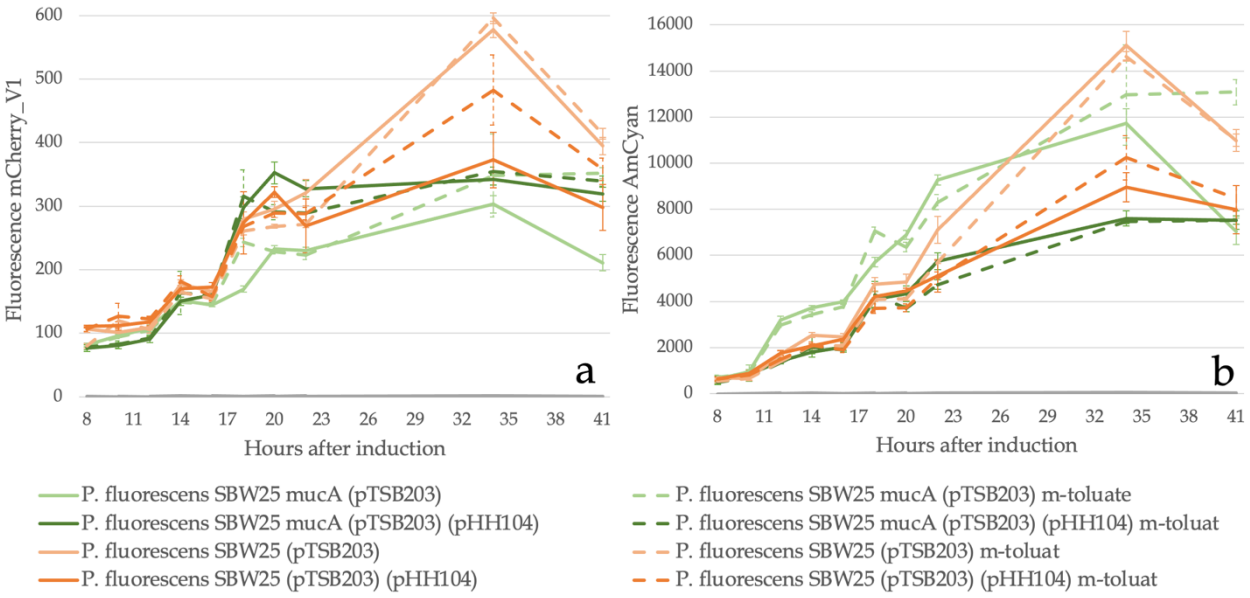


Figure 4.1-16: **Fluorescence m measurements of induced (dotted line) and uninduced (line) *P. fluorescens* SBW25 *mucA* (pTSB203), *P. fluorescens* SBW25 *mucA* (pTSB203) (pHH104), *P. fluorescens* SBW25 (pTSB203) and *P. fluorescens* SBW25 (pTSB203) (pHH104).** Uninduced *P. fluorescens* SBW25 *mucA* was used as control strain. For each data point, the average fluorescence of three biological replicates, measured in duplicates are given. The bars represent the standard deviations of each timepoint. a) mCherry_V1 fluorescence measurements. b) AmCyan florescence measurements.

Figure 4.1-16 displays the mCherry_V1 and AmCyan fluorescence measurements. For the mCherry_V1 fluorescence measurement, Figure 4.1-16 a, uninduced *P. fluorescens* SBW25 *mucA* (pTSB203) exhibit a lower fluorescence from the 16-hour timepoint than the others. Induced and uninduced *P. fluorescens* SBW25 (pTSB203) exhibit a much higher fluorescence at the 32-hour timepoint, than the other strains.

For the AmCyan fluorescence measurement (Figure 4.1-16 b), unfortunately, the first two samples (8 hours and 10 hours after induction) were measured two hours after sampling, using only 100 µL (normally 200 µL) in each well. Despite this mishap, the data from the measurements were used because they displayed legitimate numbers in relations to the 12-hour sampling and the increase in fluorescence.

P. fluorescens SBW25 strains with pTSB203 display a have a higher *AmCyan* amount than the strains containing the *mucR* overexpressing plasmid pHH104. *P. fluorescens* SBW25 *mucA* (pTSB203) both induced and uninduced, show higher *AmCyan* levels earlier in the exponential growth phase than the other strains.

Expected results for non-functional Bc3-5 riboswitch would have been largely similar curves for the florescence of AmCyan and mCherry_V1 (Figure 4.1-16), because the translation of mCherry would not have been repressed by the riboswitch. Expected results of non-functional activating of the riboswitch by c-di-GMP, would have been no fluorescence of mCherry_V1. Fluorescent results display differences in the fluorescent curves, especially for the not-mucR overexpressed *P. fluorescens* SBW25 *mucA* (pTSB203).

When comparing the fluorescence of mCherry_V1 and AmCyan, induced and uninduced *p. fluorescens* SBW25 *mucA* (pTSB203) is prominent, in being in the lower level of Figure 4.1-16 a, and in the upper level of Figure 4.1-16 a, including having a low OD_{600} towards the stationary phase (Figure 4.1-15).

To evaluate the intracellular c-di-GMP levels, the relative fluorescence intensity (RFI) was calculated by dividing fluorescence of mCherry by the fluorescence of AmCyan (Figure 4.1-17). This gives a ratio between the translation of *AmCyan* and a value of the c-di-GMP activated translation of *mCherry_V1*.

Figure 4.1-17: **The RFI-value calculated of induced (dotted line) and uninduced (line) *P. fluorescens* SBW25 *mucA* (pTSB203), *P. fluorescens* SBW25 *mucA* (pTSB203) (pHH104), *P. fluorescens* SBW25 (pTSB203) and *P. fluorescens* SBW25 (pTSB203) (pHH104).** For each data point, the average RFI value are given. The bars represent the standard deviations of each timepoint.

Induced and uninduced *P. fluorescens* SBW25 *mucA* (pTSB203) stands out from the rest of the strains, by exhibiting low RFI from the 12-hour timepoint values. This is expected when examining Figure 4.1-17. Whereas the difference between induced and uninduced strains are barely detectable. There is also a slight difference between the mucR overexpressing strains and the mucR normal-expressing strains, where the mucR overexpressing strains exhibits overall higher RFI values. Standard curves intersect at 8 and 10 hours after induction.

4.1.4 Alginate Production Measurement by *P. fluorescens* SBW25 *mucA* Conjugates

The alginate concentration of the *P. fluorescens* SBW25 *mucA* strains was measured to investigate the effect *mucR* overexpression has on alginate synthesis. Four biological replicates were made for each strain, two induced by m-toluate at the beginning of cultivation, and the cultivation was done according to the methods. The strains were tested in triplicates (Figure 4.1-18). Samples of the control *P. fluorescens* SBW25 *mucA* were taken at another time-point.

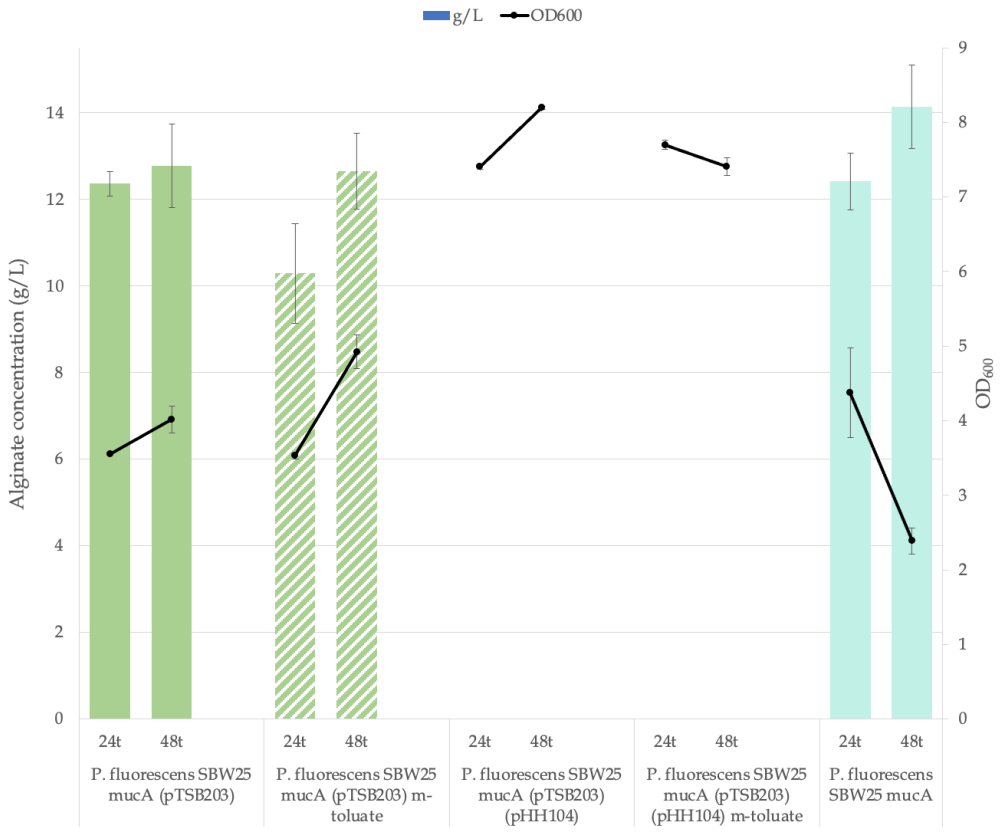


Figure 4.1-18: **Alginate concentration in g/L and OD_{600} for induced and uninduced *P. fluorescens* SBW25 *mucA* (pTSB203) and *P. fluorescens* SBW25 *mucA* (pTSB203) (pHH104).** Each bar represents the average of 2 biological replicates tested in triplicates, with standard deviations. Each dot represents the average OD_{600} with standard deviations. Lines between the dots represent the growth of the cultivates strain. *P. fluorescens* SBW25 *mucA* as a positive control for illustration, sampled at another timepoint from 3 biological replicates, tested in triplicate.

Of the four conjugated strains, only the *P. fluorescens* SBW25 *mucA* (pTSB203) strains produced alginate. The *mucR* overexpressing strains produced no alginate. The alginate producing strains exhibited less growth than the non-alginate mucR-overexpressing strain.

P. fluorescens SBW25 *mucA* exhibits a fall in OD_{600} between 24 and 48 hours, which indicated that the strain was in the death phase. *P. fluorescens* SBW25 *mucA* (pTSB203) exhibits higher growth at 48-hour timepoint, indicating that the cells were in the stationary phase.

4.2 The effect of Inactivating *lsc* in *Pseudomonas fluorescens* SBW25 and its *mucA* Mutant.

In this section, the results of constructing *lsc* negative strains (Figure 4.2-1) of the alginate overproducing *P. fluorescens* SBW25 *mucA* and the non-alginate-producing *P. fluorescens* SBW25 are presented. A new system using the I-sceI endonuclease to select for homologue recombined *P. fluorescens* was tested. Production of M- and G-lyases for usage in alginate quantification measurements are given, including the alginate measurements of the *lsc*-negative strains.

4.2.1 Constructing pTSB404

In order to construct *lsc* knockouts, first a recombination vector was constructed, with a kanamycin resistance gene (*Kan*^{r}), counter-selection gene *I-sceI* and its recognition site (for the second homologous recombination event), and the selection marker gene *lacZ* (Figure 4.2-1). It was also favourable to use a high-copy number plasmid, as it is easier to clone. Then, a plasmid containing DNA sequences up- and downstream *lsc* was made. These plasmids were assembled, and the origin of transfer *oriT* was included at the last step (Figure 4.2-1Figure 1.2-1). An overview of the stages in the plasmid construction is presented in Figure 4.2-1. This plasmid was then conjugated into *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA*, and *lsc* was removed from the genome by homologous recombination. To examine the effects on alginate synthesis, an alginate assay was performed.

The plasmids constructed are listed in Appendix B.

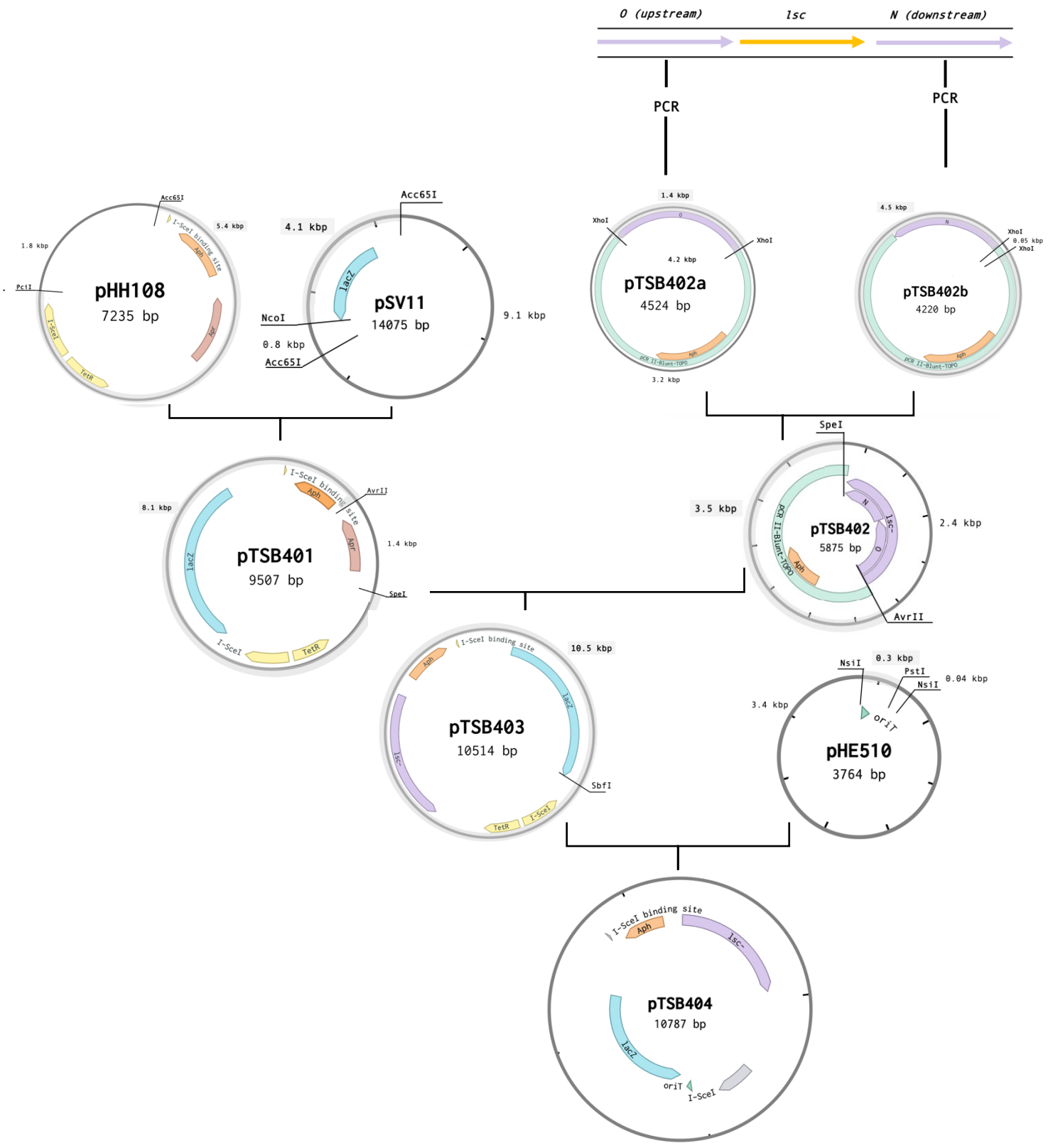


Figure 4.2-1**: Illustration of the steps in constructing pTSB404**. The fragments to be ligated in the next step is marked in grey.

4.2.1.1 Construction of the Recombiation Vector pTSB401

The recombination vector was to include *Kan*^{r}, the counter-selection gene *I-sceI* and its recognition site, and the selection marker gene *lacZ* (Figure 4.2-1). *lacZ* was isolated from pSV11 and *Kan*^{r}, *I-sceI* and the recognition site were isolated from pHH108 (Figure 4.2-1). In this section, the results of the isolation and ligation of these elements are presented.

The two plasmids, pSV11 and pHH108 was isolated. pSV11 was digested by the restriction enzymes Acc65I and NcoI. pHH108 was digested by the restriction enzymes Acc65I and PciI. The digested plasmids were separated on an agarose gel (Figure 4.2-2 a).

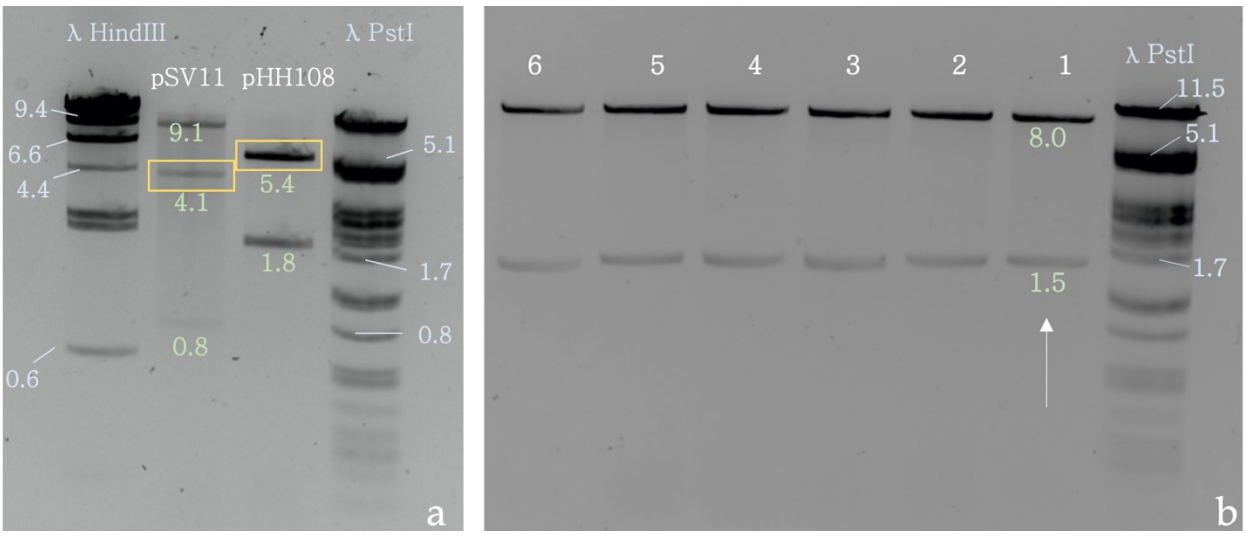


Figure 4.2-2: Fragment sizes in kbp. **a) Separation of digested pSV11 and pHH108.** λ PstI and λ HindIII as DNA standards. **b) Separation of plasmids isolated from 6 transformants, annotated 1 to 6, digested by AvrII.** λ PstI as DNA standard.

The marked 4.1 kbp fragment from pSV11 and the marked 5.3 kbp fragment from pHH108 were cut out, purified, and ligated.

The ligation mix was transformed to *E. coli* S17-1 *λpir* and selected on LA Kan. Plasmids from 6 transformants were isolated and tested by digesting with the restriction enzyme XbaI and separated on an agarose gel. (Figure 4.2-2 b) The expected fragment sizes of pTSB401 were 8.0 kbp and 1.5 kbp. Ligation of the parent plasmid pHH108 would have expected fragment sizes of 5.8 kbp and 1.5 kbp. The fragments were of the expected sizes of the wanted plasmid, and the plasmid from transformant number 1 was chosen and named pTSB401 (Figure 4.2-2 b).

4.2.1.2 Identification of *lsc* and the Adjacent DNA Sequences

The first step to create an *lsc*-negative strain is to make a template for recombination where chromosomal DNA sequences up-and downstream of the sequence that ought to be removed, *lsc*, are cloned and ligated. This section describes the results of identifying the *lsc* gene in *P. fluorescens* SBW25.

lsc was located by searching for the gene *lsc* in the UniProt database. This resulted in two reviewed entries from *Pseudomonas* with gene names *lsc*. An entry from *Pseudomonas syringae* with accession number O68609 (41) was chosen, as is has the same functions as levansucrase in *p. fluorescens* (0)(41).

The sequence of this entry was used as a query in the BLAST software tblastn. In tblastn, *Pseudomonas fluorescens* SBW25 was set as the target organism. The tblastn search resulted in only one alignment, accession number OV986001.1, with protein ID CAI2796546.1. The result had an E. value of 0.0 and a 98% query cover. The *lsc* gene was identified in *P. fluorescens* SBW25.

To identify the sequences up- and downstream *lsc*, the software Benchling was used. The alignment found in BLAST was used as a query when searching for *lsc* in the genome of *P. fluorescens* SBW25. This resulted in the identification of the sequences up- and downstream *lsc* in *P. fluorescens* SBW25.

4.2.1.3 Construction of pTSB402

DNA sequences up- and downstream *lsc* needed to be amplified, ligated, and cloned into a selection vector in the process of making *lsc* negative strains (Figure 4.2-1). The results of this process are presented in this section.

The sequences up-and downstream of *lsc* were amplified by PCR using the primer pairs dellscN and dellscO. The PCR reactions were conducted using the genomic DNA isolated from *P. fluorescens* SBW25 as template DNA, 55 °C as annealing temperature and 1 minute elongation time. Initially, the elongation time was set to 40 seconds, and the annealing temperature was set to 57 °C. This was changed to 1 minute and 55 °C, respectively, due to initial unsuccessful results.

The PCR fragments were separated on an agarose gel (Figure 4.2-3). The expected fragments size of upstream (annotated O) and downstream (annotated N) PCR products were 1340 bp and 1036 bp, respectively. The fragments were of the expected size (Figure 4.2-3).

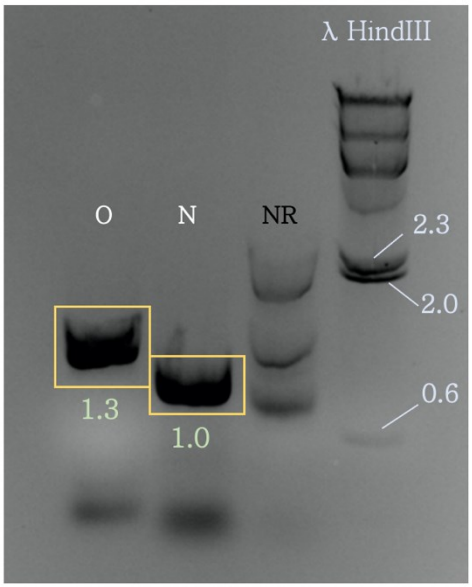


Figure 4.2-3: **Separation of up- and downstream lsc PCR products, annotated O and N respectively.** λ HindIII as DNA standard. Fragment sizes in kbp.

The blunt end fragments of 1.0 kbp and 1.3 kbp, marked in Figure 4.2-3, were cut out and purified. Each fragment was cloned separately, utilizing the Zero Blunt^{R} TOPO^{R} PCR Cloning system, into the vector DNA pCR^{TM}II-Blunt-TOPO^{R}. The mixtures were transformed to *E. coli* S17-1 *λpir* and selected on LA Kan.

When ligating blunt ends, the insert can orient both ways in the vector. To correctly combinate O and N, they need to have the same orientation. Therefore, each selected mutant needed to be tested for orientation before ligating together. This is illustrated in Figure 4.2-4, which shows how digestion using the restriction enzyme XhoI could be used when orienting the insert.

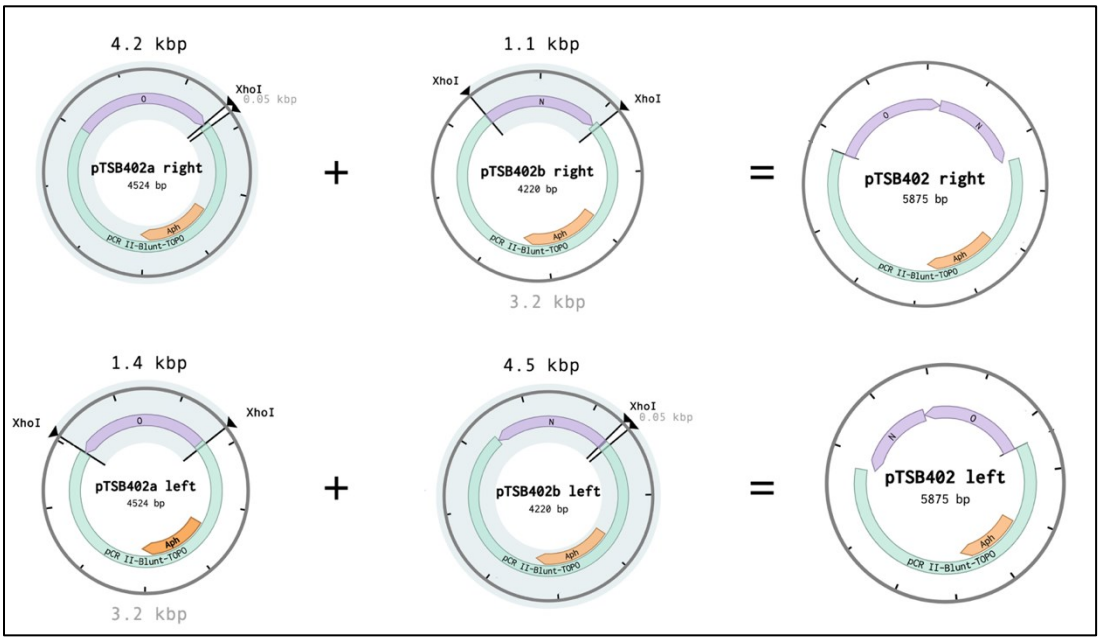


Figure 4.2-**4: An illustration of the two possible orientations (left and right) the inserts could have to be correctly combined.** The black lines illustrate the cutting site of the restriction enzyme XhoI. The grey highlights illustrate which part of the plasmid that were to be combined. The left orientation was chosen.

