Master's thesis

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Reporter genes for relative quantification of promoter strength and c-di-GMP in *Pseudomonas fluorescens*

Master's thesis in Biotechnology
Supervisor: Helga Ertesvåg
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Preface

This thesis was completed from January 2020 to May 2021 at the Department of Biotechnology and Food Science at NTNU Trondheim with Professor Helga Ertesvåg as supervisor.

First and foremost, I would like to offer my sincere thanks to Professor Helga Ertesvåg for the help and guidance I have received throughout the writing of this thesis, and for always being available whenever I had questions. In addition, I would like to thank David Zamorano-Sánchez and his research group for letting me use the plasmid pFY4535 in my experiments.

I would also like to thank my family for encouragement and support, as well as my friends and fellow students in the lab, for great conversations and good advice.

Trondheim, May 2021

Susanna Vold

Summary

Alginate is an industrially important polymer composed of the two monomers mannuronic and guluronic acid. The polymer can be used in drug delivery systems, wound healing and possibly in tissue regeneration. Alginate is mainly derived from brown algae, where it serves as a structure-forming component. However, in recent years, several studies on the production of alginate by bacteria like *Pseudomonas* and *Azotobacter* has been performed. The goal is to be able to modify and tailor the bacterial alginate to the industry's needs. This study aims to contribute to this by using reporter genes to study the strength of promoters, which could be used to create a mutant that in turn could be used to control alginate production tightly. Reporter genes could also be used to evaluate a method for relative quantification of c-di-GMP in *P. fluorescens.*

In the experimental part of this project, a reporter gene assay was carried out as a tool in order to investigate different promoters. Reporter genes tested were RFP, CFP, YFP and GFP. The promoters investigated were P_{BAD}, P_{trc}, P_m, P_{mG5} and P_m ML1- 17. P_{trc}, which is induced by IPTG, was found to be transcribed in the stationary phase. An alginate-producing strain of *P. fluorescens* SBW25 with this promoter in front of *algC*, a gene important for synthesis of the precursor in the biosynthesis pathway, was constructed through homologous recombination. The concentration of alginate was measured in this strain in cultures without IPTG, and with addition of IPTG at different time points. No conclusions could be drawn from this measurement and it was recommended to repeat the assay. However, it was observed that induction of P_{trc} with IPTG may not have had the desired effect.

A system for measuring c-di-GMP, important for regulation of the biosynthesis, was tested in *P. fluorescens*. The results from this study indicated that it likely functions well in *P. fluorescens* SBW25. However, a MucR-overproducing strain would be necessary to confirm this as it would serve as a positive control considering MucR is a c-di-GMP synthesizing enzyme. Attempts at making such a strain were unsuccessful due to problems with cloning. It was discovered that the PFP2 promoter which regulates the fluorescent genes in the c-di-GMP reporter system, was also transcribed in the stationary phase. A possible correlation between P_{trc} and PFP2 was recommended to explore in further studies.

Sammendrag

Alginat er en industrielt viktig polymer som består av de to monomerene mannuronsyre og guluronsyre. Denne polymeren kan brukes til å transportere legemidler inn i kroppen, sårheling og muligens i vevsregenerering. Alginat kommer hovedsakelig fra brunalger, der det fungerer som en strukturell komponent. I senere år har det blir gjort flere studier på produksjon av alginat av bakterier fra arter som *Pseudomonas* og *Azotobacter.* Målet er å kunne modifisere og skreddersy alginatet som bakteriene produserer slik at det oppfyller kravene industrien har til alginatet. Målet med denne oppgaven var å lage en mutant som kunne brukes til å kontrollere alginatproduksjonen i *Pseudomonas fluorecens.* Reportergen var et viktig verktøy og ble også brukt til å evaluere en metode for relativ kvantifisering av c-di-GMP i *P. fluorescens.*

I den eksperimentelle delen av prosjektet ble det utført en reporter gen studie som et verktøy i vurderingen av promotorstyrke. Reportergen som ble testet var RFP, CFP, YFP og GFP. Promotorene som ble undersøkt var P_{BAD}, P_{trc}, P_m, P_{mG5} og P_m ML1- 17. Den IPTG-induserbare promotoren P_{trc}, viste seg å også bli transkribert i stasjonærfasen. En alginatproduserende stamme av *P. fluorescens* med denne promotoren foran *algC*, et gen viktig for dannelsen av forløperen i alginat biosyntesen, ble konstruert gjennom homolog rekombinering. Alginatkonsentrasjonen i den nye stammen ble målt i kulturer der P_{trc} både var uindusert, og indusert til ulike tidspunkt. Det ble konkludert med at målingen burde gjentas på grunn av uforventede resultat. Likevel ble det observert at induksjon med IPTG muligens ikke hadde den ønskede effekten.