Preculture of the transformed cells were made, and the plasmid was isolated and digested by the restriction enzyme XhoI. The digested plasmids were separated on an agarose gel (Figure 4.2-5). The expected sizes of digested pTSB402a and pTSB402b are illustrated in Figure 4.2-4. The expected fragment size after religation of the parent plasmid pCR™II-Blunt-TOPO® would have been one 3.5 kbp fragment.

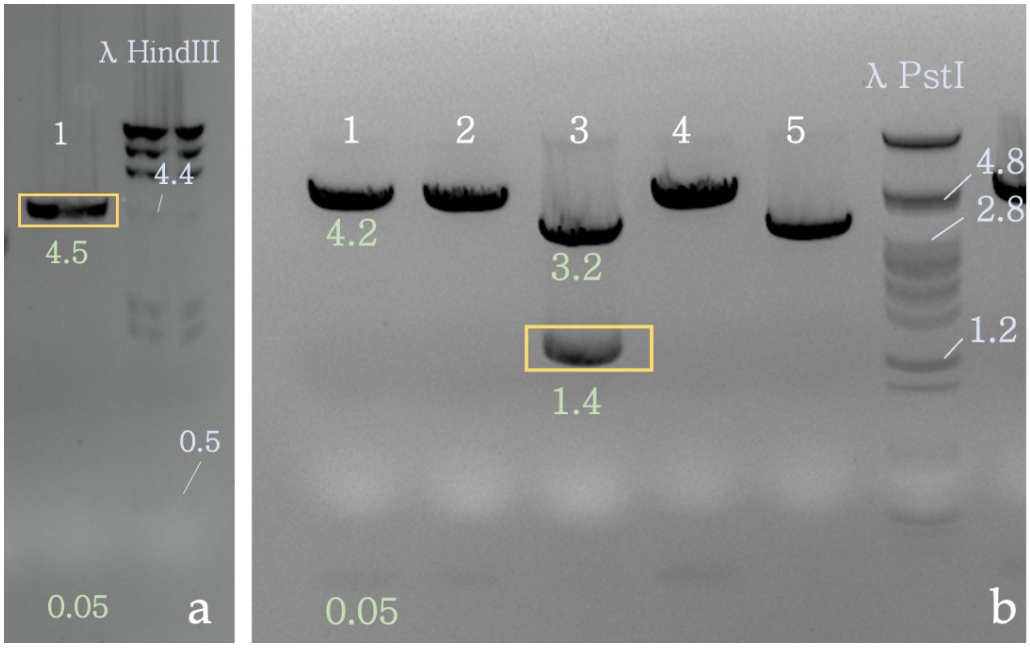


Figure 4.2-5: Fragment sizes in kbp. **a) Digested plasmid isolated from one N-transformant.** λ HindIII as DNA standard. The marked fragment was cut out and isolated. **b) From left to right well, 5 digested plasmids isolated from 5 O-transformants.** λ PstI as DNA standard. Transformant number 3 seems to have left orientation. The marked 1.3 kbp fragment of transformant 3 was cut out and isolated.

The plasmids of the O transformants were mostly right-oriented, and the plasmids of the N transformants were mostly left-oriented (Figure 4.2-5). After a few cloning attempts utilizing Zero Blunt^{R} TOPO^{R} PCR Cloning system, one O transformant with a left-oriented plasmid was detected (Figure 4.2-5 b). The right orientation for the N transformants did not occur, and therefore the left orientation was chosen (Figure 4.2-4).

To prevent ligation of pTSB402b digested with XhoI to a circular plasmid, CIP was added to the cutting mixture. The marked fragment in Figure 4.2-5 a as the vector and the marked fragment in Figure 4.2-5 b as the insert, were cut out, purified, and ligated. The ligation mix was transformed into *E. coli* S17-1 *λpir* and selected on LA Kan. Plasmids from 8 transformants were isolated and tested by digesting with the restriction enzymes EcoRI and XhoI and separated on an agarose gel (Figure 4.2-6).

The expected fragment sizes of pTSB202 were 3.5 kbp, 1.3 kbp and 1 kbp. Ligation of the parent plasmid pTSB402b would have expected fragment sizes of 3.2 kbp, 1.4 kbp, 0.03 kbp and 0.02 kbp. The fragments were of the expected size for all eight plasmids (Figure 4.2-6).

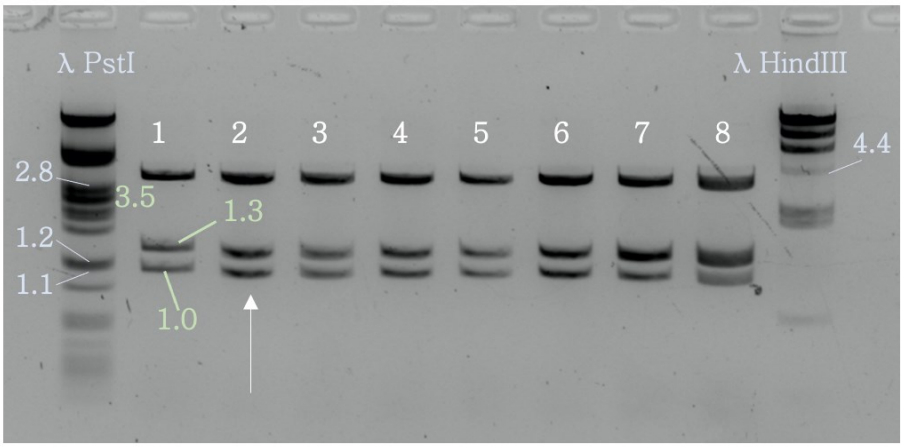


Figure 4.2-6: **Separation of plasmids from 8 transformants, annotated 1 to 8, digested by EcoRI and XhoI.** λ PstI and λ HindIII as DNA standards. Fragment sizes in kbp.

Plasmid number 2 was sequenced (Figure 4.2-6). Sequencing results (Appendix H) showed that the combined sequence upstream and downstream of *lsc* in transformant number two was successfully inserted in the vector. It also revealed that primer dellscNR had lost two nucleotides, which resulted in losing a recognition site of the restriction enzyme NotI. As a result, the initial proposal of utilizing NotI for digestion in the next step was abandoned, and the restriction enzyme SpeI was used instead. The sequencing results revealed the orientation of the insert in pTSB201, which is shown in Figure 4.2-1.

The plasmid of transformant number 2 (Figure 4.2-6) was chosen and called pTSB402.

4.2.1.4 Construction of pTSB403

The combined DNA sequence from up- and downstream *lsc* from pTSB402 was to be inserted into pTSB402. This section presents the results of this process.

pTSB402 and pTSB401 were digested by the restriction enzymes AvrII and SpeI. The digested plasmids were separated on an agarose gel (Figure 4.2-7 a). The expected fragment sizes of pTSB202 were 3.5 kbp and 2.4 kbp. The expected fragment sizes of pTSB401 were 8.1 kbp and 1.4 kbp. pTSB401 was treated with CIP to prevent religation. The marked fragments of 2.4 kbp and 8.1 kbp (Figure 4.2-7 a) were cut out, purified, and ligated.

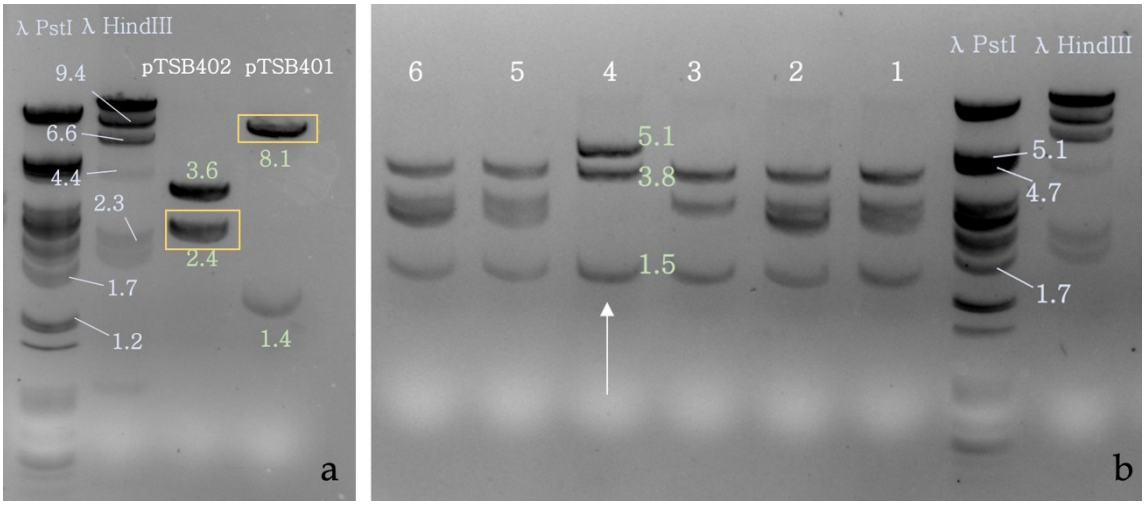


Figure 4.2-7: Fragment sizes in kbp. λ PstI and λ HindIII as DNA standards. **a) Separation of digested pTSB401 and pTSB402. b) Separation of plasmids from 6 transformants, annotated 1 to 6, digested by EcoRI.**

The ligation mix was transformed to *E. coli* S17-1 *λpir* and selected on LA Kan. Transformed colonies were replicated onto LA Kan and LA Apr. The latter was to ensure that the transformants were not re-ligated pTSB401.

Plasmids from 6 transformants were isolated and tested by digesting with the restriction enzyme EcoRI. (Figure 4.2-7 b) The expected fragment sizes of pTSB403 were 5.1 kbp, 3.8 kbp and 1.5 kbp. Religation of the parent plasmid pTSB401 would have given fragment sizes of 3.8 kbp, 2.8 kbp, 1.5 kbp and 1.4 kbp. The plasmid from transformant number 4 (Figure 4.2-7 b) showed the wanted DNA fragments and was selected as pTSB403.

4.2.1.5 Construction of pTSB404

In order to transfer the plasmid by conjugation, *oriT* had to be cloned into it. This section presents the results of inserting *oriT* from pHE510 into pTSB403.

pHE510 was digested by the restriction enzymes NsiI and PstI. pTSB403 was digested by the restriction enzyme SbfI. pHE510 was treated with CIP to prevent religation. The digested plasmids were separated on an agarose gel (Figure 4.2-8 a).

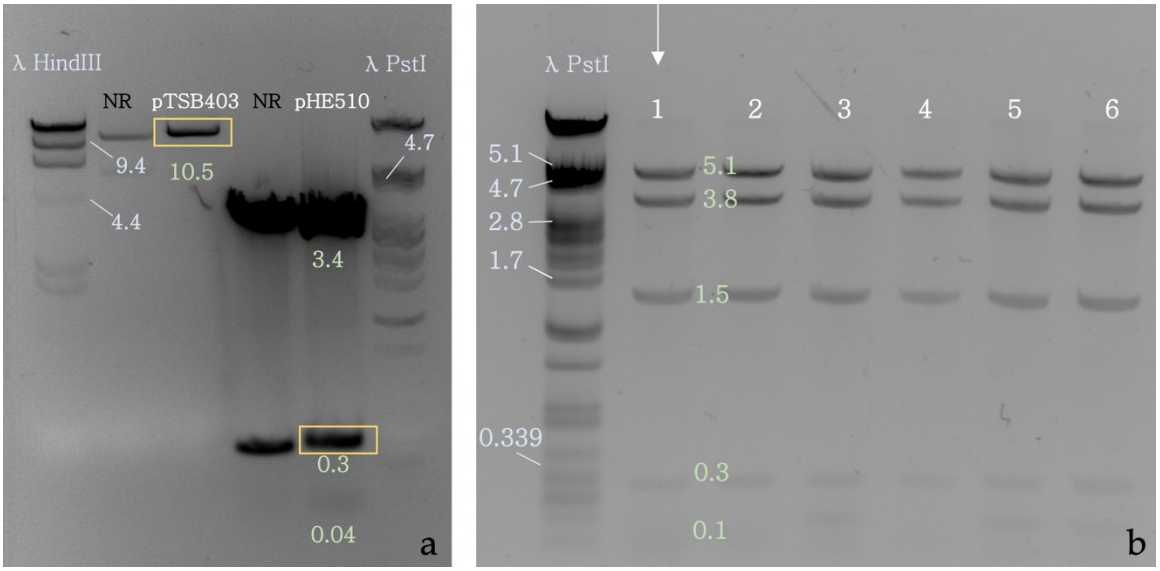


Figure 4.2-8: Fragment sizes in kbp. **a) Separation of digested pHE510 and pTSB403.** λ PstI and λ HindIII as DNA standards.NR: Not Relevant. **b) Separation of plasmids from 6 transformants, annotated 1 to 6, digested by EcoRI.** λ PstI as DNA standard.

The marked 0.3 kbp fragment from pHE510 and the marked 10.5 kbp fragment from pTSB403 (Figure 4.2-8 a) were cut out, purified, and ligated. The ligation mix was transformed to *E. coli* S17-1 *λpir* and selected on LA Kan.

Plasmids from 6 transformants were isolated and tested by digesting with the restriction enzyme EcoRI. The expected fragment sizes of pTSB404 were 5.1 kbp, 3.8 kbp, 1.5 kbp, 0.3 kbp and 0.1 kbp. Religation of the parent plasmid pTSB403 would have given fragment sizes of 3.8 kbp, 2.8 kbp, 2.4 kbp and 1.6 kbp. The transformants were of the expected sizes for the wanted plasmid.

Transformant number 1(Figure 4.2-8) was chosen, and the plasmid was called pTSB404.

4.2.2 Construction of *lsc*-negative Strains

lsc-negative strains were constructed by conjugating pTSB404 into *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA* and homologue recombining the combined sequence up and downstream *lsc*. This section presents the results of this process.

pTSB404 (Figure 4.2-8) was conjugated into *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA* and the dilution series was selected on PIA Kan X-Gal. Blue colonies expressing *lacZ,* therefore undergone the first homologous recombination event, were picked for overnight cultures in LB.

For the second homologous recombination event to occur, the first overnight culture was plated on no-salt LA plates containing 20% sucrose and 0.5 ug/mL ATc for I-SceI counter-selection (47). ATc is an inducer for the TetR promoter, controlling the expression of the endonuclease I-SceI (1.4.1), which expression causes lethal double-strand DNA breaks in the presence of the recognition. The expected results were cells undergone the second homologous recombination event (white colonies without LacZ) However, it resulted in the overgrowth of blue colonies, which indicateed that the counter-selection was unsuccessful. The recombination experiment was therefore repeated with twice the amount of ATc (1 ug/mL), which also resulted in blue-colony overgrowth. It was concluded that the I-SceI counter-selection system did not work, and therefore, selection for conjugation was performed by culturing overnight cultures over six nights and testing the consecutive cultures on LA X-Gal.

White colonies not expressing *lacZ* were tested by making replica plates on LA and LA Kan. 32 colonies that did not grow on LA Kan were tested by colony PCR. In the colony PCR reaction, dellscOF and dellscNR were used as primers, the annealing temperature was 55 °C, and the elongation time was 2 minutes. The expected fragment of the mutant was 2.4 kbp, and the wild-type fragment was 3.5 kbp. The PCR products were separated on an agarose gel (Figure 4.2-9).

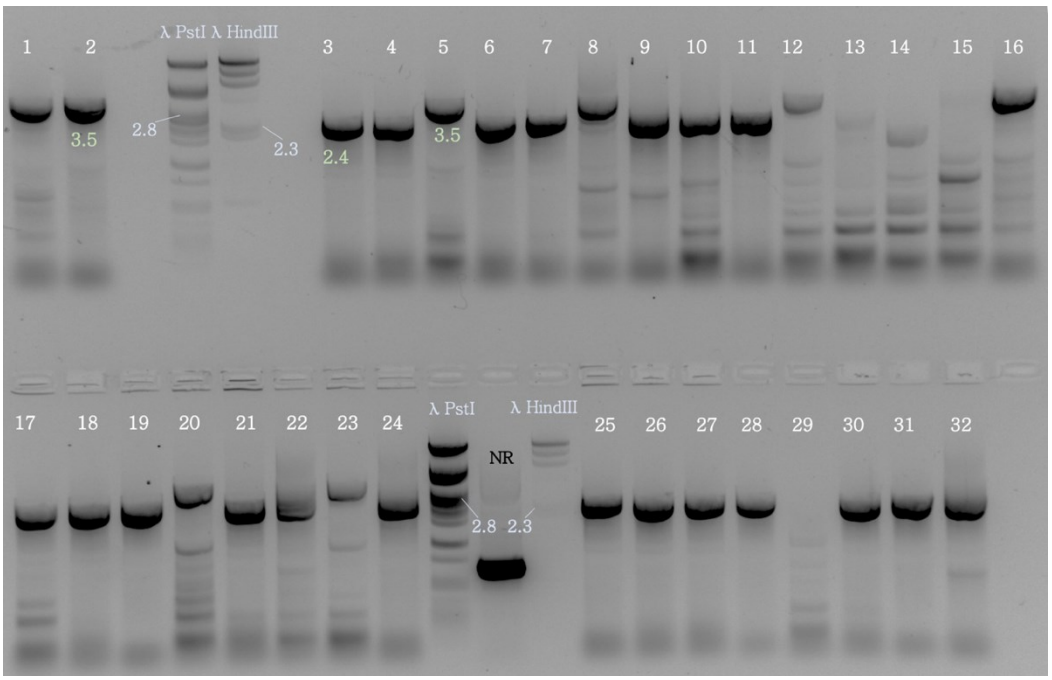


Figure 4.2-9: **Separated colony PCR products from 32 colonies.** The wells numbered 1 to 16 is loaded with PCR products from potential recombinant P. fluorescens SBW25 lsc- mutants, and wells numbered 17-32 is loaded with PCR products from potential recombinant P. fluorescens SBW25 mucA lsc- mutants. λ PstI and λ HindIII as DNA standards. Fragment sizes in kbp.

Analysis of Figure 4.2-9 revealed at least 20 mutants and 8 wildtypes.

To obtain a better separation between the fragment sizes 3.5kbp and 2.4kbp, 14 PCR products were separated on a new agarose gel (Figure 4.2-10)

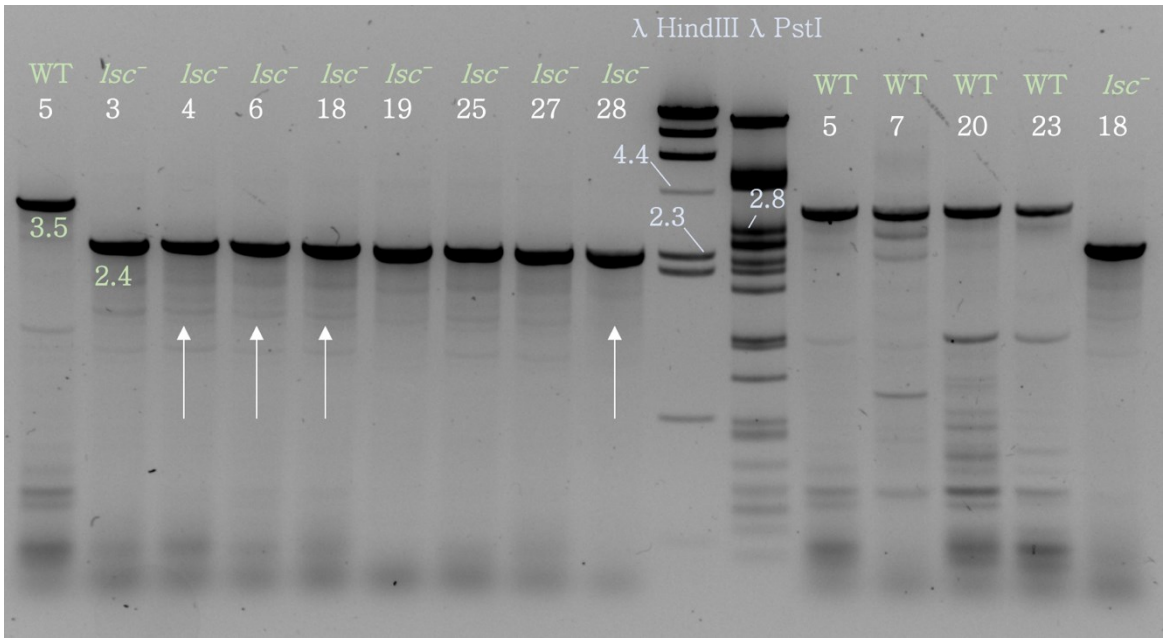


Figure 4.2-10: **Separated colony PCR products from 14 colonies**, numbered as from Figure 4.2-9. λ PstI and λ HindIII as DNA standards. Fragment sizes in kbp.

P. fluorescens SBW25 *mucA lsc⁻ m*utants number 4 and 6 (annotated A and B respectively), and *P. fluorescens* SBW25 *lsc⁻ m*utants number 18 and 28 (annotated A and B respectively) were chosen for further analysis.

4.3 Production and Purification of G- and M-lyase

In order to measure the amount of alginate present in cultured cells, degradation of alginate by G- and M-lyases are necessary. In this section, the results of producing G-lyase and M-lyase and testing them for lyase activity are presented.

G-lyase and M-lyase were produced by the recombinant strains *E. coli* RV308 (pAT77) and *E. coli* RV308 (pLe3), respectively. The recombinant gene expression was induced by m-toluate using the XylS/pm promotor system. After cultivation, the cells were lysed by sonication. The proteins were partially purified through an anion exchange chromatography column in an FPLC system and eluted with a NaCl gradient. The M- and G-lyase activity of the eluted proteins was analysed by measuring the absorbance at 235 nm every 10 seconds for 10 minutes.

4.3.1 Purification and Elution using Phosphate buffers A and B

Phosphate buffers A and B were used for elution through an anion exchange chromatography column in an FPLC system (Figure 4.3-1).

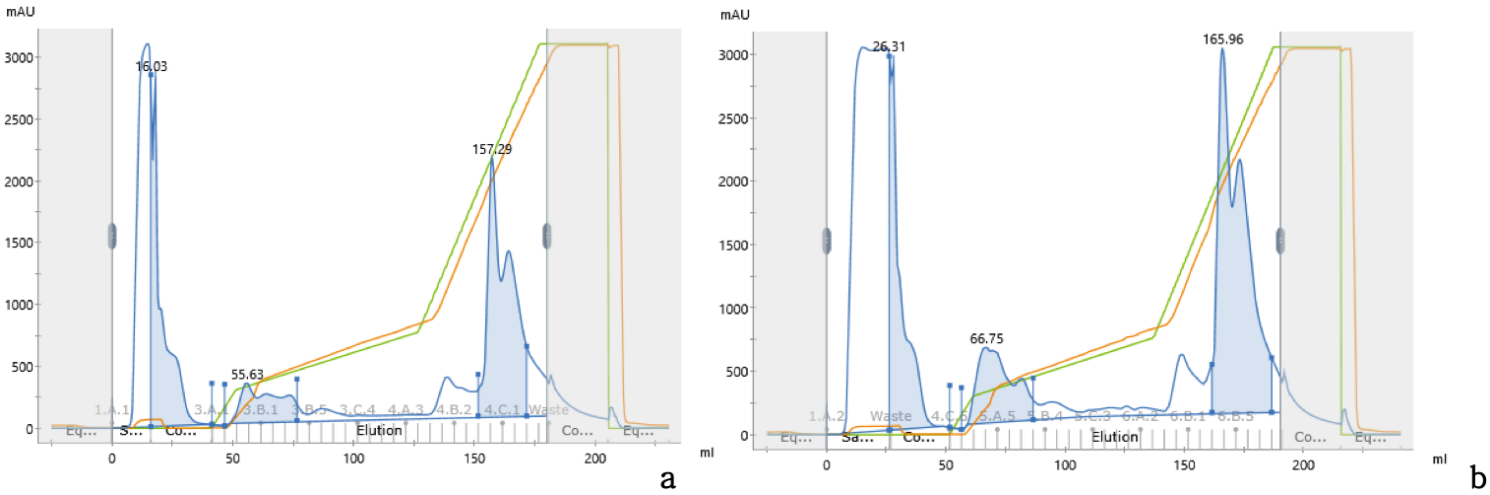


Figure 4.3-1: **Chromatograms of the elution fractions collected during an anion-exchange chromatography.** Proteins harvested from *E. coli* RV308 (pLe3) (a) and E*. coli* RV308 (pAT77) (b) eluted in Phosphate Buffer A with Phosphate Buffer B (1M NaCl) gradually from 0M to 1M NaCl. Percentage of elution with Phosphate Buffer B is given by the green graph. Conductivity is given by the orange graph. The absorbance is given in mAU (blue graph, left axis) with peaks defining large fractions of eluted proteins. mAU is plotted against the flowthrough volume (mL). Elution fraction labels, which are referred to in subsequent graphs, are given at the bottom. The fractions with the highest peaks were further investigated in an enzyme activity analysis.

The fractions containing in the highest peaks (Figure 4.3-1) were tested for activity by mixing eluate fractions and alginate, and measuring the absorbance at 235 nm every 10 seconds for 10 minutes, as described in 3.20.

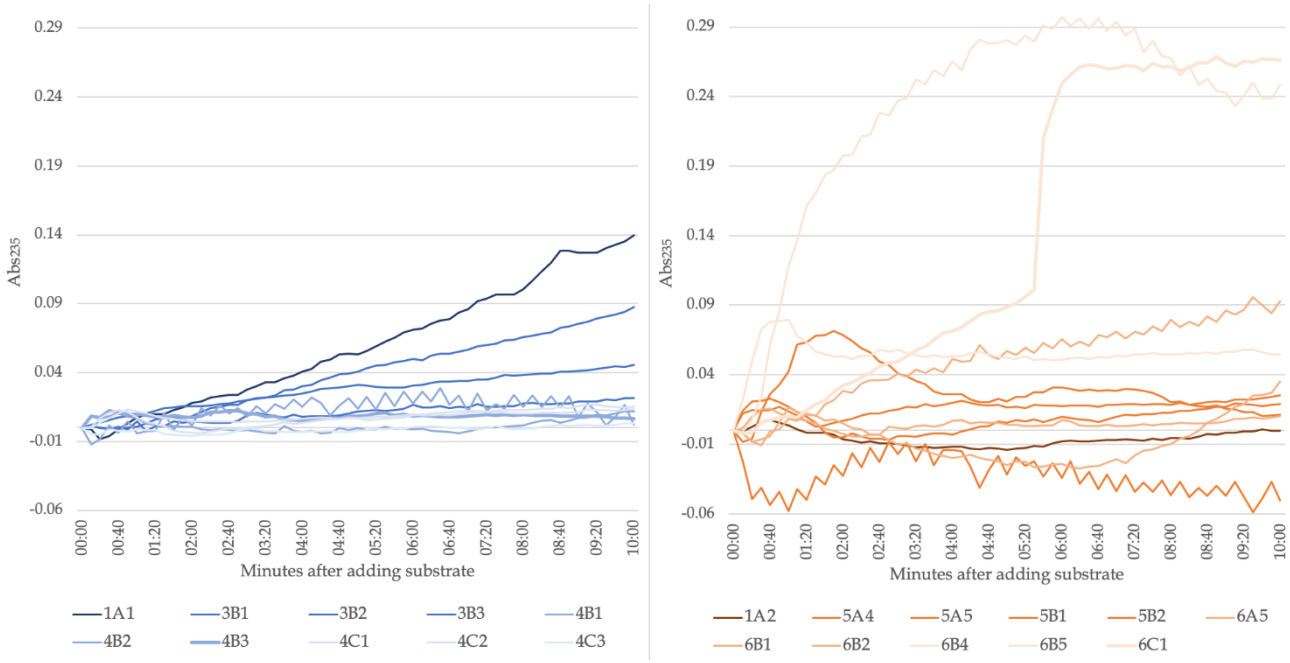


Figure 4.3-2: **Delta Abs_{235}, measured every 10 seconds for 10 minutes.** Orange shades are fractions from *E. coli* RV308 (pLe3), each shade representing each peak in (Figure 4.3-1 a). Blue shades are fractions from *E. coli* RV308 (pAT77), each shade representing each peak in (Figure 4.3-1 b).

Figure (Figure 4.3-2) indicated that elutes had overall low activity. But, in comparison with the others, *E. coli* RV308 (pAT77) had the highest G-lyase activity in the first two peaks, 1A1, 3B1, 3B2 and 3B3. The figure also indicated that fractions from *E. coli* RV308 (pLe3) had the highest M-lyase activity from the last peak, 6B4, 6B5 and 6C1.

New OD_{235} measurements were conducted (Appendix F) and U/mL were calculated (Table 4.3-1) of the peaks with the highest activity, including fragments before and after the peak, to specify further which fractions have the most enzyme. The two first peaks (Figure 4.3-1) of *E. coli* RV308 (pAT77) fractions and the last peak (Figure 4.3-1) of *E. coli* RV308 pLe3 fractions were investigated further in this new measurement. The graph is listed in Appendix F.

The U/mL of each fraction is listed in Table 4.3-1.

Table 4.3-1 U/mL from the first two peaks (Figure 4.3-1) of *E. coli* RV308 (pAT77) fractions and the last peak (Figure 4.3-1) of *E. coli* RV308 (pLe3) fractions in Phosphate Buffer calculated. The average of two technical measurements and the standard deviations (SD) are given.

M-lyase fraction	**Average**	**SD**	**G-lyase fraction**	**Average**	**SD**
6B3	0.097	±0.024	1A1	16.340	±0.086
6B4	0.082	±0.235	3A3	10.888	±1.573
6B5	0.834	±0.665	3A4	47.012	±24.179
6C1	-0.002	±1.782	3A5	12.735	±1.861
			3B1	5.273	±0.731
			3B2	1.872	±1.652

Figure in Appendix F indicated that the fractions have higher activity than the previous measurement (Figure 4.3-2). The fractions 1A1, 3A3, 3A4 and 3A5 from *E. coli* RV308 (pAT77) (Figure 4.3-1) display high activity (Table 4.3-1), usable in alginate assay. Although, this does not seem to be the case for *E. coli* RV308 (pLe3), which showed no activity. When repeating the assay several times for *E. coli* RV308 (pLe3) fractions, no activity was registered.

Therefore, a new elution and purification through an FPLC column were conducted using 0.05M Tris-HCl buffer A and B.

4.3.2 Purification and Elution using 0.05M Tris-HCl Buffer A and B.

0.05M Tris-HCl Buffer A and B were used for elution and purification through an anion exchange chromatography column in an FPLC system. Because of an interest in harvesting more G-lyase, proteins from *E. coli* RV308 pAT77 was also eluted (Figure 4.3-3)

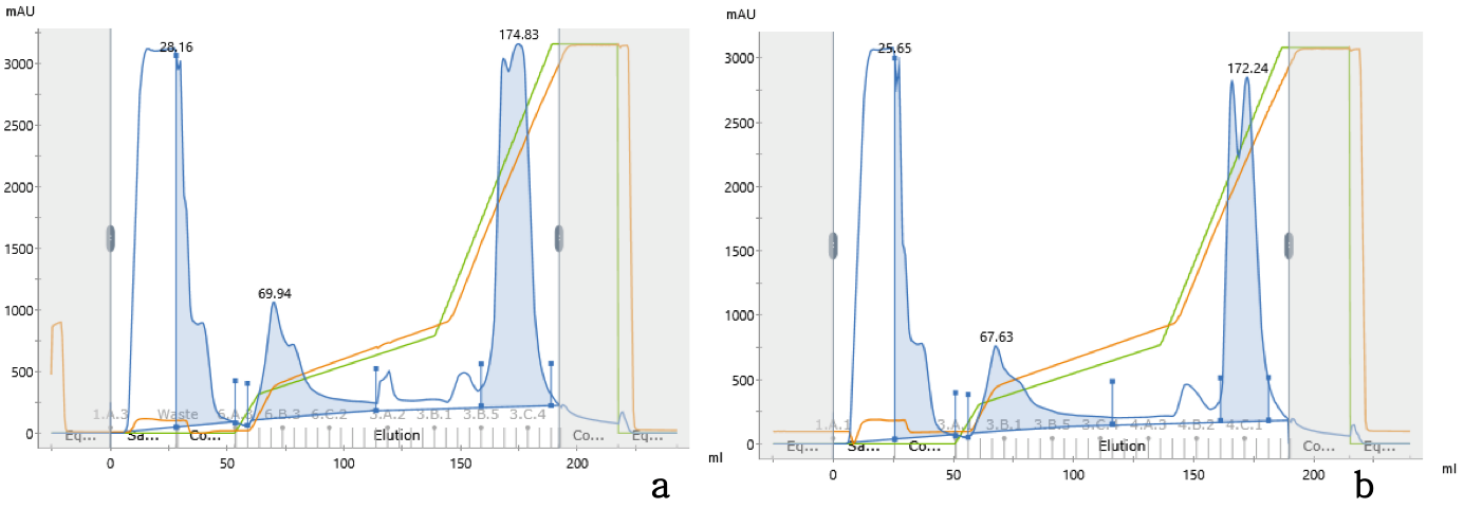


Figure 4.3-3: **Chromatograms of the elution fractions collected during anion-exchange chromatography.** Proteins harvested from *E. coli* RV308 (pLe3) (a) and *E. coli* RV308 (pAT77((b) eluted in 0.05M Tris-HCl buffer A with 0.05M Tris-HCl Buffer B(1M NaCl) gradually from 0M to 1M. Percentage of elution with 0.05M Tris-HCl Buffer B is given by the green graph. Conductivity is given by the orange graph. The absorbance is given in mAU (blue graph, left axis) with peaks defining large fractions of eluted proteins. mAU is plotted against flowthrough volume (mL). Elution fraction labels, which are referred to in subsequent graphs, are given at the bottom. The fractions with the highest peaks were further investigated in an enzyme activity analysis.

The fractions eluted in the highest peaks (Figure 4.3-3) were tested for activity by mixing eluate fractions and alginate and measuring OD_{235} every 10 seconds for 10 minutes, as described in 3.20 (Figure 4.3-4).

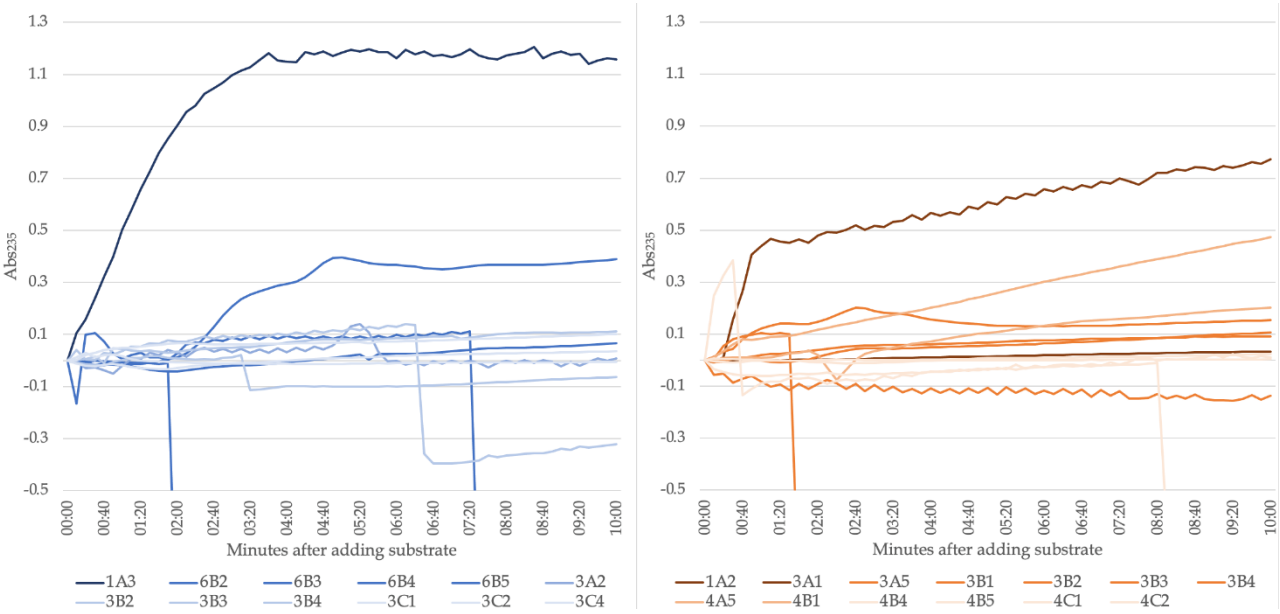


Figure 4.3-4: **Delta Abs_{235}, measured every 10 seconds for 10 minutes.** Blue shades are fractions from *E. coli* RV308 (pAT77), each shade representing each peak in Figure 4.3-3. Orange shades are fractions from *E. coli* RV308 (pLe3), each shade representing each peak in Figure 4.3-3. The steep graphs pointing down is probably air bubbles in the wells.

Figure (Figure 4.3-4) indicated that fractions from *E. coli* RV308 (pAT77) had the highest G-lyase activity in the two first peaks, 1A3, 6B2, 6B3, 6B4 and 6B5, as for the elutes in Phosphate Buffer A and B.

Figure 4.3-4 also indicated that fractions from *E. coli* RV308 (pLe3) had the highest M-lyase activity in the first and second peak. This differs from the fractions in Phosphate Buffer A and B, where the highest activity was measured from the last peak.

For the Tris-HCl Buffer A and B fractions, new Abs_{235} measurements (Appendix F) and calculation of U/mL (Table 4.3-2) were also conducted of the peaks with the highest activity, including fragments before and after the peak. The two first peaks (Figure 4.3-3) of *E. coli* RV308 (pAT77) and *E. coli* RV308 (pLe3) fractions were investigated further.

The graph is listed in Appendix F.

Table 4.3-2: **U/mL** from the first two peaks (Figure 4.3-3) of *E. coli* RV308 (pLe3) fractions and the first peak (Figure 4.3-1) of *E. coli* RV308 (pAT77) fractions in 0.05 Tris-HCl Buffer calculated. The average of two technical duplicates and the standard deviations (SD) are given for M-lyase 1A2, 3B1 and 4B1. 4A5 only measured once because of flaw in the well.

M-Lyase	**Average**	**SD**	**G-Lyase**	**U/mL**
1A2	2.948	± 2.695	1A3	20.056
3B1	2.556	± 2.084	6A4	2.459
4A5	0.842		6A5	5.217
4B1	1.928	± 0.095	6B1	7.967
			6B2	3.048

Table 4.3-2 indicated that the *E. coli* RV308 pAT77 fractions 1A3, 6A5 and 6B1 good activity, as for the elutes in Phosphate Buffer A and B. For *E. coli* RV308 pLe3 fractions, the M-lyase activity was too poor to use in further measurements.

The G-lyases produced from *E. coli* RV308 pAT77 in Phosphate Buffer A and B and Tris-HCl Buffer A and B were gathered and used in further alginate assays. The low-activity M-lyases were discarded. One possible explanation for the low activity is that the samples were tested and thawed too many times, so the enzyme may have lost its activity.

4.4 Measurement of the Amount of Alginate Synthesised by the Mutants *P. fluorescens* SBW25 *mucA lsc*⁻ and *P. fluorescens* SBW25 *lsc*⁻

The amount of alginate synthesised by the *lsc*⁻ mutant *P. fluorescens* SBW25 strains was measured by cultivating the cells, sampling, and performing an alginate assay. Two assays were performed, where the *lsc*- mutants and control strains were cultivated in either fructose or sucrose as the sole carbon source. In this section, the results from the assays are presented.

The cells were cultivated in DEF3 medium containing fructose or sucrose as sole carbon source. Three biological replicates, in each carbon source, were made from *P. fluorescens* SBW25 *mucA lsc*⁻ A and *lsc*⁻ B and *P. fluorescens* SBW25 *mucA* as a reference and positive control. The cells were cultivated for 24 and 48 hours, and an alginate assay was conducted (Figure 4.4-1).

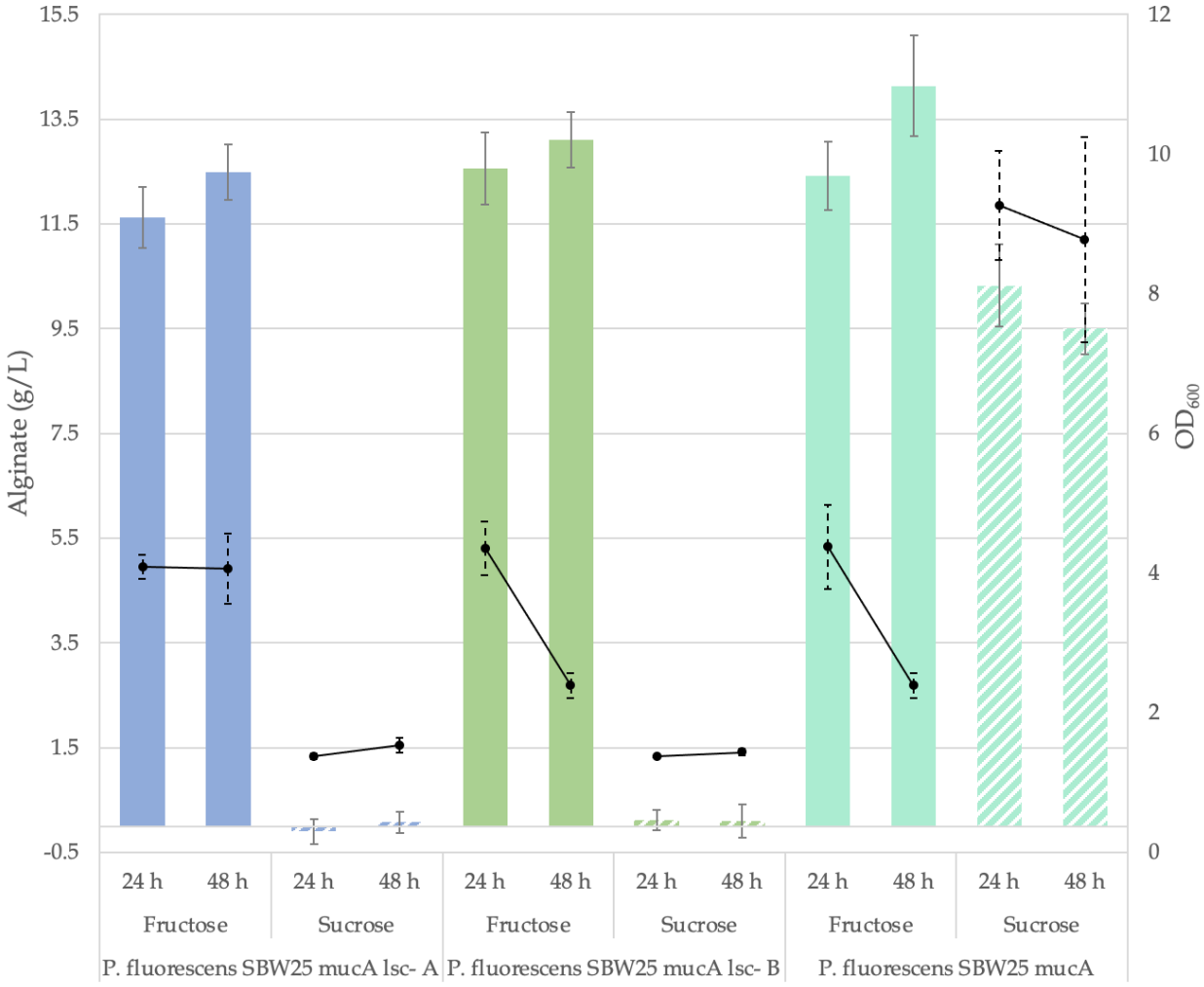


Figure 4.4-1: **Alginate concentration in g/L and OD_{600} for *P. fluorescens* SBW25 mucA cultivated in fructose and sucrose.** Each bar represents the average g/L of 3 biological replicates tested in triplicates with standard deviations. Each dot represents the average OD_{600} with standard deviations. Lines between the dots represent the growth of the cultivates strain.

Figure 4.4-1 shows that there is no substantial difference between *P. fluorescens* SBW25 *mucA and the lsc*$^-$ mutants when growing in fructose. When growing in sucrose, the *lsc*$^-$ mutants do not produce any alginate, and have substantial less growth than *P. fluorescens* SBW25 *mucA*. Because of the surprisingly low alginate yield of the *lsc*$^-$ mutants, the cultivation in sucrose and subsequent alginate assay was repeated, resulting in the same results. *P. fluorescens* SBW25 *mucA* exhibit a higher alginate yield when growing in fructose as opposed to sucrose but exhibit a higher growth in sucrose. As alginate production is energy demanding, it is expected that a lower alginate yield results in higher OD_{600}. A graph of g/L per OD_{600} is listed in Appendix G.

Cultivation in sucrose and fructose, and the subsequent alginate assay were conducted for the non-alginate producing *P. fluorescens* SBW25 and the *lsc* mutants *P. fluorescens* SBW25 *lsc*$^-$ A and B. It is not expected that *P. fluorescens* SBW25 produce alginate, as the synthesis is tightly controlled by mucA (22).

In fructose, three biological replicates, were made from *P. fluorescens* SBW25 *lsc*$^-$ A and *lsc*$^-$ B. Two biological replicates were made from *P. fluorescens* SBW25. In sucrose, three biological replicates were made from *P. fluorescens* SBW25 *lsc*$^-$ A and *P. fluorescens* SBW25. The cells were cultivated for 24 and 48 hours, and an alginate assay were conducted. No alginate was measured for either of the samples, therefore only the OD_{600} values is shown in Figure 4.4-2.

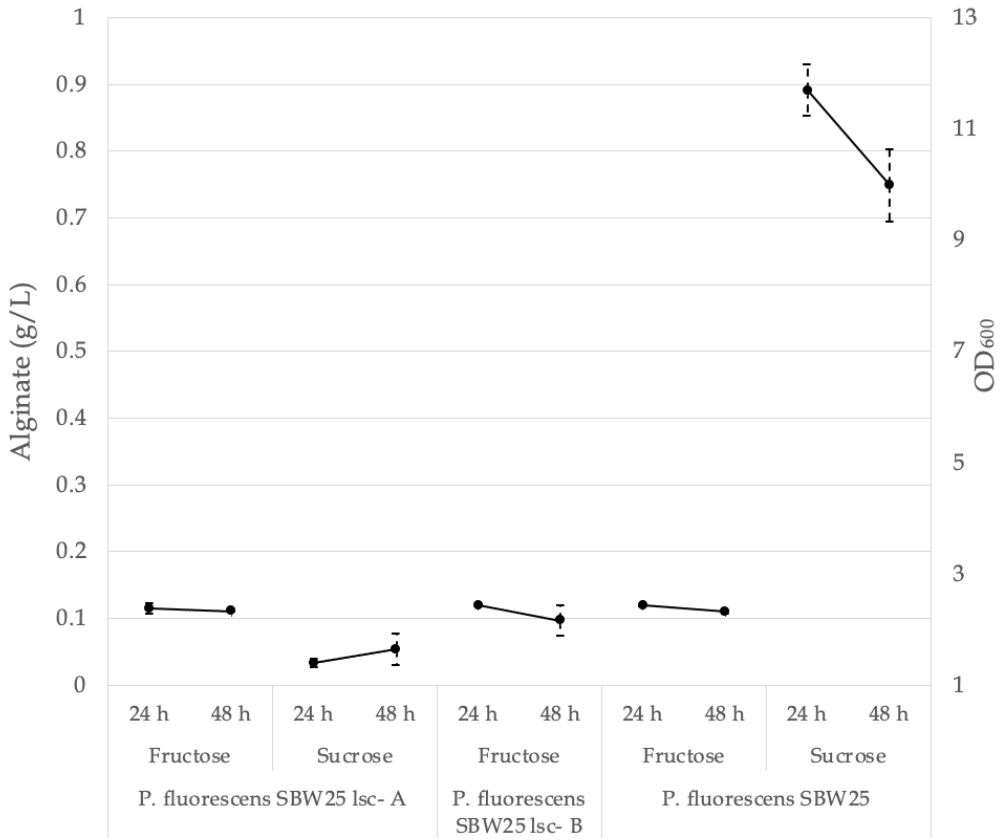


Figure 4.4-2: **OD_{600} for *P. fluorescens* SBW25 cultivated in fructose and sucrose.** Each dot represents the average OD_{600} of the biological replicates with standard deviations. Lines between the dots represent the growth of the cultivates strain.

Figure 4.4-1 shows that *P. fluorescens* SBW25 and the *lsc* mutant, grown in fructose and sucrose, do not produce any alginate, as expected. When grown in sucrose, OD_{600} of *P. fluorescens* SBW25 is substantially higher than the *lsc*$^-$ mutant. When grown in fructose, the OD_{600} were not substantial different amongst the strains. As for *P. fluorescens* SBW25 *mucA* (Figure 4.4-1), *P. fluorescens* SBW25 exhibit higher growth when cultivated in sucrose than when cultivating in fructose.

5 Discussion and Further Work

5.1 Testing the Triple-tandem Bc3-5 Riboswitch Dual-fluorescence c-di-GMP Detecting System

The altered triple-tandem riboswitch Bc3-5 dual-fluorescence c-di-GMP detecting system was constructed. It was tested using alginate-producing and non-producing *P. fluorescens* SBW25 strains, including *mucR* overexpressing strains. mCherry_V1, a derivative of mCherry constructed by Fages-Lartaud *et al.* (34), was chosen as the alternative fluorescent protein because it doesn't contain the alternative RBS. mCherry_V1 was found not to interfere with the autofluorescence of *P. fluorescens* SBW25 (Figure 4.1-1).

The function of the triple-tandem riboswitch Bc3-5 dual-fluorescence c-di-GMP was tested. From the RFI values (Figure 4.1-17), it is suggested that the triple-tandem riboswitch Bc3-5 functions as a c-di-GMP detector, as the RFI differs among the strains.

5.2 Testing the c-di-GMP Levels in *P. fluorescens* SBW25 and the *mucA* Mutant

The triple-tandem riboswitch Bc3-5 c-di-GMP dual-fluorescence detecting system was tested in wildtype strain *P. fluorescens* SBW25 and the alginate overproducing mutant *P. fluorescens* SBW25 *mucA* to monitor the difference in c-di-GMP levels.