Et system for å måle c-di-GMP, et molekyl viktig for regulering av biosyntesen av alginat, ble testet i *P. fluorescens*. Resultatene viste at det sannsynligvis fungerer bra, men at en stamme som overproduserer MucR vil være viktig for å verifisere resultatene ettersom MucR er et c-di-GMP produserende enzym og vil da fungere som en positiv kontroll. En slik stamme ble ikke laget på grunn av problemer i kloningsprosessen. Det ble oppdaget at promotoren PFP2, som regulerer de fluorescerende genene i c-di-GMP reportersystemet, også transkriberes i stasjonærfasen. En mulig korrelasjon mellom denne og P_{trc} ble anbefalt å undersøke videre.

List of abbreviations

Amp	Ampicillin
Km	Kanamycin
Tc	Tetracycline
Gm	Gentamycin
Apr	Apramycin
Tric	Triclosan
GFP	Green fluorescent protein
YFP	Yellow fluorescent protein
CFP	Cyan fluorescent protein
RFP	Red fluorescent protein
OD	Optical density
Bp	Base pair
DNA	Deoxyribonucleic acid
SLIC	Sequence- and ligation-independent cloning
PCR	Polymerase chain reaction
DMSO	Dimethyl sulfoxide
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
DGC	Diguanylate cyclase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
PIA	Pseudomonas Isolation Agar
PIM	Pseudomonas Isolation Medium
LB	Lysogeny broth
SDS	Sodium dodecyl sulfate
dNTPS	Deoxyribonucleotides
ddNTPs	Dideoxyribonucleotides

Contents

Preface 1

Summary 2

Sammendrag 3

List of abbreviations 4

1. Aims 7

2. Introduction 9

2.*1 Pseudomonas fluorescens* 9
2.2 Alginate and its biosynthesis 9
2.2.1 Alginate biosynthesis 10
2.2.2 MucR and its regulation of alginate biosynthesis 12
2.2.3 Quantification of c-di-GMP 13
2.2.4 Regulation of the alginate operon through AlgU and MucA 14
2.3 Gene expression and promoters 15
2.3.1 Regulation of gene expression 15
2.3.2 Promoter systems 16
2.4 Transposons and their use in genetic engineering 18
2.5 Characterization of reporter genes used in this study 18

3. Materials and Methods 20

3.1 Media and solutions 20
3.2 Growth of bacteria and bacterial strains and plasmids used in this study 22
3.3 Analysis of reporter genes 24
3.4 Plasmid isolation 25
3.5 Measure concentration of DNA with Nanodrop 26
3.6 Restriction cutting of plasmids 26
3.7 Agarose gel electrophoresis 27
3.8 Purification of DNA restriction fragments from gel 28
3.9 Ligation 28
3.10 Making competent cells and transformation 29
3.11 PCR 30
3.12 Topo cloning 30
3.13 One step sequence- and ligation- independent cloning (SLIC) 31
3.14 Conjugation 32
3.15 Homologous recombination for gene replacement 33
3.16 Sequencing 34
3.17 Alginate assay 35
3.18 Calculation of standard deviation 36
3.19 Bioinformatics tools 36
3.19.1 Programs used to simulate cloning 36
3.19.2 Programs used to visualize sequencing results 37

4. Results 38

4.1 Evaluation of reporter genes 38
4.1.1. Construction of plasmid pSV1 39
4.1.2 Measurement of reporter genes 39
4.2 Evaluation of the strength of different promoters in *P. fluorescens* 43
4.2.1 Construction of vectors with promoters and RFP 43
4.2.2 Measurement of promoter strength using RFP as a reporter 47
4.2.3 New measurement to investigate a possible stationary phase promoter 50
4.4 Test of a c-di-GMP reporter system in *P. fluorescens* 52
4.3 Cloning of MucR 55
4.3.1 Clone mucR into TOPO-vector and partial digestion to insert mucR 56

4.3.2 Construction of transposon vector with the P_{trc} promoter 57
4.3.3 Construction of transposons with mucR through one-step SLIC 57
4.5 Studies on the effect of a stationary phase promoter on *algC* and this system´s effect on alginate production........ 58
4.5.1 Construction of vector with algC under control of the P_{trc} promoter 59
4.5.2 Construction of P. fluorescens SBW25 mucA strain with algc under control of P_{trc}. 61
4.5.3 Measurement of alginate production by P. fluorescens SBW25 mucA Ptrc algC 63

5. Discussion 67
5.1 Reporter gene study 67
5.2 Promoter study 68
5.3 Cloning of *mucR* 69
5.4 Testing a c-di-GMP reporter system in *P. fluorescens* 70
5.5 Study effect of stationary phase promoter-regulated *algCs* effect on alginate production 71
5.6 Further work 72

6. Conclusion 74

References 75

Appendix 81
Appendix A – Map of plasmids constructed in this study 81
Appendix B – DNA ladders 83
Appendix C – PCR program 84
Appendix D – Primers 85
Appendix E – Gel image of colonies after homologous recombination 86
Appendix F – Raw data from the promoter study 87
Appendix G – Raw data from the measurement of AmCyan and TurboRFP 89
Appendix H – Alginate assay data and calculations 91
Appendix I – Sequencing of pSV6 93
Appendix J – Sequencing of pSV7 95