P. fluorescens SBW25 *mucA* exhibited low detected c-di-GMP levels as opposed to *P. fluorescens* SBW25 (Figure 4.1-17). c-di-GMP positively regulates the alginate synthesis by binding as a dimer and activating Alg44, and therefore it is expected that c-di-GMP levels rises in alginate-producing strains. Also, it is suggested by Monds *et al.* (90) that less c-di-GMP leads to loss of biofilm formation. However, c-di-GMP is up- and down regulated by DCGs and PDEs throughout the cell in many different signalling pathways (28), and this system measures the total sum of c-di-GMP and not only the local c-di-GMP produced by MucR in close proximity to Alg44 (24). Therefore, it is difficult to point out a specific reason for the low c-di-GMP levels in the *mucA* mutant.

mucR overexpressing *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA* were tested for c-di-GMP levels, and alginate production was measured in the *mucA* mutants. The *mucR* overexpressing gene was controlled by the inducible promoter P_m-*G5*, previously found to have low background expression (51). From the OD_{600} measurements (Figure 4.1-15), the induced replicates exhibit lower OD_{600} than the not-induced replicates, which can indicate that m-toluate or mucR overexpression led to a metabolic burden for the cells (91). From the fluorescence measurement results (Figure 4.1-16), there is no substantial difference between the induced and not-induced strains containing pHH104 (P_5-*G5* controlling *mucR*).

A reason could be that enhancing the transcription of *mucR* does not result in considerably higher c-di-GMP levels because of post-translational modulation in response to high c-di-GMP levels, can result in PDE activity (28), which could mean that the c-di-GMP levels were at equilibrium.

The MucR overexpressing *P. fluorescens* SBW *mucA* exhibits higher c-di-GMP levels than *P. fluorescens* SBW *mucA* from 12 hours after induction (Figure 4.1-17). From this time point, the growth of the *mucR* overexpressing strain increases more than the mucR-normal-expressing strain (Figure 4.1-15). This is towards the end of the exponential phase, where alginate is starting to be produced (13), and for alginate-producing strains, the growth is poorer than of the non-alginate-producing strains. This is expected because alginate production is energy-demanding (1, 13).

As seen in Figure 4.1-18, the *mucR* overexpressing *mucA* mutant strain produces no alginate, but the c-di-GMP levels was raised (Figure 4.1-17). These results can indicate that the mucR overexpression negatively affects the alginate synthesis in *P. fluorescens* SBW25 *mucA* (pTSB203) (pHH104). As found by Hay *et al.*, overexpressing *mucR* leads to a sevenfold increase in alginate production in the *mucA* isogenic mutant of *Pseudomonas aeruginosa* strain PDO300 (24). However, for *P. fluorescens* SBW25 *mucA,* overexpressing *mucR* seems to have the opposite effect. An explanation could be the dual PDE/DGE activity MucR exhibits. Overexpressing c-di-GMP could lead to an enzymatic shift in MucR, leading to enhanced PDE activity and the subsequent loss of alginate production.

For future work, testing whether MucR exhibits PDE activity in the *mucR* overexpressing strains, could lead to explanations in why MucR overexpressing strains do not produce alginate. This could be done by genetically inactivate the EAL active site domain by inserting a stop-codon, as the EAL domain is the C-teminus of the protein. It could also be achieved by disrupt the glutamate residue (E), suggested by Valentini *et al.* (28) to be essential in the EAL signature motif. Then, c-di-GMP levels and alginate measurements should be tested and compared to the MucR wild type.

Inactivation of the PDE activity of *mucR* in the genomic DNA of *P. fluorescens* SBW25 could result in higher alginate yield, if the enzymatic shift would lead to an activation of the PDE domain, and not an inactivation of the DGC domain. This could be worth testing for future work.

The metabolic burden of having two expressing plasmids in a cell could also be a reason for the loss of alginate production (91, 92). A solution to assess this objective could be to insert an empty vector containing the components of pHH104, except the *mucR* overexpressing gene, and measure the alginate production.

5.3 Testing the Counter-selection Marker I-SceI in Homologous Recombination

The counter-selection marker gene *I-SceI* was tested under homologue recombination of the *lsc*-negative *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA*. The aim was to utilise the homing endonuclease I-SceI to induce double-stranded cuts at the I-sceI recognition site in the integrated plasmid, leading to cell death in the cells not having undergone the second homologous recombination event. The regulation of I-SceI is controlled by the TetR repressor protein, which dissociates from the DNA upon binding with the inducer ATc, initiating transcription. If successful, the counter-selection can save time in finding the correct mutants.

However, this system did not seem to be functioning, resulting in the overgrowth of cells not having undergone the second homologous recombination event (4.2.2). However, if it functions, it can reduce the time of obtaining homologous recombined mutants. Therefore, there is interest in finding if this counter-selection system can function in *Pseudomonas*. I-SceI, as a counter-selection marker, has previously been found to be functioning in *Pseudomonas putida* under control by the RhaRS/P_{rhaB} system, induced by L-rhamnose (60). The counter-selection system could be tested by changing the TetR promoter to the RhaRS/P_{rhaB} system. This could also overcome problems by using ATc as an inducer, as ATc is light sensitive.

LA no salt and 20% sucrose plates were used (47), where sucrose is used to compensate for the osmolarity when NaCl is not present. In liquid culture (Figure 4.4-1, Figure 4.4-2), the growth of lsc-negative cells in sucrose is not as high as the lsc-positive strains. It may be that cultivating in sucrose favourites the presence of *lsc*, therefore resulting in no mutants. Simply plating the cells on LA ATc instead could easily and quickly be tested to overcome this problem.

5.4 Evaluation of the Effect *lsc*-negative Strains Have on Alginate Production and Growth

lsc-negative *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA* was constructed by constructing a recombination vector, and *lsc* was deleted by homologous recombination.

The results presented in Figure 4.4-1 demonstrate that when fructose is the sole carbon source available, there is no significant difference observed in growth and alginate synthesis between the *lsc*-negative strains and the *lsc*-positive strain. From these results, it is possible that levan synthesis is not draining on the cells' energy and carbon, neither does the absence of *lsc* impact the growth or alginate synthesis. The results could also indicate that *lsc* is not active when growing fructose because of little sucrose available. To evaluate this hypothesis, an investigation could be conducted to assess *lsc* activity in *P. fluorescens* SBW25 *mucA* while cultivating the strain on fructose (93).

Figure 4.4-1 also shows that the growth, when cultivating in sucrose, of the *lsc* negative strains is much lower than for the alginate-producing *P. fluorescens* SBW25 *mucA*. A suggestion to these results is that if sucrose is not broken down to its monomers, as

levansucrase is able to do according to Gross *et. al.* (39), or to levan and glucose, the monomers are not available for the rest of the metabolism either, and therefore the cell could be stressed and grow slowly. *P. fluorescens* SBW25 *mucA* exhibits higher OD_{600} when growing in sucrose than in fructose, suggesting that *lsc* is highly involved in supplying the Entner-Doudoroff pathway with glucose. A slight increase in growth from 24-hour to 48-hour timepoint in the *lsc* negative strains cultivated in sucrose can suggest that the cells grow slowly, as the other strains exhibit a decrease in OD_{600}, which suggest that they are in the death phase (Figure 4.4-1).

In *P. putida*, Löwe *et al*. (94) found that the native sucrose porins for transport of sucrose through the outer membrane, were not sufficient for unlimited growth on sucrose. This could be the case for *P. fluorescens.* Löwe *et al*. (94) introduced the sucrose porin CscY for sucrose uptake in *P. putida*, which resulted in sufficient growth when cultivating on sucrose as the sole carbon source. For further work, this could be tested for functioning in *p. fluorescens* SBW25 *mucA* lsc^-, and monitor if growth, and the subsequent alginate yield gets improved.

When sucrose was the only available carbon source, the results (Figure 4.4-1) show a large difference in both alginate production and growth between the *lsc* negative strains and *P. fluorescens* SBW25 *mucA*. The alginate production in the *lsc* negative strains is not detectable (Figure 4.4-1). Because of the low growth, this is expected, as cells prioritize growth over alginate production (1).

For the non-alginate-producing strains (Figure 4.4-2), the growth of *P. fluorescens* SBW25 when cultivating in sucrose as the sole carbon source, the growth is much higher than when utilising fructose as carbon source. As for *P. fluorescens* SBW25 *mucA* lsc^-, *P. fluorescens* SBW25 lsc^- grow poorly in sucrose. This indicated that also for *P. fluorescens* SBW25, lsc^- is important for supplying the cell with carbon.

6 Conclusions

This study aimed to evaluate different regulatory areas in the alginate synthesis of *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA*. Two different regulatory areas were evaluated.

The altered version of the triple-tandem Bc3-5 riboswitch dual-fluorescence c-di-GMP detecting system showed to be functioning (Figure 4.1-17) and can be used in further assessments. This thesis used it to test the c-di-GMP levels in alginate production and growth, and in relation to *mucR* overexpression. *P. fluorescens* SBW25 exhibited a higher c-di-GMP level than the alginate overproducing mutant *P. fluorescens* SBW25 mucA (Figure 4.1-17). The mucR overproducing *P. fluorescens* SBW25 mucA produced no alginate, indicating that overexpression of mucR is negative for alginate production (Figure 4.1-18).

The homing endonuclease I-sceI, used for counter-selection of cells having undergone the second homologous recombination event with the ATc inducible promoter TetR, showed not to be functioning in the given environment. However, by changing the promoter or cultivating on a different medium, the counter-selection system could still be promising.

lsc negative strains were constructed, cultivated on sucrose or fructose as the sole carbon source, and alginate and growth were measured. These findings (Figure 4.4-1) suggest that *lsc* is needed for growth, and subsequent alginate production when cultivating in sucrose. However, these results also imply that the presence or absence of *lsc* does not affect growth or alginate production when cultivated in fructose.

Bibliography

1. Maleki S, Mærk M, Hrudikova R, Valla S, Ertesvåg H. New insights into *Pseudomonas fluorescens* alginate biosynthesis relevant for the establishment of an efficient production process for microbial alginates. N Biotechnol. 2017;37(Pt A):2-8.
2. Trippe K, McPhail K, Armstrong D, Azevedo M, Banowetz G. *Pseudomonas fluorescens* SBW25 produces furanomycin, a non-proteinogenic amino acid with selective antimicrobial properties. BMC Microbiol. 2013;13:111.
3. Silby MW, Cerdeno-Tarraga AM, Vernikos GS, Giddens SR, Jackson RW, Preston GM, et al. Genomic and genetic analyses of diversity and plant interactions of *Pseudomonas fluorescens*. Genome Biol. 2009;10(5):R51.
4. Tank N, Rajendran N, Patel B, Saraf M. Evaluation and biochemical characterization of a distinctive pyoverdin from a *pseudomonas* isolated from chickpea rhizosphere. Braz J Microbiol. 2012;43(2):639-48.
5. Maleki S, Mærk M, Valla S, Ertesvåg H. Mutational Analyses of Glucose Dehydrogenase and Glucose-6-Phosphate Dehydrogenase Genes in *Pseudomonas fluorescens* Reveal Their Effects on Growth and Alginate Production. Appl Environ Microbiol. 2015;81(10):3349-56.
6. Garrido-Sanz D, Meier-Kolthoff JP, Goker M, Martin M, Rivilla R, Redondo-Nieto M. Genomic and Genetic Diversity within the *Pseudomonas fluorescens* Complex. PLoS One. 2016;11(2):e0150183.
7. Rainey PB, Bailey MJ. Physical and genetic map of the *Pseudomonas fluorescens* SBW25 chromosome. Mol Microbiol. 1996;19(3):521-33.
8. Jackson RW, Preston GM, Rainey PB. Genetic characterization of *Pseudomonas fluorescens* SBW25 rsp gene expression in the phytosphere and in vitro. J Bacteriol. 2005;187(24):8477-88.
9. Heredia-Ponce Z, de Vicente A, Cazorla FM, Gutierrez-Barranquero JA. Beyond the Wall: Exopolysaccharides in the Biofilm Lifestyle of Pathogenic and Beneficial Plant-Associated *Pseudomonas*. Microorganisms. 2021;9(2).
10. Meyer JM, Abdallah MA. The Fluorescent Pigment of *Pseudomonas fluorescens*: Biosynthesis, Purification and Physicochemical Properties. Microbiol. 1978;107(2):319-28.
11. Spiers AJ, Bohannon J, Gehrig SM, Rainey PB. Biofilm formation at the air-liquid interface by the *Pseudomonas fluorescens* SBW25 wrinkly spreader requires an acetylated form of cellulose. Mol Microbiol. 2003;50(1):15-27.
12. Ertesvåg H. Alginate-modifying enzymes: biological roles and biotechnological uses. Front Microbiol. 2015;6:523.
13. Maleki S, Almaas E, Zotchev S, Valla S, Ertesvåg H. Alginate Biosynthesis Factories in *Pseudomonas fluorescens*: Localization and Correlation with Alginate Production Level. Appl Environ Microbiol. 2016;82(4):1227-36.
14. Borgos SE, Bordel S, Sletta H, Ertesvåg H, Jakobsen O, Bruheim P, et al. Mapping global effects of the anti-sigma factor MucA in *Pseudomonas fluorescens* SBW25 through genome-scale metabolic modeling. BMC Syst Biol. 2013;7:19.

15. Mann EE, Wozniak DJ. *Pseudomonas* biofilm matrix composition and niche biology. FEMS Microbiol Rev. 2012;36(4):893-916.
16. Mærk M, Jakobsen OM, Sletta H, Klinkenberg G, Tøndervik A, Ellingsen TE, et al. Identification of Regulatory Genes and Metabolic Processes Important for Alginate Biosynthesis in *Azotobacter vinelandii* by Screening of a Transposon Insertion Mutant Library. Front Bioeng Biotechnol. 2019;7:475.
17. Pereira L. Alginates - Recent Uses of This Natural Polymer. Blumenberg M, editor. London, United Kingdom: IntechOpen; 2020 February 5th, 2020.
18. Skjåk-Bræk G, Donati I, Paoletti S. Alginate hydrogels: Properties and applications. 2016. p. 449-98.
19. Slabov V, Jain G, Chernyshova I, Kota HR, Ertesvåg H. Alginates as Green Flocculants for Metal Oxide Nanoparticles. Transactions of the Indian Institute of Metals. 2023.
20. Hay ID, Wang Y, Moradali MF, Rehman ZU, Rehm BH. Genetics and regulation of bacterial alginate production. Environ Microbiol. 2014;16(10):2997-3011.
21. Hay ID, Ur Rehman Z, Moradali MF, Wang Y, Rehm BH. Microbial alginate production, modification and its applications. Microb Biotechnol. 2013;6(6):637-50.
22. Ertesvåg H, Sletta H, Senneset M, Sun YQ, Klinkenberg G, Konradsen TA, et al. Identification of genes affecting alginate biosynthesis in *Pseudomonas fluorescens* by screening a transposon insertion library. BMC Genomics. 2017;18(1):11.
23. Moradali MF, Donati I, Sims IM, Ghods S, Rehm BH. Alginate Polymerization and Modification Are Linked in *Pseudomonas aeruginosa*. mBio. 2015;6(3):e00453-15.
24. Hay ID, Remminghorst U, Rehm BH. MucR, a novel membrane-associated regulator of alginate biosynthesis in *Pseudomonas aeruginosa*. Appl Environ Microbiol. 2009;75(4):1110-20.
25. Jack Preiss GA. Alginic Acid Metabolism in Bacteria: I. Enzymatic Formation Of Unsaturated Oligosaccharides And 4-deoxy-l-erythro-5-hexoselulose Uronic Acid. J Biol Chem. 1962;237(2):309-16.
26. Brown BJ, Preston JF, Ingram LO. Cloning of alginate lyase gene (alxM) and expression in *Escherichia coli*. Appl Environ Microbiol. 1991;57(6):1870-2.
27. Tøndervik A, Klinkenberg G, Aarstad OA, Drablos F, Ertesvåg H, Ellingsen TE, et al. Isolation of mutant alginate lyases with cleavage specificity for di-guluronic acid linkages. J Biol Chem. 2010;285(46):35284-92.
28. Valentini M, Filloux A. Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: Lessons from *Pseudomonas aeruginosa* and Other Bacteria. J Biol Chem. 2016;291(24):12547-55.
29. Garst AD, Edwards AL, Batey RT. Riboswitches: structures and mechanisms. Cold Spring Harb Perspect Biol. 2011;3(6).
30. Zhou H, Zheng C, Su J, Chen B, Fu Y, Xie Y, et al. Characterization of a natural triple-tandem c-di-GMP riboswitch and application of the riboswitch-based dual-fluorescence reporter. Sci Rep. 2016;6:20871.
31. Breaker RR. Prospects for riboswitch discovery and analysis. Mol Cell. 2011;43(6):867-79.
32. Zamorano-Sanchez D, Xian W, Lee CK, Salinas M, Thongsomboon W, Cegelski L, et al. Functional Specialization in Vibrio cholerae Diguanylate Cyclases: Distinct Modes of Motility Suppression and c-di-GMP Production. mBio. 2019;10(2).
33. Haaland H. A Study of c-di-GMP Levels and Alginate Production in *Pseudomonas fluorescens* SBW25 [Master]. Trondheim: NTNU; 2022.

34. Fages-Lartaud M, Tietze L, Elie F, Lale R, Hohmann-Marriott MF. mCherry contains a fluorescent protein isoform that interferes with its reporter function. Front Bioeng Biotechnol. 2022;10:892138.
35. Laue H, Schenk A, Li H, Lambertsen L, Neu TR, Molin S, et al. Contribution of alginate and levan production to biofilm formation by *Pseudomonas syringae*. Microbiol. 2006;152(Pt 10):2909-18.
36. Jathore NR, Bule MV, Tilay AV, Annapure US. Microbial levan from Pseudomonas fluorescens: Characterization and medium optimization for enhanced production. Food Science and Biotechnology. 2012;21(4):1045-53.
37. González-Garcinuño Á, Tabernero A, Sánchez-Álvarez JM, Galán MA, Martin del Valle EM. Effect of bacteria type and sucrose concentration on levan yield and its molecular weight. Microb Cell Factories. 2017;16(1):91.
38. Records AR, Gross DC. Sensor kinases RetS and LadS regulate *Pseudomonas syringae* type VI secretion and virulence factors. J Bacteriol. 2010;192(14):3584-96.
39. Gross M, Rudolph K. Studies on the Extracellular Polysaccharides (EPS) Produced in vitro by *Pseudomonas phaseolicola*. Journal of Phytopathol. 1987;119(3):206-15.
40. Krishna PS, Woodcock SD, Pfeilmeier S, Bornemann S, Zipfel C, Malone JG. *Pseudomonas syringae* addresses distinct environmental challenges during plant infection through the coordinated deployment of polysaccharides. J Exp Bot. 2022;73(7):2206-21.
41. Hettwer U, Jaeckel FR, Boch J, Meyer M, Rudolph K, Ullrich MS. Cloning, nucleotide sequence, and expression in *Escherichia coli* of levansucrase genes from the plant pathogens *Pseudomonas syringae pv. glycinea* and *P. syringae pv. phaseolicola*. Appl Environ Microbiol. 1998;64(9):3180-7.
42. Reece RJ. Analysis of Genes and Genomes. University of Manchester, UK: John Wiley & Sond, Ltd; 2009. 469 p.
43. Coupland GM, Brown AM, Willetts NS. The origin of transfer (oriT) of the conjugative plasmid R46: characterization by deletion analysis and DNA sequencing. Mol Gen Genet. 1987;208(1-2):219-25.
44. Hanahan D. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol. 1983;166(4):557-80.
45. Macgregor GR, Nolan GP, Fiering S, Roederer M, Herzenberg LA. Use of *Escherichiu coli* (*E. coli*) *lacZ* (beta-Galactosidase) as a Reporter Gene. Methods Mol Biol. 1991;7:217-35.
46. Wirth NT, Kozaeva E, Nikel PI. Accelerated genome engineering of *Pseudomonas putida* by I-SceI—mediated recombination and CRISPR-Cas9 counterselection. Microb Biotechnol. 2020;13(1):233-49.
47. Cianfanelli FR, Cunrath O, Bumann D. Efficient dual-negative selection for bacterial genome editing. BMC Microbiol. 2020;20(1):129.
48. Debowski AW, Verbrugghe P, Sehnal M, Marshall BJ, Benghezal M. Development of a tetracycline-inducible gene expression system for the study of *Helicobacter pylor*i pathogenesis. Appl Environ Microbiol. 2013;79(23):7351-9.
49. Ramos JL, Marqués S, Timmis KN. Transcriptional Control of the *Pseudomonas* TOL Plasmid Catabolic Operons is Achieved Through an Interplay of Host Factors and Plasmid-Encoded Regulators. Annu Rev Microbiol. 1997;51(1):341-73.
50. Gawin A, Valla S, Brautaset T. The XylS/Pm regulator/promoter system and its use in fundamental studies of bacterial gene expression, recombinant protein production and metabolic engineering. Microb Biotechnol. 2017;10(4):702-18.
51. Gimmestad M, Sletta H, Ertesvåg H, Bakkevig K, Jain S, Suh SJ, et al. The *Pseudomonas fluorescens* AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. J Bacteriol. 2003;185(12):3515-23.

52. Simon R, Priefer U, Pühler A. A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. Bio/Technol. 1983;1(9):784-91.
53. Grahn AM, Haase J, Bamford DH, Lanka E. Components of the RP4 conjugative transfer apparatus form an envelope structure bridging inner and outer membranes of donor cells: implications for related macromolecule transport systems. J Bacteriol. 2000;182(6):1564-74.
54. Silbert J, Lorenzo V, Aparicio T. Refactoring the Conjugation Machinery of Promiscuous Plasmid RP4 into a Device for Conversion of Gram-Negative Isolates to Hfr Strains. ACS Synth Biol. 2021;10(4):690-7.
55. Ferrieres L, Hemery G, Nham T, Guerout AM, Mazel D, Beloin C, et al. Silent mischief: bacteriophage Mu insertions contaminate products of *Escherichia coli* random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4 conjugative machinery. J Bacteriol. 2010;192(24):6418-27.
56. Matsumoto-Mashimo C, Guerout AM, Mazel D. A new family of conditional replicating plasmids and their cognate *Escherichia coli* host strains. Res Microbiol. 2004;155(6):455-61.
57. Thomason L, Court DL, Bubunenko M, Costantino N, Wilson H, Datta S, et al. Recombineering: Genetic Engineering in Bacteria Using Homologous Recombination. Curr Protoc in Mol Biol. 2007;78(1):1.16.1-1..24.
58. Heap JT, Ehsaan M, Cooksley CM, Ng YK, Cartman ST, Winzer K, et al. Integration of DNA into bacterial chromosomes from plasmids without a counter-selection marker. Nucleic Acids Res. 2012;40(8):e59.
59. Vold S. Reporter genes for relative quantification of promoter strength and c-di-GMP in *Pseudomonas fluorescens* [Master]. Trondheim: NTNU; 2021.
60. Chen Z, Ling W, Shang G. Recombineering and I-SceI-mediated *Pseudomonas putida* KT2440 scarless gene deletion. FEMS Microbiol Letters. 2016;363(21).
61. Krempl PM, Mairhofer J, Striedner G, Thallinger GG. Finished Genome Sequence of the Laboratory Strain *Escherichia coli* K-12 RV308 (ATCC 31608). Genome Announc. 2014;2(6).
62. Bakkevig K, Sletta H, Gimmestad M, Aune R, Ertesvåg H, Degnes K, et al. Role of the *Pseudomonas fluorescens* alginate lyase (AlgL) in clearing the periplasm of alginates not exported to the extracellular environment. J Bacteriol. 2005;187(24):8375-84.
63. MasterPure TM Complete DNA and RNA Purification Kit. In: Company@ I, editor.: EpiCentre; 2012.
64. Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 1979;7(6):1513-23.
65. ZR Plasmid Miniprep™-Classic Intstruction Manual. In: Research Z, editor. 1.0.4 ed: Zymo Research.
66. Ogur M, Rosen G. The nucleic acids of plant tissues; the extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid. Arch Biochem. 1950;25(2):262-76.
67. Li X, Wu Y, Zhang L, Cao Y, Li Y, Li J, et al. Comparison of three common DNA concentration measurement methods. Anal Biochem. 2014;451:18-24.
68. 260/280 and 260/230 Ratios Wilmington, Delaware USA: ThermoFisher Scientific; [Available from: https://dna.uga.edu/wp-content/uploads/sites/51/2019/02/Note-on-the-260_280-and-260_230-Ratios.pdf.
69. Snustad PSM. Principles of Genetics. 7 ed: John Wiley & Sons; 2016.
70. Nathans D, Smith HO. Restriction endonucleases in the analysis and restructuring of dna molecules. Annu Rev Biochem. 1975;44:273-93.

71. DNA Ligation: Addgene; [Available from: https://www.addgene.org/protocols/dna-ligation/.
72. Guzaev M, Li X, Park C, Leung W-Y, Roberts L. Comparison of nucleic acid gel stains cell permeability, safety, and sensitivity of ethidium bromide alternatives. 2017.
73. Monarch DNA Gel Extraction Kit, Instruction Manual. In: BioLabs NE, editor. 1.2 ed2017. p. 14.
74. Lehman IR. DNA ligase: structure, mechanism, and function. Science. 1974;186(4166):790-7.
75. Kimoto M, Soh SHG, Tan HP, Okamoto I, Hirao I. Cognate base-pair selectivity of hydrophobic unnatural bases in DNA ligation by T4 DNA ligase. Biopolymers. 2021;112(1):e23407.
76. Panja S, Saha S, Jana B, Basu T. Role of membrane potential on artificial transformation of *E. coli* with plasmid DNA. J Biotechnol. 2006;127(1):14-20.
77. Asif A, Mohsin H, Tanvir R, Rehman Y. Revisiting the Mechanisms Involved in Calcium Chloride Induced Bacterial Transformation. Front Microbiol. 2017;8:2169.
78. R. WJDBTABSPGALML. Molecular Biology of the Gene 7ed: Pearson Deucation; 2014.
79. CloneAmp™ HiFi PCR Premix Protocol-At-A-Glance: Clontech Laboratories, Inc. A Takara Bio Company; [Available from: https://www.takarabio.com/documents/User%20Manual/CloneAmp%20HiFi%20PCR%20Premix%20Protocol/CloneAmp%20HiFi%20PCR%20Premix%20Protocol-At-A-Glance_092612.pdf.
80. Instruction Manual, Monarch PCR & DNA Cleanup Kit (5 µg) T1030 manual: NewEngland BioLabs; 2021 [3.0:[Available from: https://international.neb.com/-/media/nebus/files/manuals/manualt1030.pdf?rev=3a38f156896540df9191eef0f51eed72&hash=9BE8B7A6FD3C7543BE01A29E7DE3CB1B
81. User Guide, Zero Blunt® TOPO® PCR Cloning Kit. In: Life Technologies TFS, editor. 2014. p. 32.
82. von Wintersdorff CJ, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB, et al. Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. Front Microbiol. 2016;7:173.
83. Llosa M, Gomis-Ruth FX, Coll M, de la Cruz Fd F. Bacterial conjugation: a two-step mechanism for DNA transport. Mol Microbiol. 2002;45(1):1-8.
84. Arutyunov D, Frost LS. F conjugation: back to the beginning. Plasmid. 2013;70(1):18-32.
85. Facci P. Chapter 6 - Redox Control of Gene Expression Level. Micro and Nano Technol, Biomol Electr. 2014:189-205.
86. Thoma S, Schobert M. An improved *Escherichia coli* donor strain for diparental mating. FEMS Microbiol Lett. 2009;294(2):127-32.
87. Kuzminov A. Homologous Recombination-Experimental Systems, Analysis, and Significance. EcoSal Plus. 2011;4(2).
88. Azencott HR, Peter GF, Prausnitz MR. Influence of the cell wall on intracellular delivery to algal cells by electroporation and sonication. Ultrasound Med Biol. 2007;33(11):1805-17.
89. Cheng D, Jiang C, Xu J, Liu Z, Mao X. Characteristics and applications of alginate lyases: A review. Int J Biol Macromol. 2020;164:1304-20.
90. Monds RD, Newell PD, Gross RH, O'Toole GA. Phosphate-dependent modulation of c-di-GMP levels regulates Pseudomonas fluorescens Pf0-1 biofilm formation by controlling secretion of the adhesin LapA. Mol Microbiol. 2007;63(3):656-79.

91. Gawin A, Peebo K, Hans S, Ertesvåg H, Irla M, Neubauer P, et al. Construction and characterization of broad-host-range reporter plasmid suitable for on-line analysis of bacterial host responses related to recombinant protein production. Microb Cell Fact. 2019;18(1):80.
92. Buch AD, Archana G, Naresh Kumar G. Broad-host-range plasmid-mediated metabolic perturbations in *Pseudomonas fluorescens* 13525. Appl Microbiol and Biotechnol. 2010;88(1):209-18.
93. Li H, Ullrich MS. Characterization and mutational analysis of three allelic lsc genes encoding levansucrase in *Pseudomonas syringae*. J Bacteriol. 2001;183(11):3282-92.
94. Löwe H, Sinner P, Kremling A, Pflüger-Grau K. Engineering sucrose metabolism in *Pseudomonas putida* highlights the importance of porins. Microb Biotechnol. 2020;13(1):97-106.

Appendix

A. Restriction Enzymes

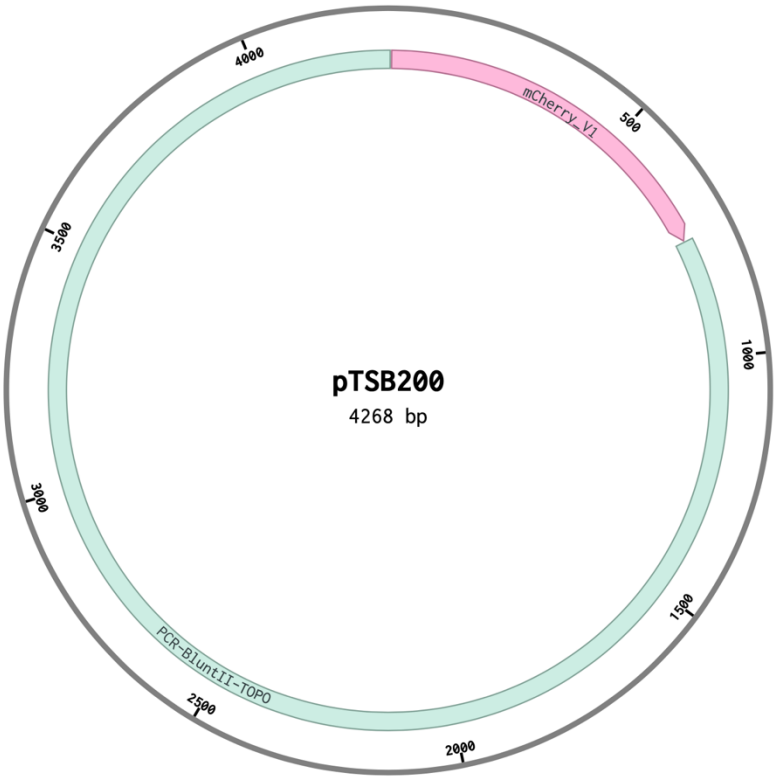
The restriction enzymes and the respective recognition site and optimal buffer are listed in Table A**.** All enzymes are from New England BioLabs.

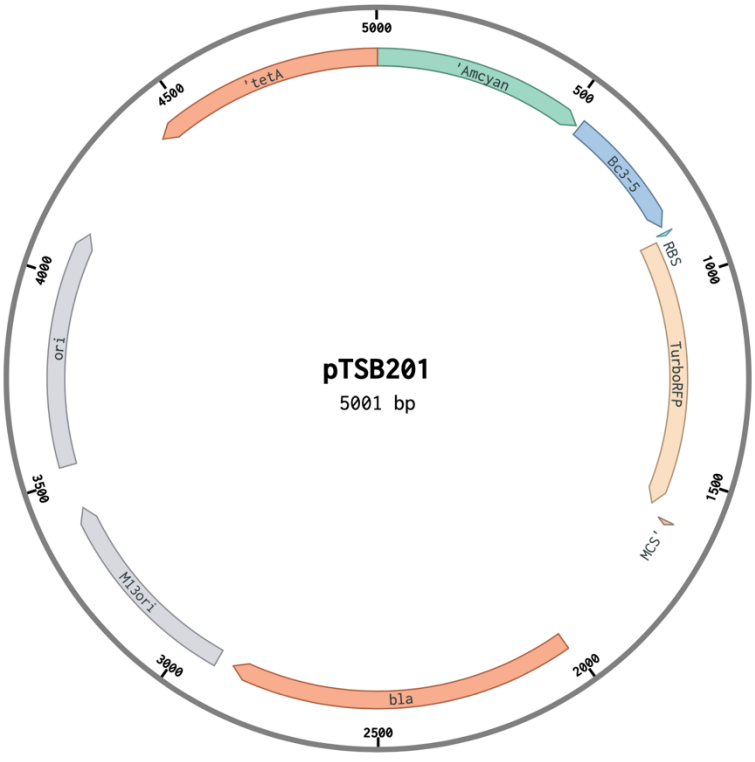
Table A: **Restriction enzymes utilised in this thesis.** Restriction sites and optimal buffer for each restriction enzyme is listed. NotI was not utilised, but it is mentioned as the restriction site was mentioned.

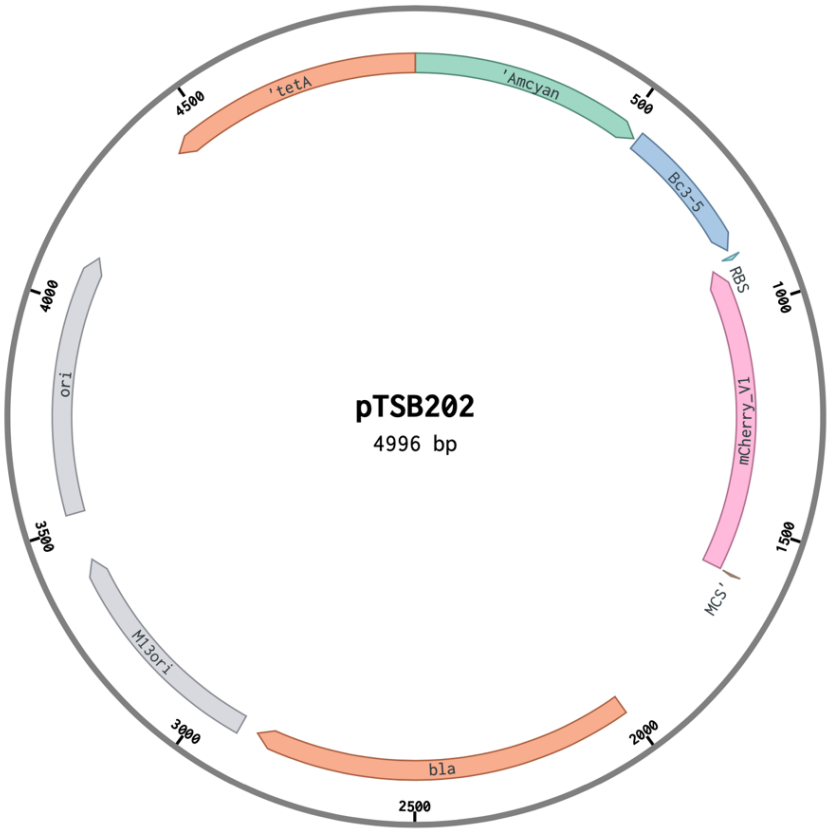
Restriction enzyme	**Recognition Site**	**Optimal buffer**
Acc65I	5′ G/GTACC 3′	3.1
AvrII	5′ C/CTAGG 3′	1.1, CutSmart
EcoRI	5′ G/AATTC 3′	CutSmart
EcoRV	5′ GAT/ATC 3′	2.1, 3.1, CutSmart
HindIII	5′ A/AGCTT 3′	2.1, CutSmart
NcoI	5′ C/CATGG 3′	1.1, 2.1, 3.1
NotI	5′ GC/GGCCGC 3′	2.1, CutSmart
NsiI	5′ ATGCA/T 3′	CutSmart
PciI	5′ A/CATGT 3′	3.1
PspOMI	5′ G/GGCCC 3′	CutSmart
PstI	5′ CTGCA/G 3′	CutSmart
SacI	5′ GAGCT/C 3′	CutSmart
SbfI	5′ CCTGCA/GG 3′	CutSmart
SpeI	5′ A/CTAGT 3′	CutSmart
XbaI	5′ T/CTAGA 3′	2.1, CutSmart
XhoI	5′ C/TCGAG 3′	2.1, 3.1, CutSmart
XmnI	5′ GAANN/NNTTC 3′	CutSmart

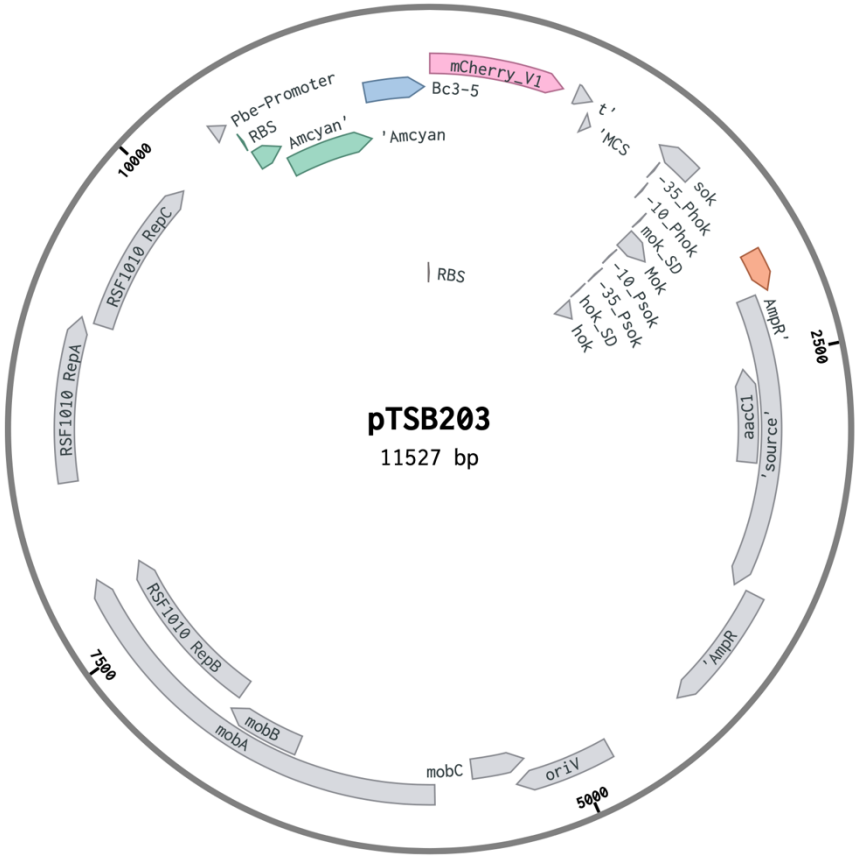
B. Plasmids constructed

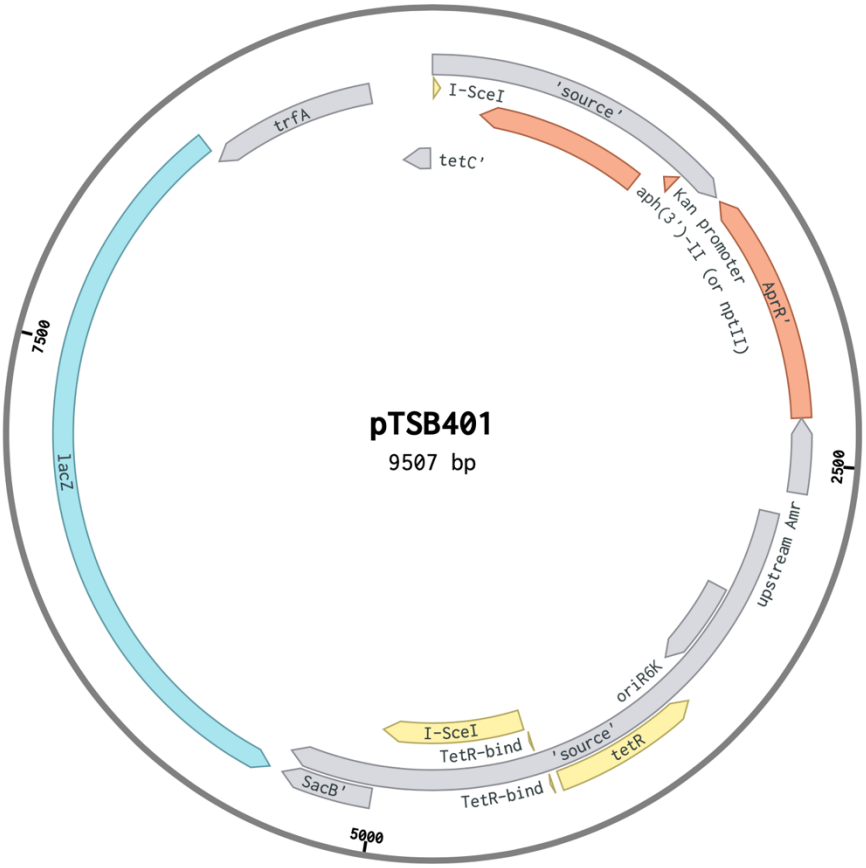
Following, a presentation of all plasmids constructed is given. The plasmids are illustrated by using Benchling.

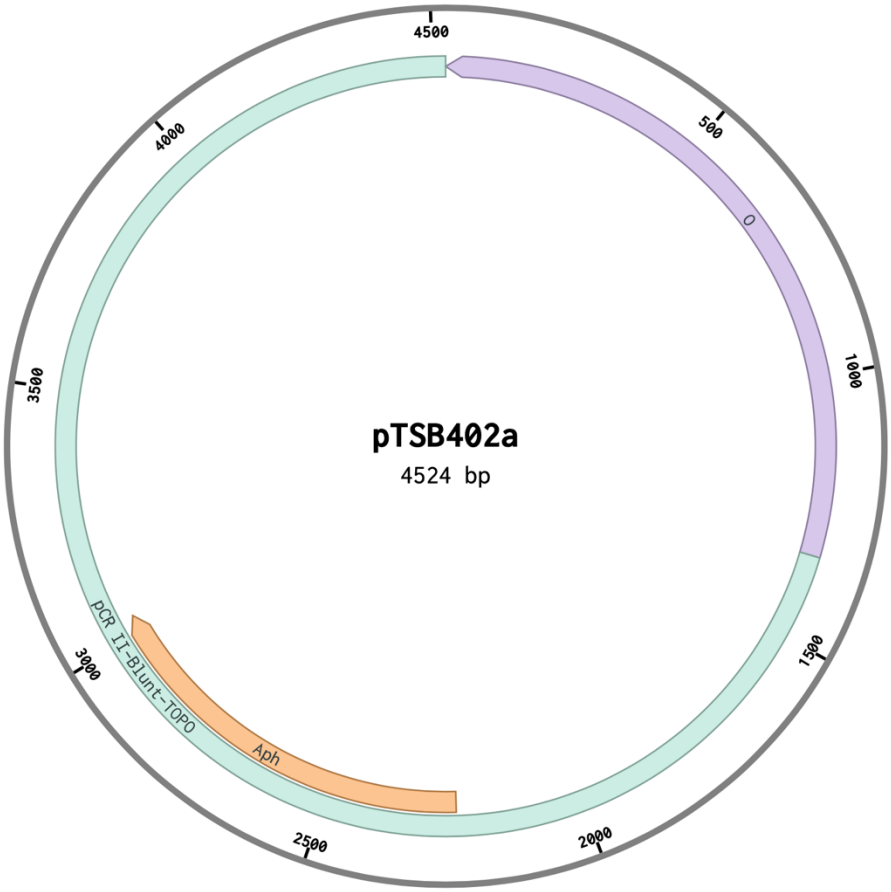


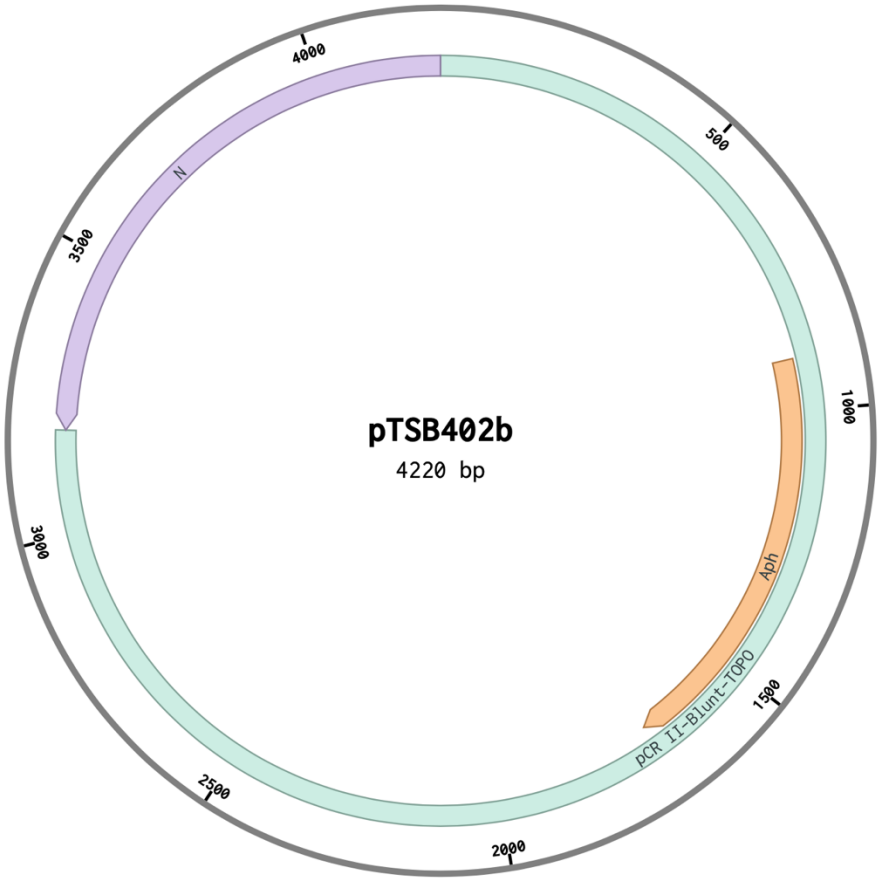


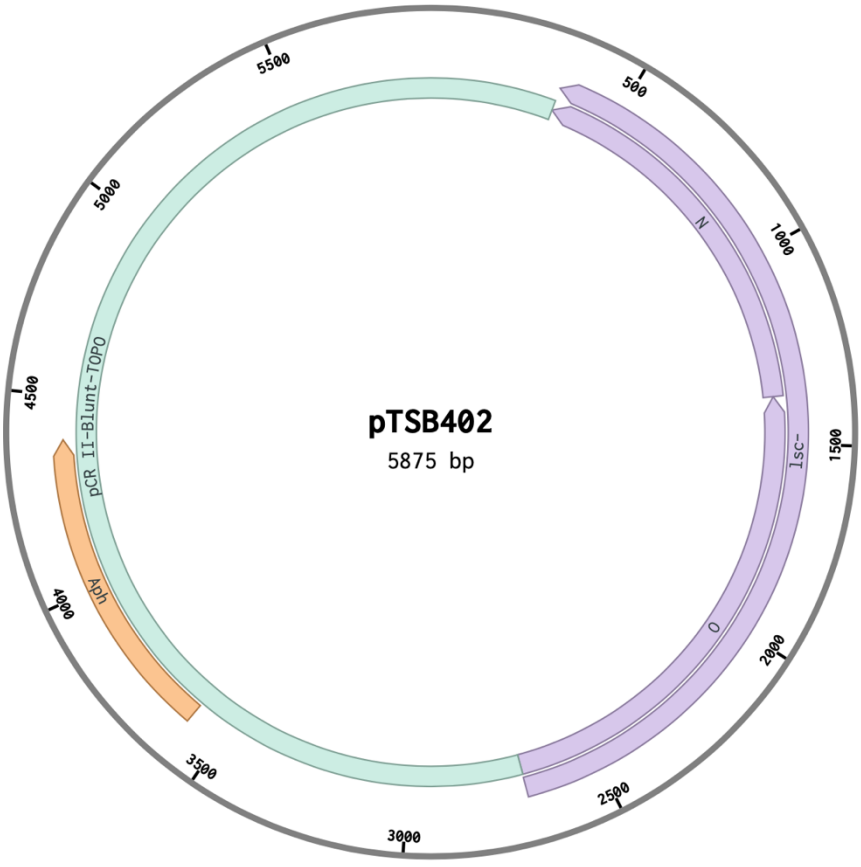

pTSB202
4996 bp
'tetA
'Amcyan
Bc3-5
RBS
mCherry_V1
MCS'
M13ori
bla
ori
500
1000
1500
2000
2500
3000
3500
4000
4500

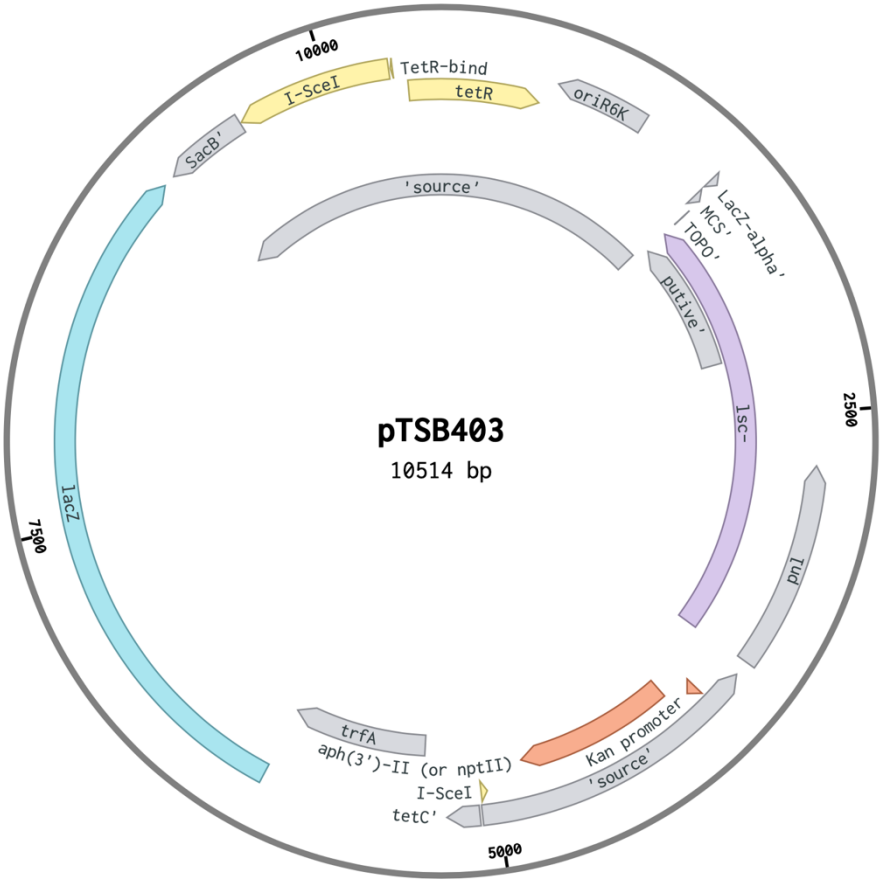

pTSB203
11527 bp
mCherry_V1
Bc3-5
Pbe-Promoter
RBS
Amcyan'
'Amcyan
t'
'MCS
sok
-35_Phok
-10_Phok
mok_SD
Mok
-10_Psok
-35_Psok
hok_SD
hok
RBS
AmpR'
aacC1
'source'
'AmpR
oriV
mobC
mobA
mobB
RSF1010 RepB
RSF1010 RepA
RSF1010 RepC
2500
5000
7500
10000

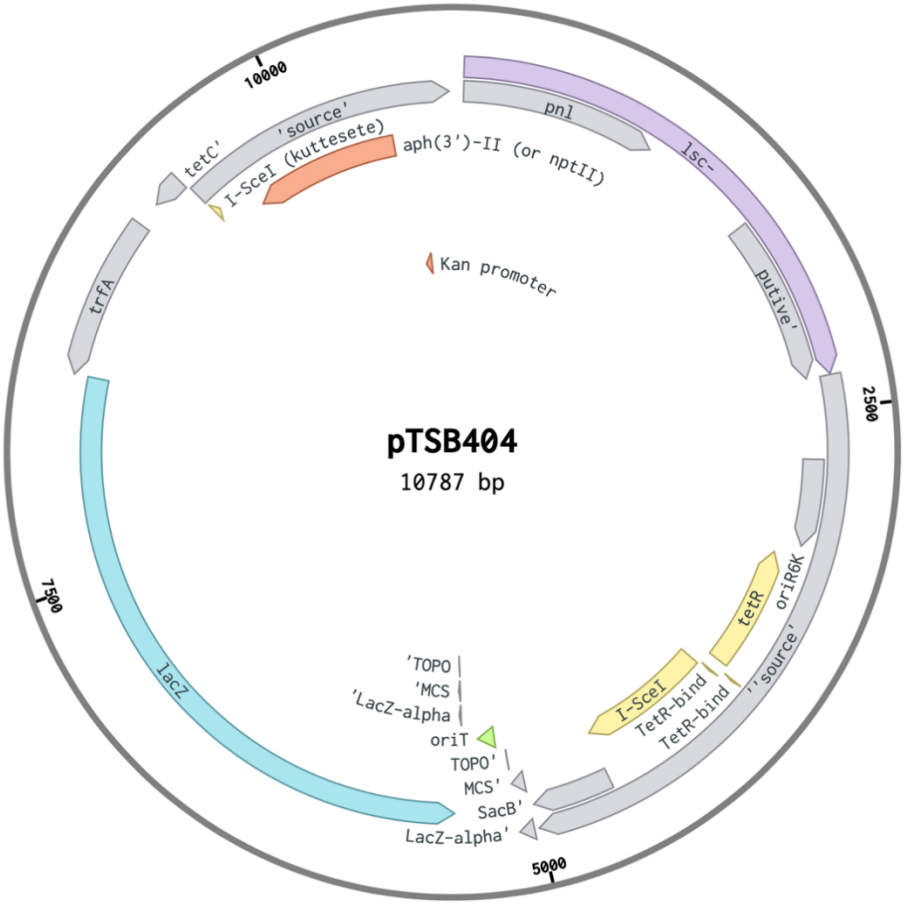

pTSB401
9507 bp
I-SceI
'source'
trfA
tetC'
Kan promoter
aph(3')-II (or nptII)
AprR'
lacZ
upstream Amr
oriR6K
I-SceI
TetR-bind
'source'
tetR
SacB'
TetR-bind
5000
7500
2500


pTSB402a
4524 bp
4500
500
1000
1500
2000
2500
3000
3500
4000
O
pCR II-Blunt-TOPO
Aph


pTSB402b
4220 bp
4000
500
1000
1500
2000
2500
3000
3500
N
Aph
pCR II-Blunt-TOPO


pTSB402
5875 bp
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
N
lsc-
O
Aph
pCR II-Blunt-TOPO


pTSB403
10514 bp
10000
2500
5000
7500
TetR-bind
I-SceI
tetR
oriR6K
SacB'
'source'
LacZ-alpha'
MCS'
TOPO'
putive'
lsc-
pnl
lacZ
trfA
aph(3')-II (or nptII)
Kan promoter
I-SceI
tetC'
'source'


pTSB404
10787 bp
10000
2500
5000
7500
tetC'
'source'
I-SceI (kuttesete)
aph(3')-II (or nptII)
pnl
lsc-
putive'
Kan promoter
trfA
lacZ
'TOPO
'MCS
'LacZ-alpha
oriT
TOPO'
MCS'
SacB'
LacZ-alpha'
I-SceI
TetR-bind
TetR-bind
tetR
oriR6K
'source'

C. DNA ladders

The DNA standards were constructed by digesting λ DNA (ThermoFisher) with the restriction enzymes HindIII or PstI. The standards were used to predict the sizes of other fragments separated on a gel. Figure A displays the separated fragment sizes of λ DNA digested with HindIII and PistI.

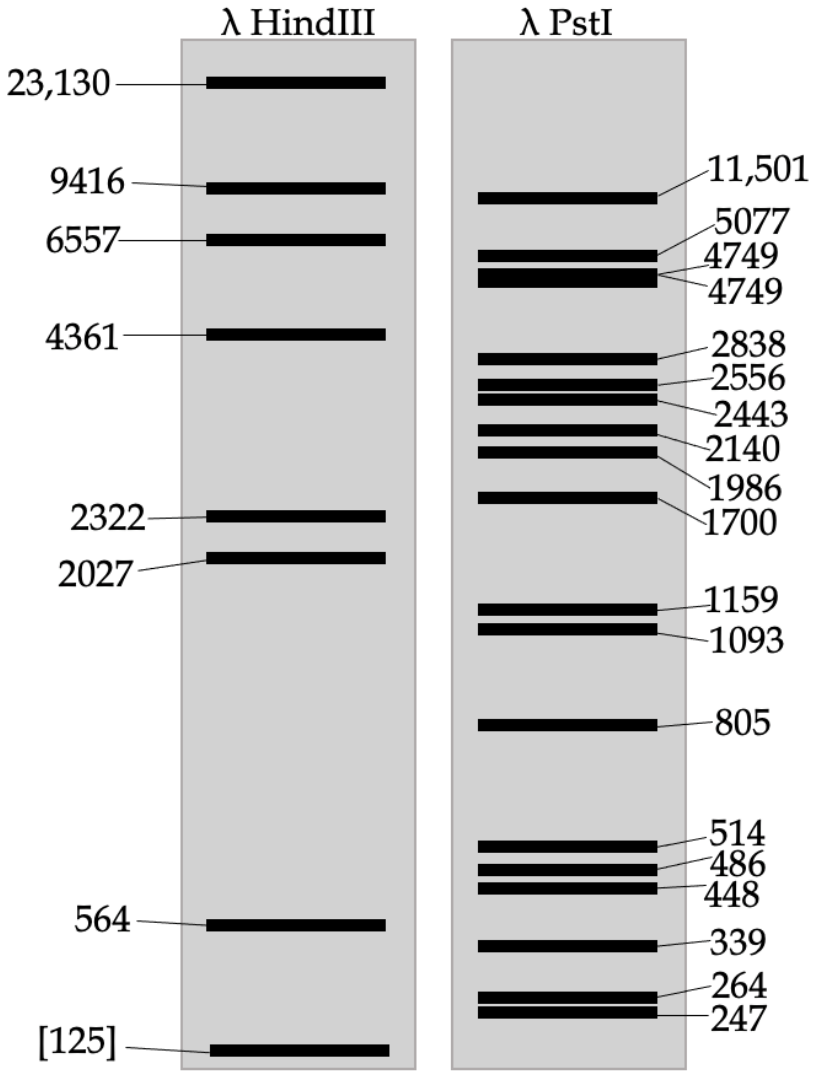


Figure A: **DNA standards used in this thesis** and their fragment sizes displayed as it would look like when separated on a gel. The figure was constructed using Microsoft PowerPoint.

D. Primers

The primers used for PCR and sequencing are listed Table **B**.

Table B: **Overview of the primers used in this thesis.** Their name, the sequence and the source are given. The first two nucleotides of dellscNR (red) revealed to be inaccurate from sequencing results. mCherryV1F and mCherryV1F2 are identical, except for one extra nucleotide at 3' end in mCherryV1F2.

Name	Sequence	Source
dellscOF	5'- ACCTAGGATGCACAGGCTCGTCTTAC -3'	ThermoFisher Scientific
dellscOR	5'- ACACCACCTCGAGTGTCGTCGTCGGGTCATTC -3'	ThermoFisher Scientific
dellscNF	5'- GACGACACTCGAGGTGGTGTTGAGCCAGTAATC -3'	ThermoFisher Scientific
dellscNR	5'- GCGGCCGCCGCGGCCAGTTTCGCTTC -3'	ThermoFisher Scientific
iscsek 1	5'- CCTGACGCTGTGCCATAACC -3'	ThermoFisher Scientific
iscsek2	5'- GAATGACCCGACGACGACAC -3'	ThermoFisher Scientific
iscsek3	5'- CTGACGCGCAAACTGCAAGG -3'	ThermoFisher Scientific
mCherryV1F	5'- CTTATTCCATGGCTATCATCAAAGAATTTATGC -3'	ThermoFisher Scientific
mCherryV1R	5'- TAACAAGAGCTCATCTAGCGAGGGCTTTACTAAG -3'	ThermoFisher Scientific
mCherryV1 sek	5'- ATGGTGTAGTCCTCGTTGTG -3'	ThermoFisher Scientific
mCherryV1F2	5'- CTTATTCCATGGCTATCATCAAAGAATTTATGCG -3'	ThermoFisher Scientific
mCherryV1R2	5'- GAGCTCATTATTATTTATACAGTTCGTCCATACCGC -3'	ThermoFisher Scientific
mCherryV1R3	5'- TATGAGCTCTGCTAGAGGGTCAGCTTTATGC -3'	ThermoFisher Scientific
M13F	5'- GTAAAACGACGGCCAG -3'	ThermoFisher Scientific Invitrogen™
M13R	5'- CAGGAAACAGCTATGAC -3'	ThermoFisher Scientific, Invitrogen™

E. Alginate standards

Alginate standards were made for each alginate assay. Three alginate standards were made and used, when conducting each experiment. That was when measuring alginate production in the *lsc*-negative strains *P. fluorescens* SBW25 and the *mucA* mutant, in fructose (Figure B) and sucrose (Figure D) as sole carbon source, and when measuring alginate production in the *mucR* overexpressing mutants in regard to c-di-GMP measurement (Figure C).

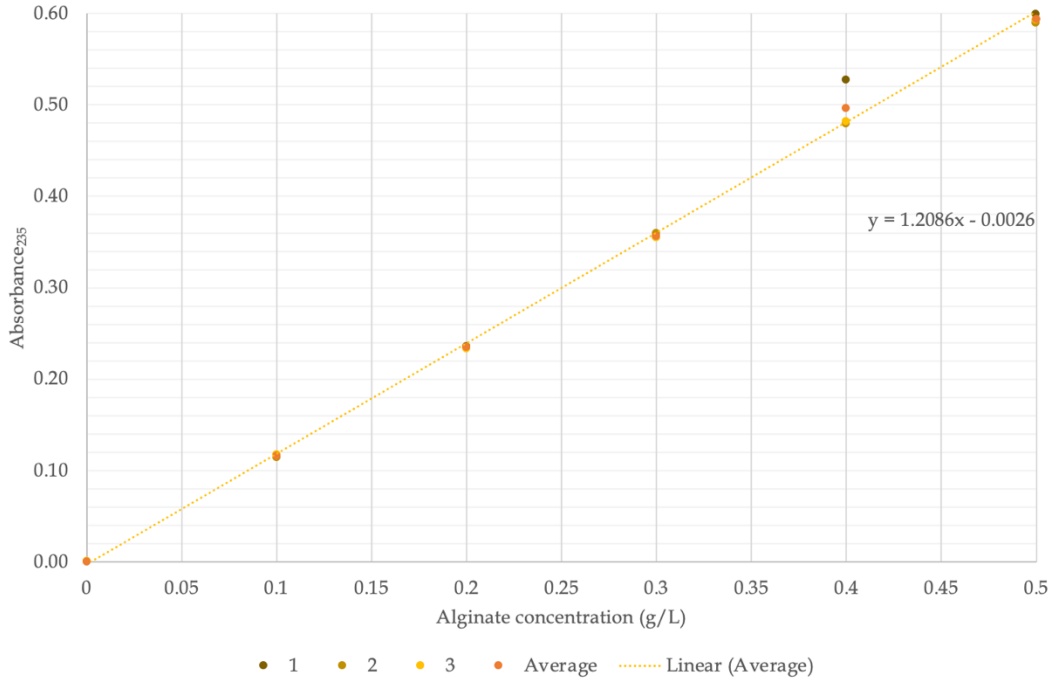


Figure B: **The standard curve showing the absorbance at 235 nm, when measuring alginate production in *Pseudomonas* strains cultivated in fructose.** for each triplicate of 0.1 g/L to 0.5 g/L LF10/60 alginate. The average of the triplicates is given, including its computed linear trendline (y = 1.286x - 0.0026).

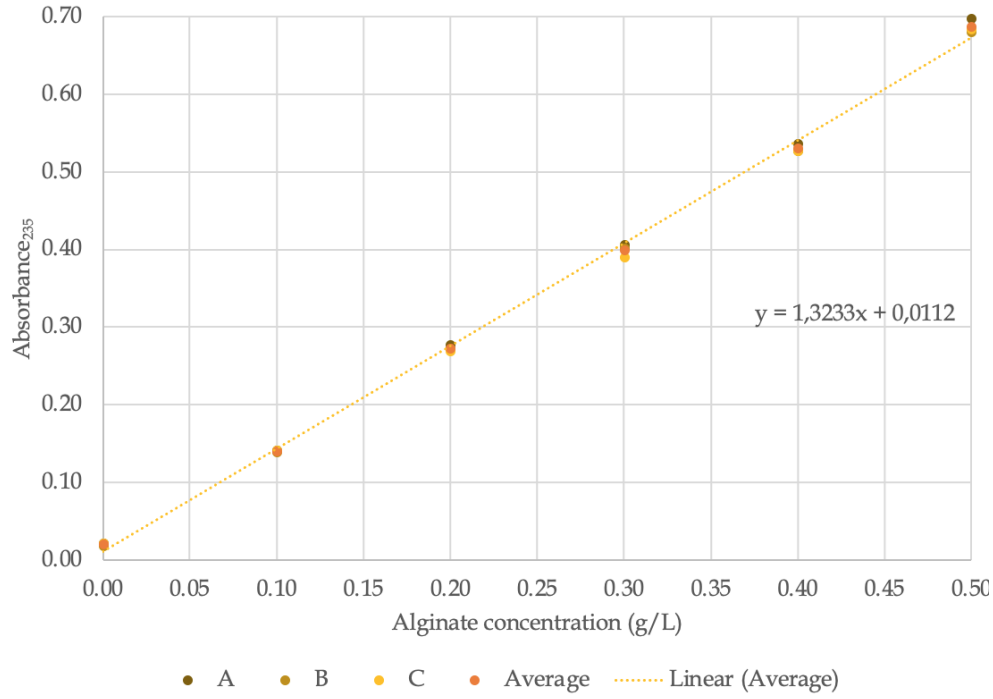


Figure C: **The standard curve showing the absorbance at 235nm, when measuring alginate production in Pseudomonas strains cultivated in fructose, in regard to c-di-GMP measurements.** for each triplicate of 0.1 g/L to 0.5 g/L LF10/60 alginate. The average of the triplicates is given, including its computed linear trendline (y = 1.3233x - 0.0112).

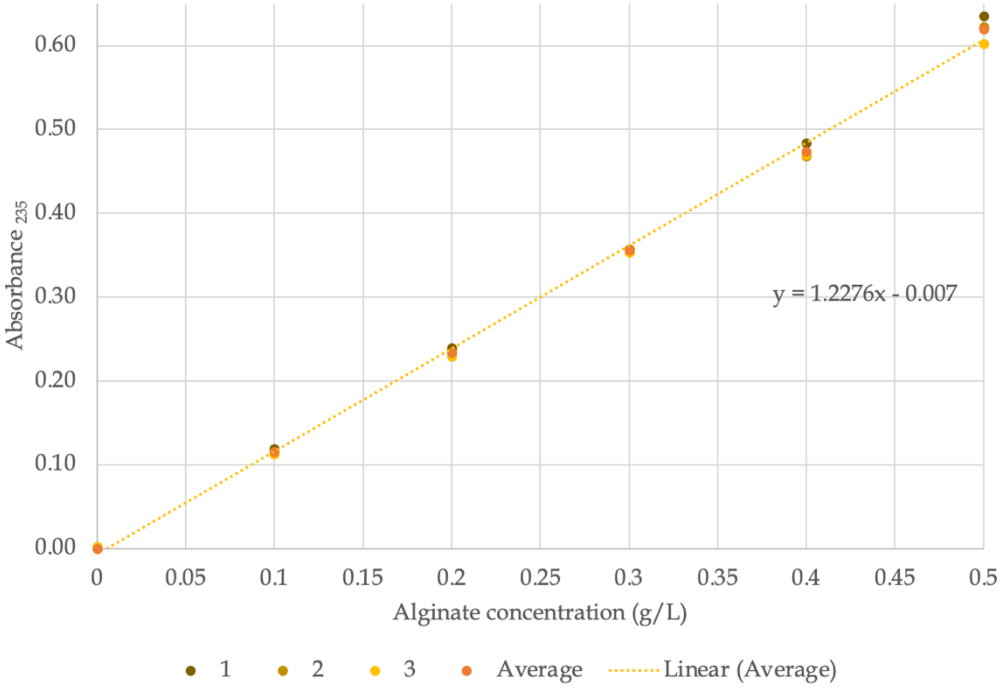


Figure D: **The standard curve showing the absorbance at 235nm, when measuring alginate production in *Pseudomonas* strains cultivated in sucrose.** for each triplicate of 0.1 g/L to 0.5 g/L LF10/60 alginate. The average of the triplicates is given, including its computes linear trendline (y = 1.2276x - 0.007).

F. M and G-lyase assay

Fractions Eluted in Phosphate Buffer A and B

Abs_{235} for the first two peaks (Figure 4.3-1) of *E. coli* RV308 (pAT77) fractions and the last peak (Figure 4.3-1) of *E. coli* RV308 pLe3 fractions were measured every 10 seconds for 10 minutes, as described in 3.20. Figure E shows the graph of the activity of the enzymes.

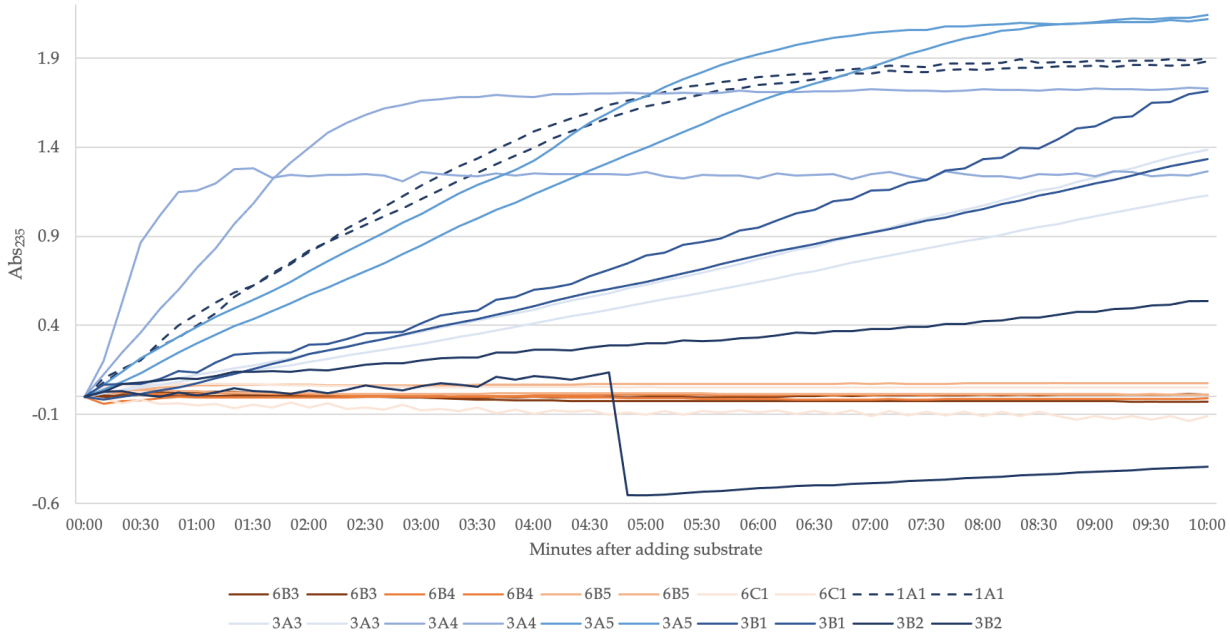


Figure E: **Delta Abs_{235}, measured every 10 seconds for 10 minutes.** Blue shades are first and second peak (Figure 4.3-1) fractions from *E. coli* RV308 (pAT77) in duplicates. Each shade representing each fraction in one peak. 1A1 has a dotted line because this is a single fraction from the first peak. Orange shades are last peak (Figure 4.3-1) fraction from *E. coli* RV308 (pLe3) in duplicates. Each shade representing each fraction in one peak.

U/mL for each fraction are listed in Table 4.3-1.

Fractions eluted in 0.05M Tris-HCl Buffer A and B

OD_{235} for the first two peaks (Figure 4.3-3) of *E. coli* RV308 (pAT77) fractions and the last peak (Figure 4.3-3) of *E. coli* RV308 pLe3 fractions were measured every 10 seconds for 10 minutes, as described in 3.20. Figure F shows the graph of the activity of the enzymes.

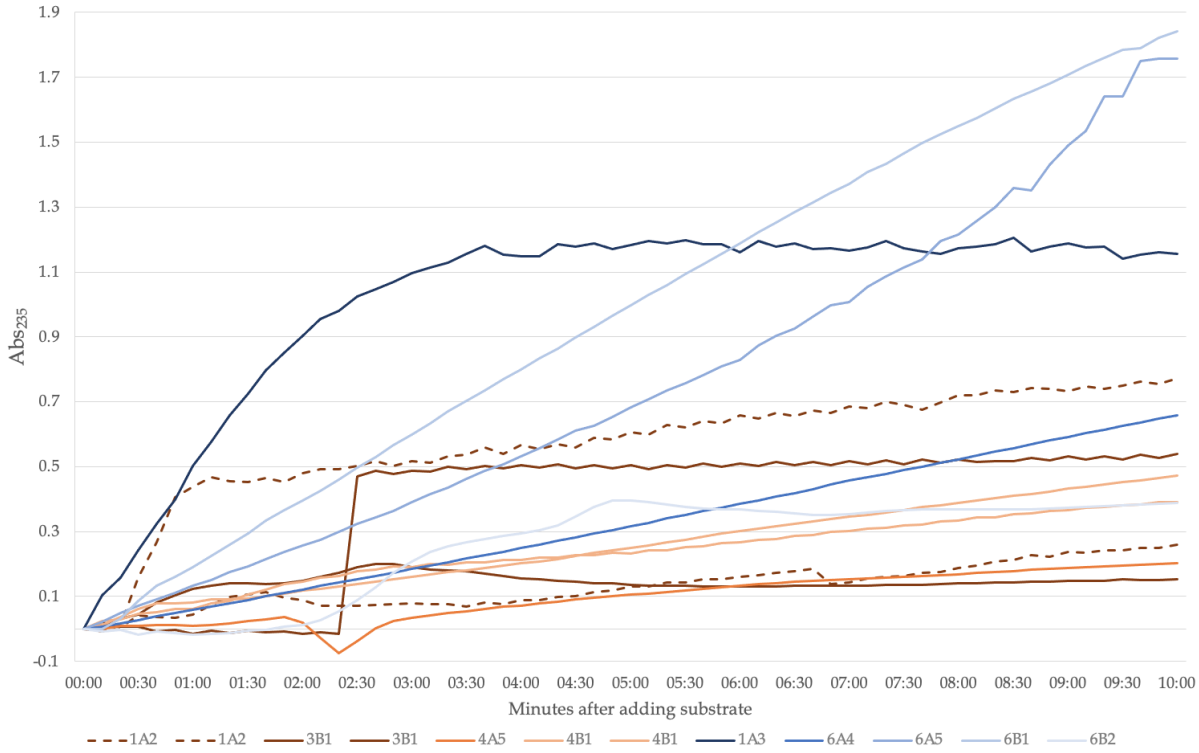


Figure F: **Delta Abs_{235}, measured every 10 seconds for 10 minutes.** Orange shades are first and second peak from *E. coli* RV308 pLe3 (Figure 4.3-3) in duplicates. 4A5 not in duplicate because of a flaw in the well. 1A2 has a dotted line because this is a single fraction from the first peak. Blue shades are the two first peaks of *E. coli* RV308 pAT77, each shade representing each fraction in one peak.

U/mL for each fraction are listed in Table 4.3-2.

G. Alginate concentrations

Alginate concentrations in g/L per OD_{600} from *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA*, and the *lsc*- negative strains when cultivated in fructose (Figure G) and sucrose (Figure H).

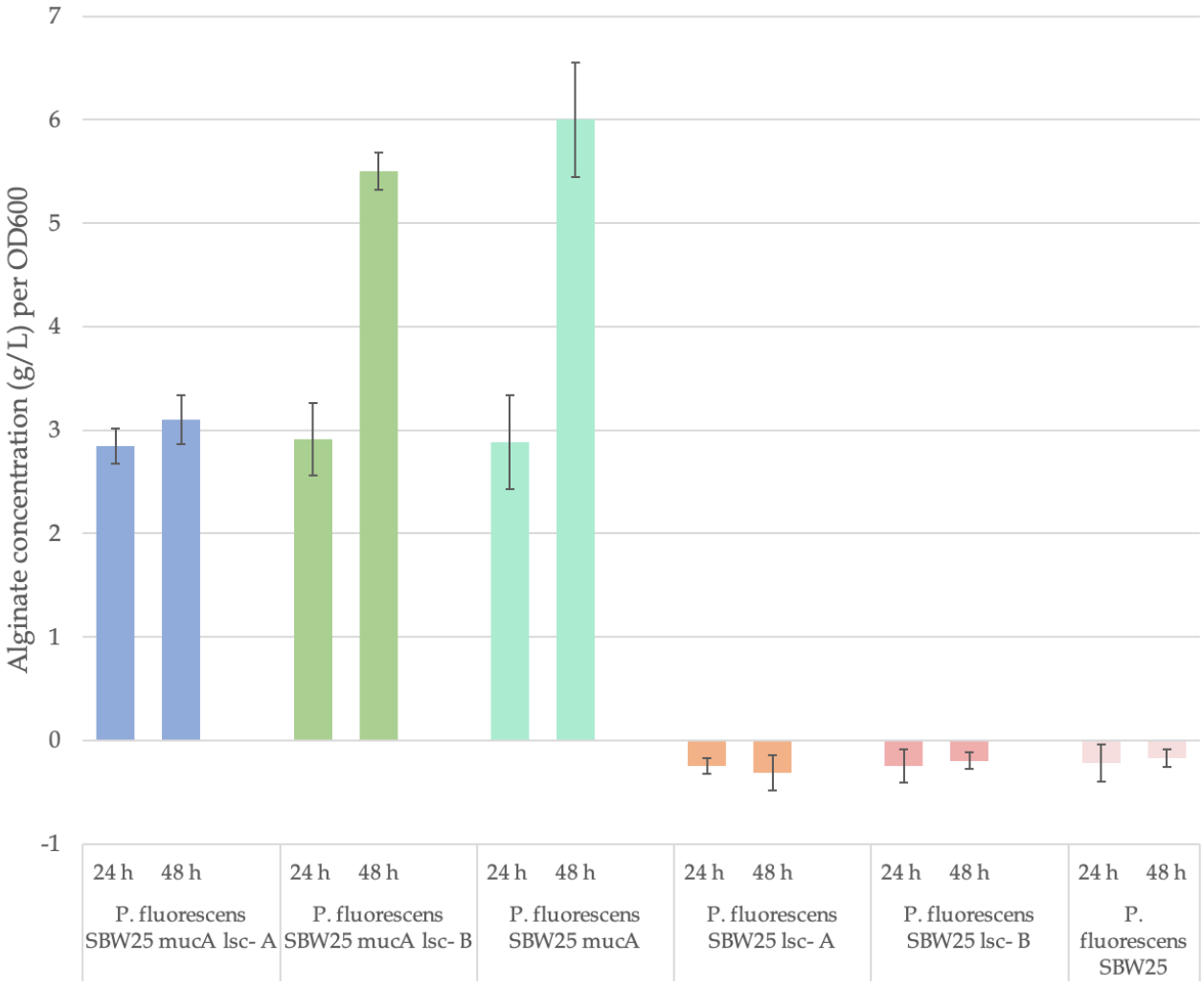


Figure G: **Alginate concentration in g/L per OD_{600} for *P. fluorescens* SBW25 strains cultivated in fructose.** Each bar represents the average of three biological replicates (two biological replicates of *P. fluorescens* SBW25), tested in triplicates, with standard deviations.

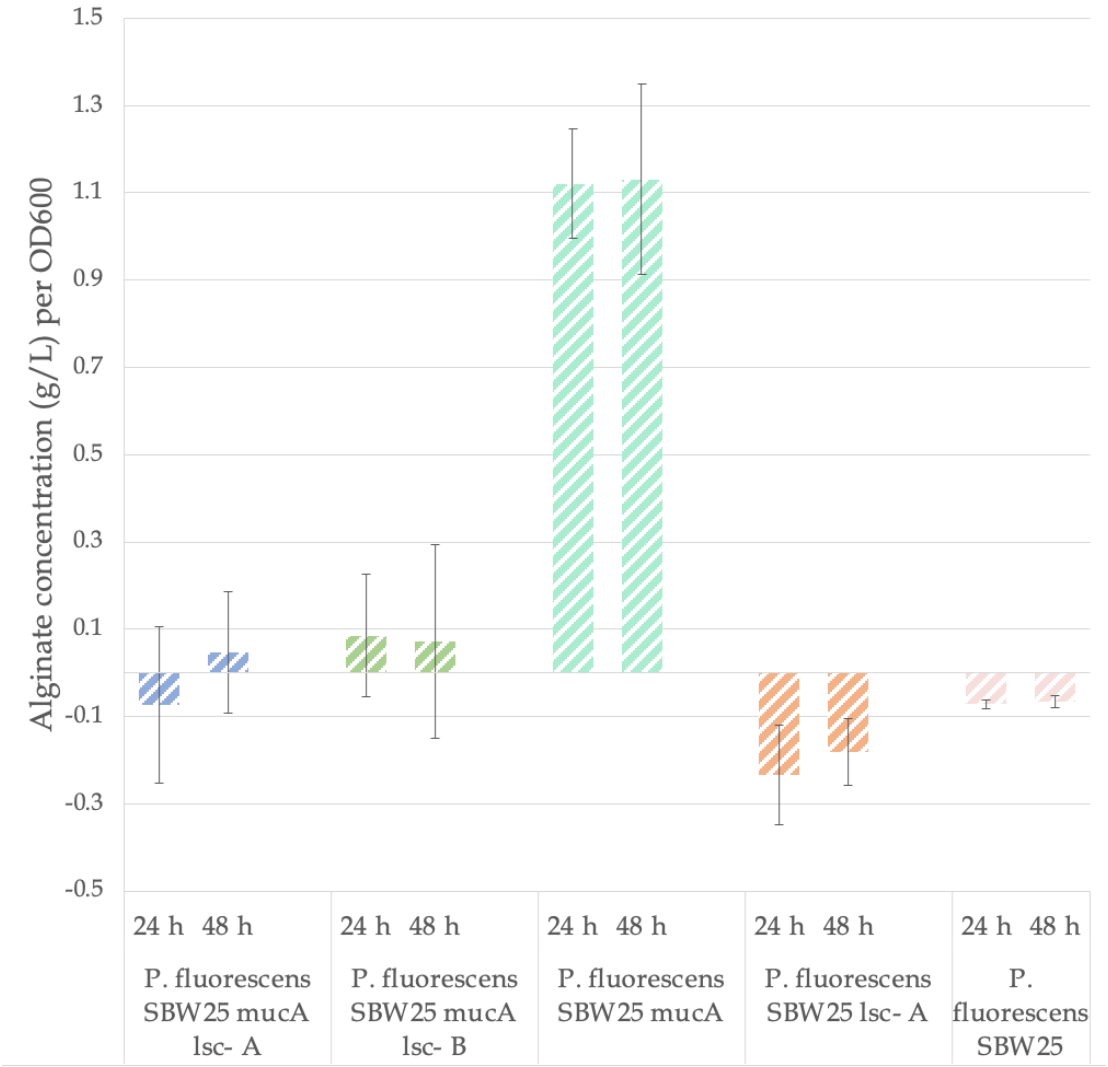
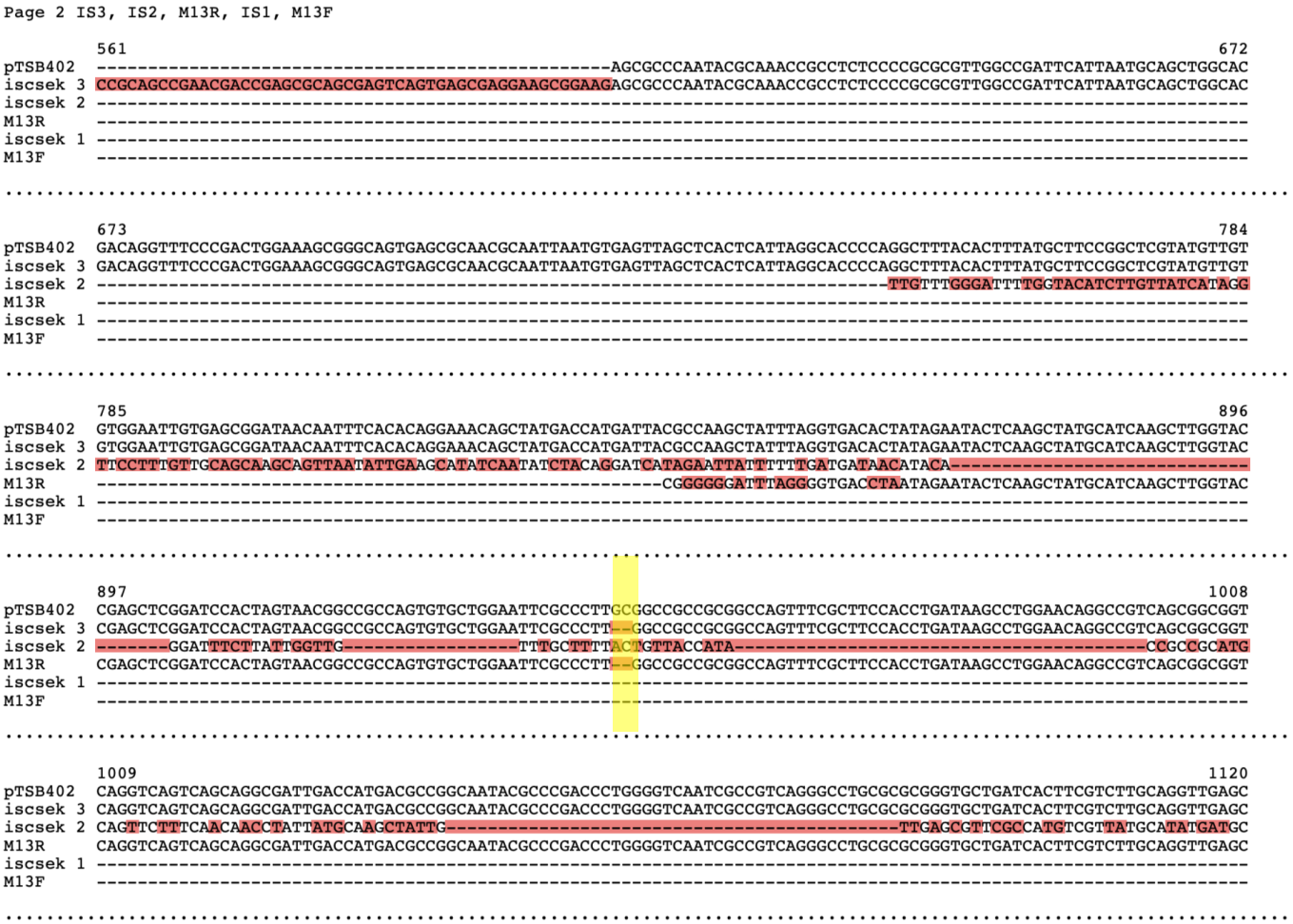


Figure H: **Alginate concentration in g/L per OD600 for P. fluorescens SBW25 strains cultivated in sucrose.** Each bar represents the average of three biological replicates, tested in triplicates, with standard deviations.

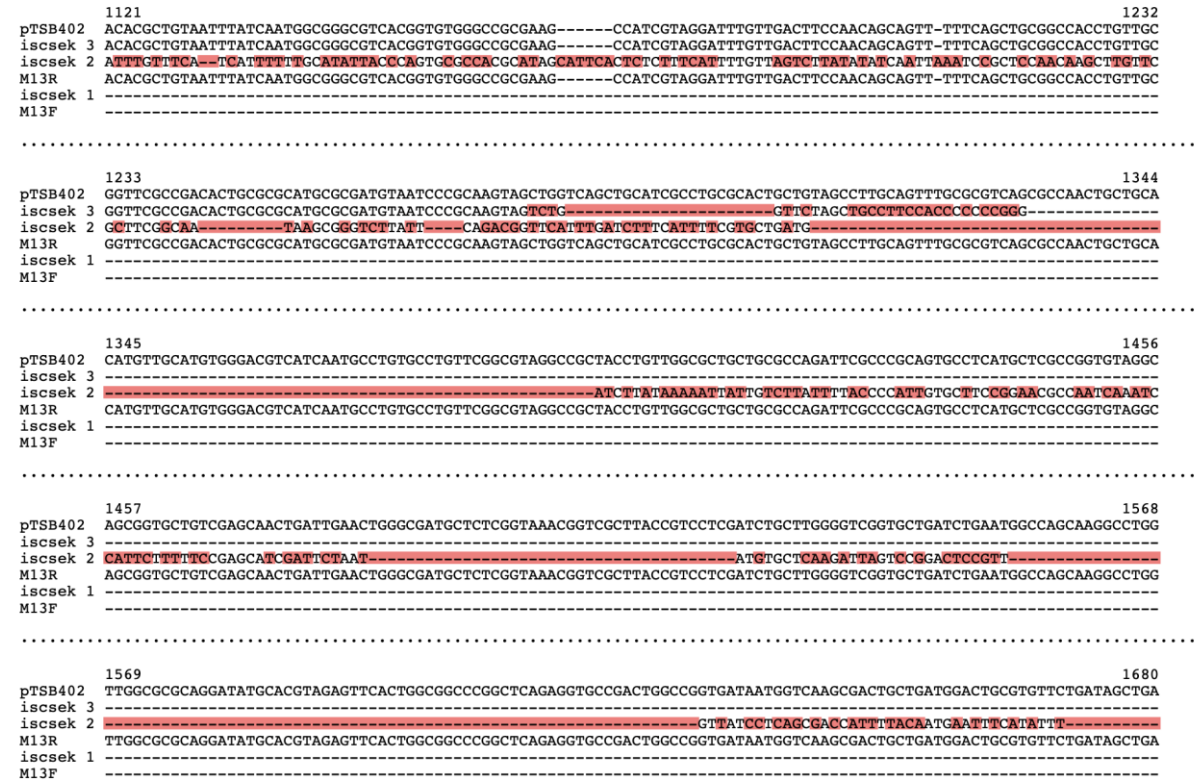
H. Sequencing results, pTSB402

The sequencing results of sequencing pTSB402. The first line shows the template DNA (pTSB402). The next show the sequencing results when using primers iscsek 3, iscsek 2, M13R, iscsek 1 and M13F.

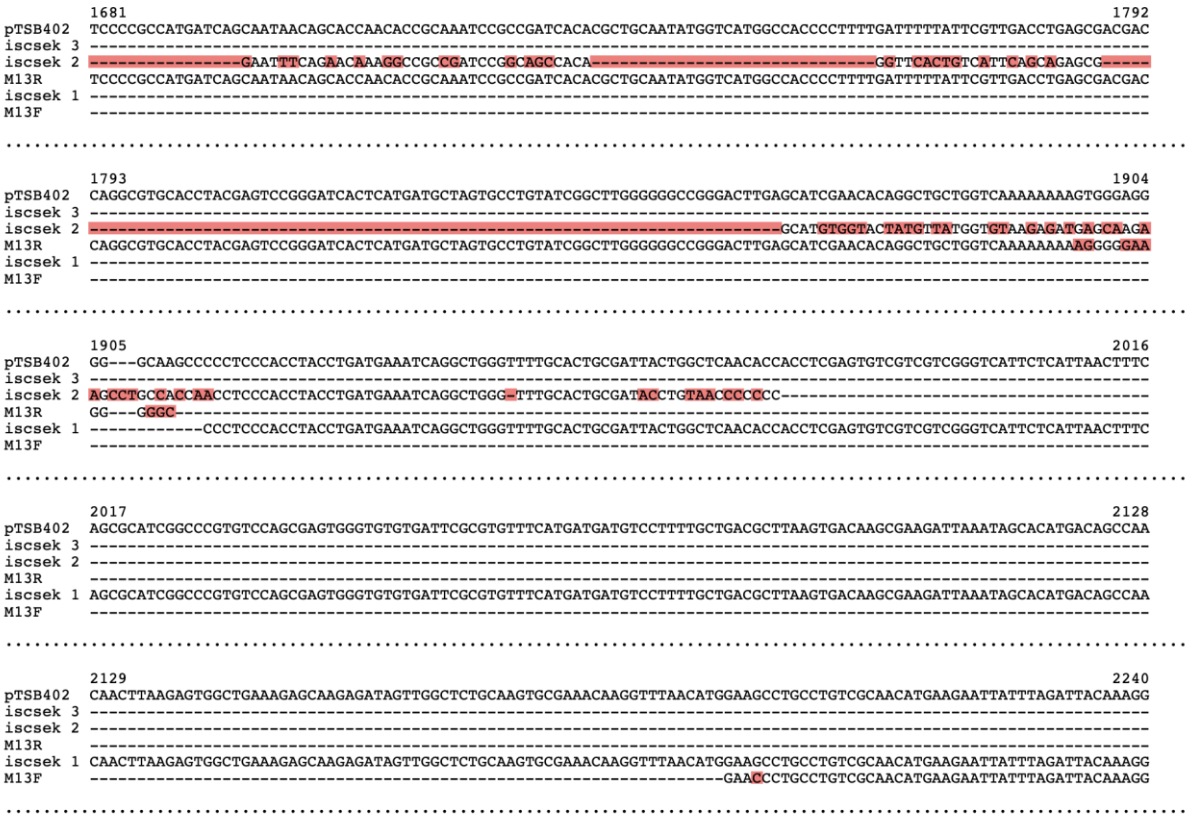
The highlighted part on Page 2, line starting at nucleotide 897, is the wrong primer sequence, mentioned in 4.2.1.3.



Page 3 IS3, IS2, M13R, IS1, M13F



Page 4 IS3, IS2, M13R, IS1, M13F



Page 5 IS3, IS2, M13R, IS1, M13F

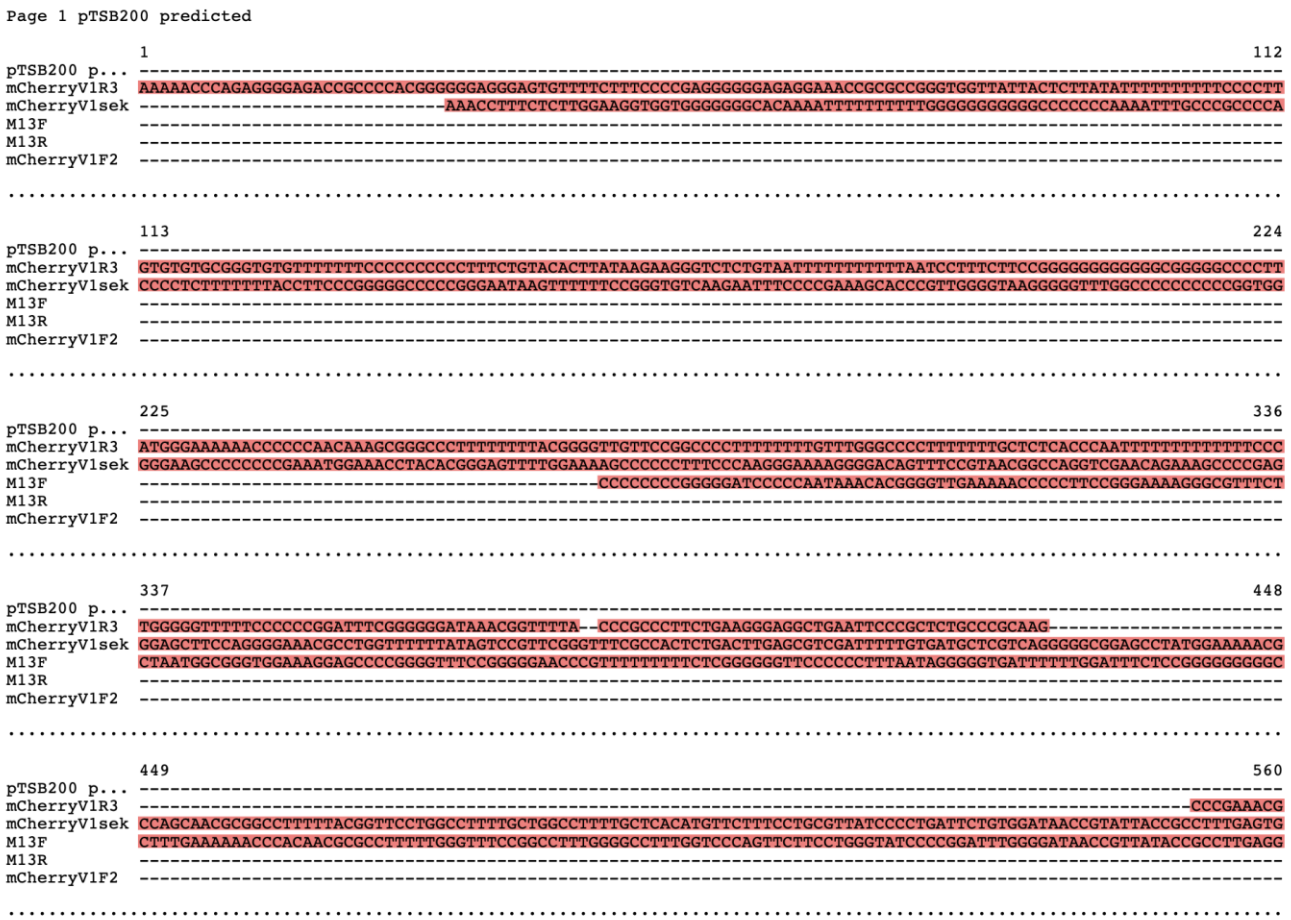
```
          2241                                                                                                2352
pTSB402   CCATCACTGTATTTATATCCTTGATATAAATACAGTGGCACCCGTATTAAATGATCGTTAAGTAGAAAGCTAGCTAATCAACTACCAAAGATCAGGGCTACCGACTGGGCGC
iscsek 3  ----------------------------------------------------------------------------------------------------------------
iscsek 2  ----------------------------------------------------------------------------------------------------------------
M13R      ----------------------------------------------------------------------------------------------------------------
iscsek 1  CCATCACTGTATTTATATCCTTGATATAAATACAGTGGCACCCGTATTAAATGATCGTTAAGTAGAAAGCTAGCTAATCAACTACCAAAGATCAGGGCTACCGACTGGGCGC
M13F      CCATCACTGTATTTATATCCTTGATATAAATACAGTGGCACCCGTATTAAATGATCGTTAAGTAGAAAGCTAGCTAATCAACTACCAAAGATCAGGGCTACCGACTGGGCGC

..................................................................................................................................

          2353                                                                                                2464
pTSB402   CGGCATTCTTCTGGGTAAAGTCCTTGGCTTGTGCGGCGGTCTTCAAGGTATAGCCATAGTTGGAAAGGGCCGTCCATTGCGCTTTAGGCGACTTCTGGTTGGTGATCGGCGG
iscsek 3  ----------------------------------------------------------------------------------------------------------------
iscsek 2  ----------------------------------------------------------------------------------------------------------------
M13R      ----------------------------------------------------------------------------------------------------------------
iscsek 1  CGGCATTCTTCTGGGTAAAGTCCTTGGCTTGTGCGGCGGTCTTCAAGGTATAGCCATAGTTGGAAAGGGCCGTCCATTGCGCTTTAGGCGACTTCTGGTTGGTGATCGGCGG
M13F      CGGCATTCTTCTGGGTAAAGTCCTTGGCTTGTGCGGCGGTCTTCAAGGTATAGCCATAGTTGGAAAGGGCCGTCCATTGCGCTTTAGGCGACTTCTGGTTGGTGATCGGCGG

..................................................................................................................................

          2465                                                                                                2576
pTSB402   GACACTGTTGGTATCGGTGAACGTGCCAGTGCCTTTGTCGTCCAACATGCCTTTATTTTGGCTGCCTTCGCCAAAATAGTTACTTTCAGAAAGGATATTGGCGTTTTGTGCC
iscsek 3  ----------------------------------------------------------------------------------------------------------------
iscsek 2  ----------------------------------------------------------------------------------------------------------------
M13R      ----------------------------------------------------------------------------------------------------------------
iscsek 1  GACACTGTTGGTATCGGTGAACGTGCCAGTGCCTTTGTCGTCCAACATGCCTTTATTTTGGCTGCCTTCGCCAAAATAGTTACTTTCAGAAAGGATATTGGCGTTTTGTGCC
M13F      GACACTGTTGGTATCGGTGAACGTGCCAGTGCCTTTGTCGTCCAACATGCCTTTATTTTGGCTGCCTTCGCCAAAATAGTTACTTTCAGAAAGGATATTGGCGTTTTGTGCC

..................................................................................................................................

          2577                                                                                                2688
pTSB402   AGGGTAAAACCGAGGTGGAAGTTATTGATGTAGTTGTTGTACACATGAAAATAACCGTAACGCATCAAACCAGGCGCACGCACTTCCATGTTGTCGAAGCGGTTATGGCACA
iscsek 3  ----------------------------------------------------------------------------------------------------------------
iscsek 2  ----------------------------------------------------------------------------------------------------------------
M13R      ----------------------------------------------------------------------------------------------------------------
iscsek 1  AGGGTAAAACCGAGGTGGAAGTTATTGATGTAGTTGTTGTACACATGAAAATAACCGTAACGCATCAAACCCAGGCGCACGCAACTTCCCCCTTC----------------
M13F      AGGGTAAAACCGAGGTGGAAGTTATTGATGTAGTTGTTGTACACATGAAAATAACCGTAACGCATCAAACCAGGCGCACGCACTTCCATGTTGTCGAAGCGGTTATGGCACA

..................................................................................................................................

          2689                                                                                                2800
pTSB402   GCGTCAGGCGCGGGTAACCATTGAACTCCGCTTTATTATCATCCGCCGGGTGGCCGAAGATCAGGCCATACTTGTGGCTGCCAAAGAAGCAGTTGCTGATGGTGGCATAATC
iscsek 3  ----------------------------------------------------------------------------------------------------------------
iscsek 2  ----------------------------------------------------------------------------------------------------------------
M13R      ----------------------------------------------------------------------------------------------------------------
iscsek 1  ----------------------------------------------------------------------------------------------------------------
M13F      GCGTCAGGCGCGGGTAACCATTGAACTCCGCTTTATTATCATCCGCCGGGTGGCCGAAGATCAGGCCATACTTGTGGCTGCCAAAGAAGCAGTTGCTGATGGTGGCATAATC

..................................................................................................................................
```

Page 6 IS3, IS2, M13R, IS1, M13F

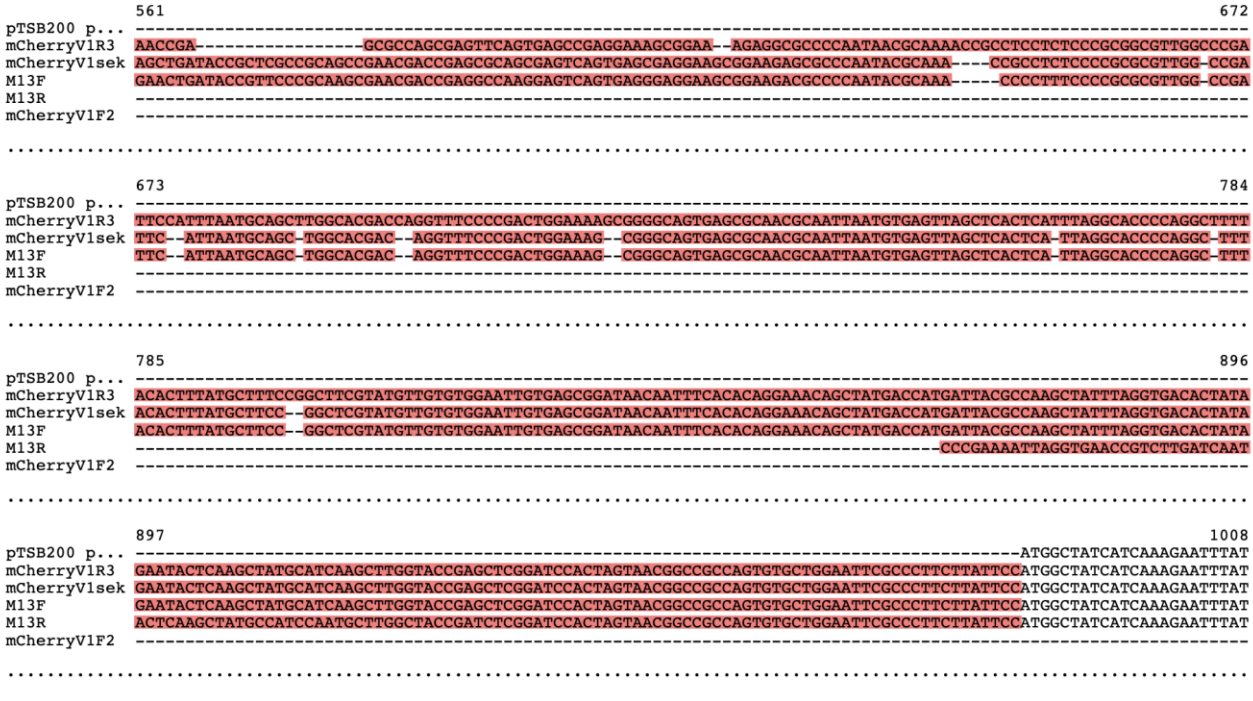
```
          2801                                                                                                2912
pTSB402   GGCCTTTTCACCGATATACAGCAGTTTATCTTCACTGCCGTCAGTGGTGGACCAGCTGTGCCCGACGAAAGAGCAATGGTCAATCCAATACTTGCTGCCGTAGTTCAAGTAG
iscsek 3  ----------------------------------------------------------------------------------------------------------------
iscsek 2  ----------------------------------------------------------------------------------------------------------------
M13R      ----------------------------------------------------------------------------------------------------------------
iscsek 1  ----------------------------------------------------------------------------------------------------------------
M13F      GGCCTTTTCACCGATATACAGCAGTTTATCTTCACTGCCGTCAGTGGTGGACCAGCTGTGCCCGACGAAAGAGCAATGGTCAATCCAATACTTGCTGCCGTAGTTCAAGTAG

..................................................................................................................................

          2913                                                                                                3024
pTSB402   ACCTGGATATCGTCATTGGCCTTGATACTTGCCGAATGCTTGAAGATAAGATTCTGCAGAATAATGTTCTGCGACTGCGCCGTTGCACGCAGGTGAATATTCTCCAGGGTGC
iscsek 3  ----------------------------------------------------------------------------------------------------------------
iscsek 2  ----------------------------------------------------------------------------------------------------------------
M13R      ----------------------------------------------------------------------------------------------------------------
iscsek 1  ----------------------------------------------------------------------------------------------------------------
M13F      ACCTGGATATCGTCATTGGCCTTGATACTTGCCGAATGCTTGAAGATAAGATTCTGCAGAATAATGTTCTGCGACTGCGCCGTTGCACGCAGGTGAATATTCTCCAGGGTGC

..................................................................................................................................

          3025                                                                                                3136
pTSB402   GGTTCTGGAACGAGCCAATCAGGGTCTTGTTGGCGCCCATGTTGACTTTGGTCAGGCTCGACGCCGAGATATTACTGTTGATCACCAGTACCCGTGGGGTGGTGTCGCCGAT
iscsek 3  ----------------------------------------------------------------------------------------------------------------
iscsek 2  ----------------------------------------------------------------------------------------------------------------
M13R      ----------------------------------------------------------------------------------------------------------------
iscsek 1  ----------------------------------------------------------------------------------------------------------------
M13F      GGTTCTGGAACGAGCCAATCAGGGTCTTGTTGGCGCCCATGTTGACTTTGGTCAGGCTCGACGCCGAGATATTACTGTTGATCACCAGTACCCGTGGGGTGGTGTCGCCGAT

..................................................................................................................................

          3137                                                                                                3248
pTSB402   ATTGGCCTTGAGTTGATCGAGTGTGGTGATACTGACCACAGGGCCGGACCAGCCACCTGTCACTTTTGCCGCTTGGGCGAAACCGATCAAACCGGTGAGTTTGCTTTCTGGA
iscsek 3  ----------------------------------------------------------------------------------------------------------------
iscsek 2  ----------------------------------------------------------------------------------------------------------------
M13R      ----------------------------------------------------------------------------------------------------------------
iscsek 1  ----------------------------------------------------------------------------------------------------------------
M13F      ATTGGCCTTGAGTTGATCGAGTGTGGTGATACTGACCACAGGGCCGGACCAGCCACCTGTCACTTTTGCCGCTTGGGCGAAACCGATCAAACCGGTGAGTTTGCTTTCTGGA

..................................................................................................................................

          3249                                                                                                3360
pTSB402   TAGGACATGAGTTCCCTCTCTATCAATAAAATGGCTCGGTAAGACGAGCCTGTGCATCCTAGGTAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCAT
iscsek 3  ----------------------------------------------------------------------------------------------------------------
iscsek 2  ----------------------------------------------------------------------------------------------------------------
M13R      ----------------------------------------------------------------------------------------------------------------
iscsek 1  ----------------------------------------------------------------------------------------------------------------
M13F      TAGGACATGAGTTCCCTCTCTATCAATAAAATGGCTCGGTAAGACGAGCCTGTGCATCCTAGGTAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCAT

..................................................................................................................................
```

I. Sequencing results, pTSB200

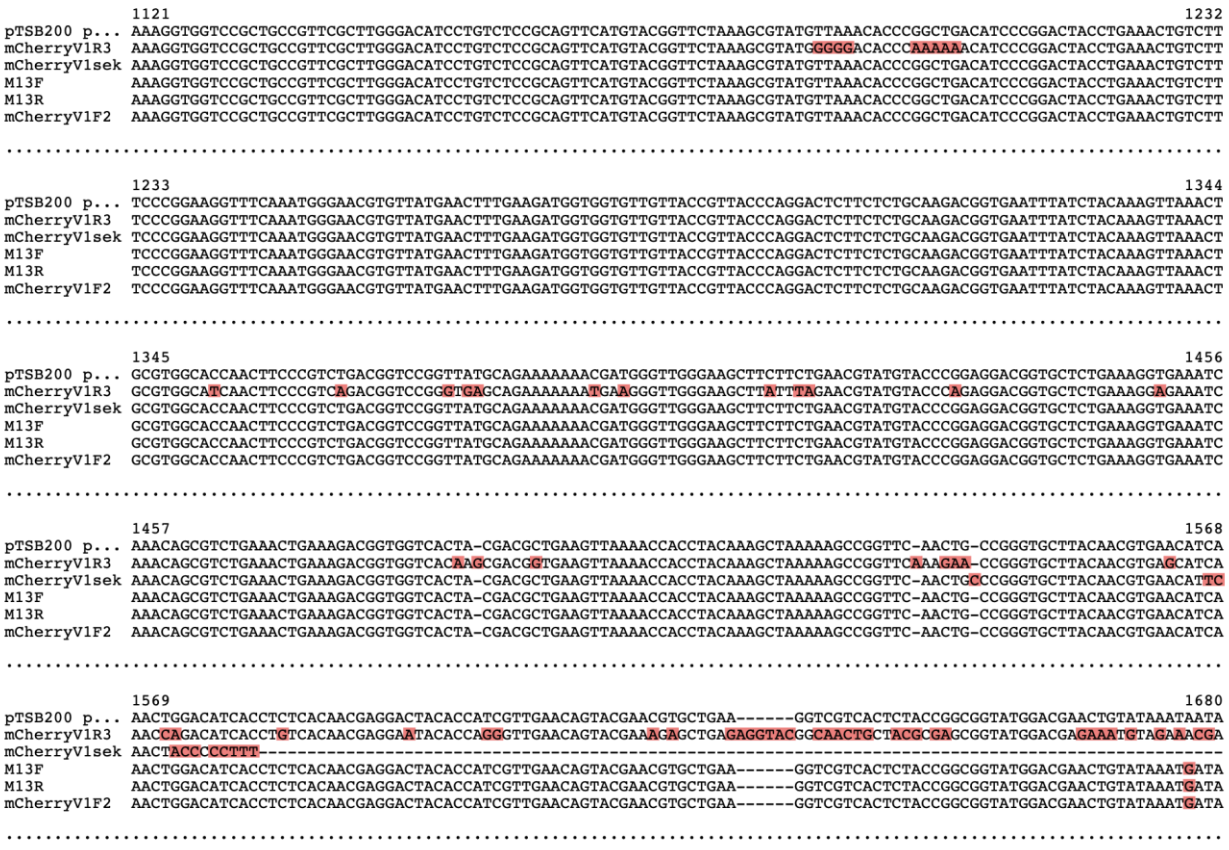
Sequencing results of sequencing pTSB200. The first line shows the template DNA (pTSB200) The next show the sequencing results when using primers mCherryV1F2, M13R, M13F, mCherryV1R3, mCherryV1sek. As seen from line starting with nucleotdise 1681 (Page 4), the sequence that looked like it was removed from the initial sequence was the terminator sequence from RK2-mCherry_V1.



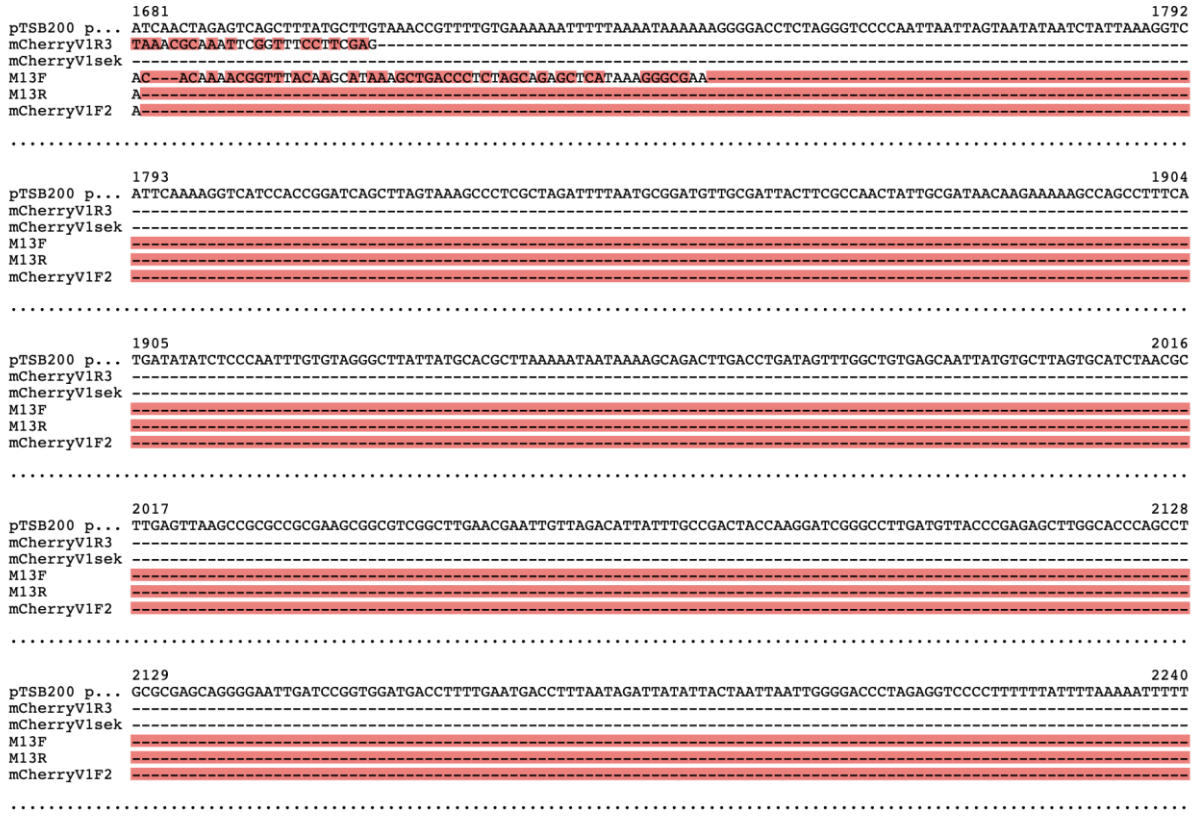
Page 2 pTSB200 predicted



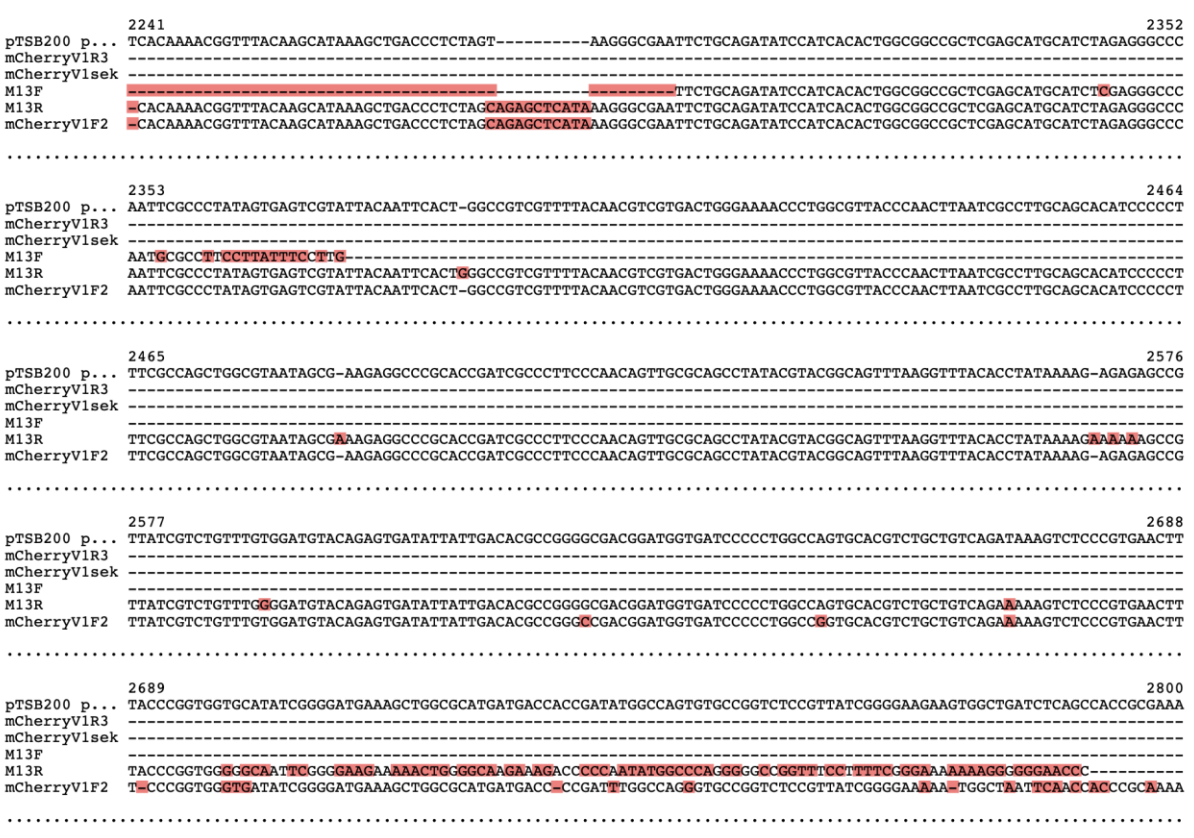
Page 3 pTSB200 predicted



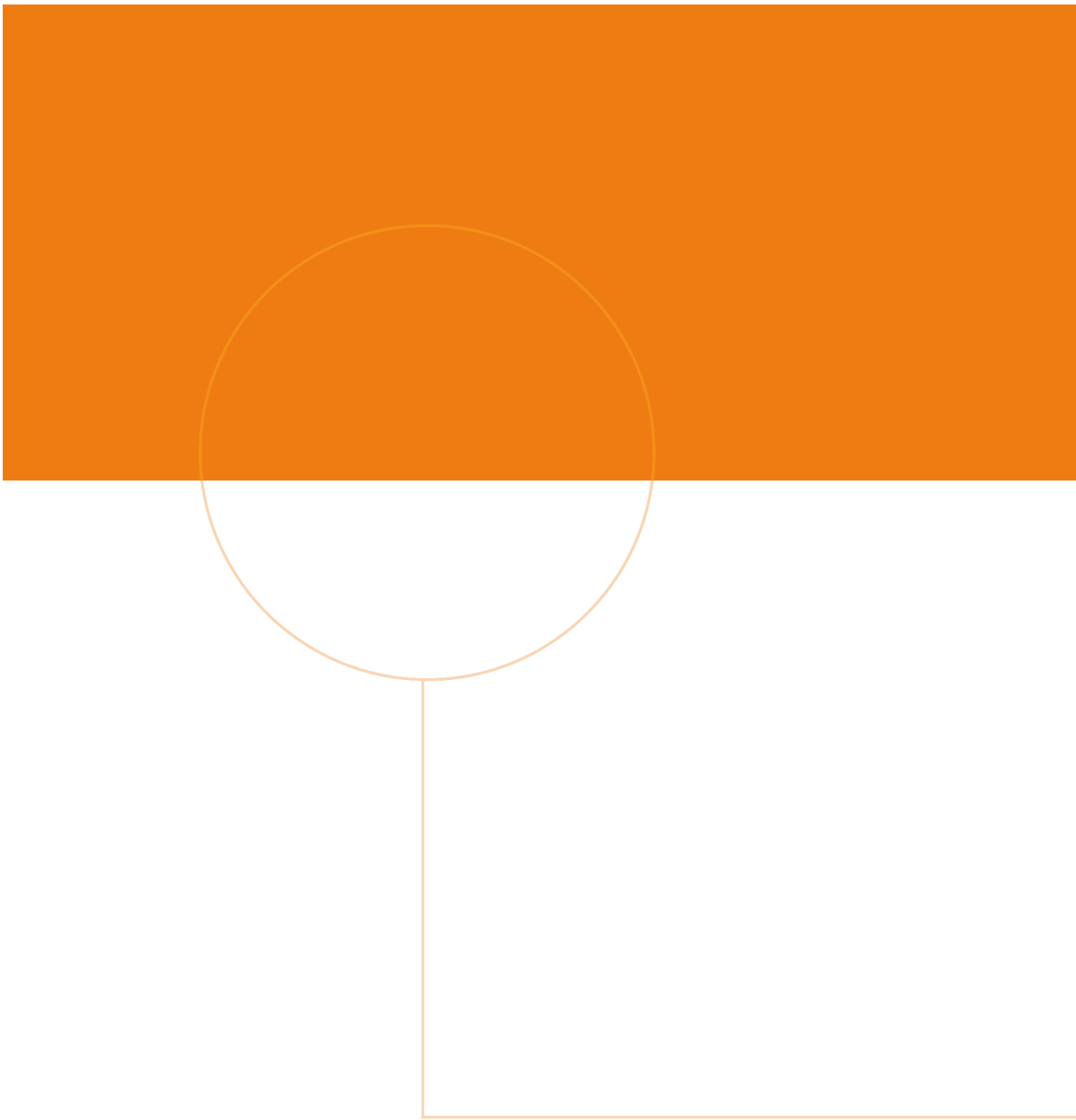
Page 4 pTSB200 predicted



Page 5 pTSB200 predicted



```
            2801                                                                                                 2912
pTSB200 p... ATGACATCAAAAACGCCATTAACCTGATGTTCTGGGGAATATAAATGTCAGGCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTCACGTAGAAAGCCAGTCCGCA
mCherryV1R3  ----------------------------------------------------------------------------------------------------------------
mCherryV1sek ----------------------------------------------------------------------------------------------------------------
M13F         ----------------------------------------------------------------------------------------------------------------
M13R         ----------------------------------------------------------------------------------------------------------------
mCherryV1F2  TGACTTCAAAACGCCTTTACCTGATTTCTGGGGAATTAAAGGTCGGGAGAAATTTAAAAAAGAATTCTCCTAAATCTTTTTCCCGAAAAAACCCTCCCGAAAAAGGGTTGC

.....

            2913                                                                                                 3024
pTSB200 p... GAAACGGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTA
mCherryV1R3  ----------------------------------------------------------------------------------------------------------------
mCherryV1sek ----------------------------------------------------------------------------------------------------------------
M13F         ----------------------------------------------------------------------------------------------------------------
M13R         ----------------------------------------------------------------------------------------------------------------
mCherryV1F2  CCCGGAAGAATTTATTTTTTGGGTATATTGGAAAAAGGAAAAAACACAACCCAAAAAAAAAAGGGTATTTTGGGGGCTTTTTTGGGAATATATTAATGGGTGTTTTTTAAAA

.....

            3025                                                                                                 3136
pTSB200 p... GACTGGGCGGTTTTATGGACAGCAAGCGAACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTCGCCGCCAAGGA
mCherryV1R3  ----------------------------------------------------------------------------------------------------------------
mCherryV1sek ----------------------------------------------------------------------------------------------------------------
M13F         ----------------------------------------------------------------------------------------------------------------
M13R         ----------------------------------------------------------------------------------------------------------------
mCherryV1F2  CCAACAAACAATATTTCCTGGGCCCGCCCTCGAGGGGGAAAACCCCAAAAAAAAGGTGTTTTTTGCCCCAAATTGTGAGGCGGGAATATTTAAAAAAAAAGAAAATATTTTCT

.....

            3137                                                                                                 3248
pTSB200 p... TCTGATGGCGCAGGGGATCAAGCTCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCT
mCherryV1R3  ----------------------------------------------------------------------------------------------------------------
mCherryV1sek ----------------------------------------------------------------------------------------------------------------
M13F         ----------------------------------------------------------------------------------------------------------------
M13R         ----------------------------------------------------------------------------------------------------------------
mCherryV1F2  GTCTAATAAAGATTACTCGCCCTTCCCCGTGGGAGGAGATATAAATAAAAAAACATTTGTCGCGCGCGTGTGGGCGGCTTTTTTTTATCGCGGAAGAAAGAGGTGTTTTTCT

.....

            3249                                                                                                 3360
pTSB200 p... ATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCC
mCherryV1R3  ----------------------------------------------------------------------------------------------------------------
mCherryV1sek ----------------------------------------------------------------------------------------------------------------
M13F         ----------------------------------------------------------------------------------------------------------------
M13R         ----------------------------------------------------------------------------------------------------------------
mCherryV1F2  TTT-------------------------------------------------------------------------------------------------------------

.....
```



NTNU

Norwegian University of
Science and Technology