1. Aims

The biopolymer alginate has many pharmaceutical, biomedical and industrial applications, and the control of its production would be favorable. Alginate is normally produced by brown seaweeds, but the bacteria belonging to the genera *Pseudomonas* and *Azotobacter* are also able to produce it. By gaining a wider understanding of the biosynthesis of alginate in these bacteria, it could become easier to control the production, tailor and modify the structure, and make it more applicable in the industries mentioned above. This study aims to take a closer look upon genes important for alginate biosynthesis, in order to create mutants where alginate production could be controlled tightly. Another goal was to find a promoter whose transcription could be turned on in the stationary phase. *algC,* a gene necessary for synthesis of the precursor GDP-mannuronic acid in the alginate biosynthesis pathway (1), has previously been put under control of the inducible P_m promoter. However, this was not optimal as P_m is turned off in the stationary phase (Ertesvåg, personal communication). Some long-term goals would be to be able to regulate alginate production in such a way that it is not activated until optimal growth rate is achieved, as alginate production reduces cell growth. Other long-term goals would be to have the production independent on regulation from the host, as well as to be able to control alginate production in the stationary phase.

Part 1

Before making the mutant, a promoter study would be necessary to perform. As a tool in the promoter study, a reporter gene study would be carried out to investigate which reporter genes were easiest to measure in *P. fluorescens.* This would be done by constructing plasmids with different reporter genes, transfer them into *P. fluorescens* and measure their fluorescence and optical density. The results from this measurement would then be used to study promoters, with the goal of finding a stationary phase promoter. Finding a strong promoter with a low uninduced level that is also transcribed in the stationary phase would be good for studying different gene´s influences on alginate synthesis in this phase. By using the reporter gene found easiest to measure, plasmids with different promoters could be constructed and fluorescence and optical density would be measured. If a stationary phase promoter was found, this could be used to see if *algC* could be regulated, and to study its effect on alginate biosynthesis. Homologous recombination would be used to insert this promoter into the genome of an alginate-producing strain of *P. fluorescens.* The last step would be to measure alginate.

Part 2

The gene *mucR* codes for a diguanylate cyclase, enzymes that increase levels of c-di-GMP. The alginate biosynthesis is dependent on c-di-GMP (2). By using promoters of different strengths, it would be possible to study the expression of the *mucR* gene and its influence on alginate production in *P. fluorescens. mucR* has an internal NdeI-site, so partial digestion or SLIC were methods that could be used to overcome this problem. Transposon mutagenesis in an alginate-producing strain of *P. fluorescens* would be used to construct a strain that overproduced MucR. A method for measurement of c-di-GMP would also be investigated. The c-di-GMP reporter system constructed by Zhou et al. (3) would be tested and evaluated to see if this system was possible to use in *P. fluorescens.* This reporter system could be used to find out if there was a measurable difference in c-di-GMP levels in *P. fluorescens* SBW25 *mucA* and *P. fluorescens SBW25*. Also, if a MucR-overproducing strain was made, it could be used as a positive control to verify that the c-di-GMP reporter system could be used in *P. fluorescens*.

2. Introduction

2.1 *Pseudomonas fluorescens*

P. fluorescens are nonpathogenic, gram negative motile rods. The bacteria releases a pigment called pyoverdine, which fluoresces yellow-green under certain conditions, notably when there is little iron available to the bacteria. When iron concentration is low, the siderophore pyoverdine is synthesized as this pigment can chelate iron. This is then transferred to the bacterial cell, where ferric iron is reduced to ferrous iron (4). *P. fluorescens* is obligate aerobe, although some strains can use nitrate as final electron acceptor. The bacterium can be found in both soil and water. It can easily be cultured in the lab by supplying the media with a wide range of carbon sources as well as mineral salts. *P. fluorescens* is a rhizobacterium, meaning it can grow on roots and live in symbiosis with many plants (5). Although regarded as nonpathogenic, some strains of *P. fluorescens* can cause opportunistic infections, such as in the bloodstream of humans with a compromised immune system (6). The strain SBW25, which was first isolated from the leaf surface of a sugar beet plant, contains 6 009 coding sequences and has become an important model organism (7). *P. fluorescens* has the ability to produce alginate. However the wild-type strains, like SBW25, does not produce alginate under laboratory conditions (1).

2.2 Alginate and its biosynthesis

The naturally occurring polymer alginate has unique properties and versatile applications. It is non-toxic, biocompatible, relatively cheap to produce and can form hydrogels with divalent cations like Ca^{2+}. It is derived from brown algae and bacteria like *Pseudomonas* and *Azotobacter.* Alginate has applications in multiple industries, including pharmaceutical, medical, agriculture and food industries. Future possibilities could include using alginate in drug delivery systems, tissue engineering, tumor studies and wound healing. Alginate consists of two monomers: β-D-mannuronic acid (M) and α-L guluronic acid (G) residues in different ratios linked together with 1,4-linkages. However, alginate produced by *Pseudomonas* species has been found not to contain stretches of G-residues, only M- and MG-residues. Long stretches of G-residues enable the alginate to form gels by binding divalent cations, like Ca^{2} , in a structure that resembles an egg box (8).

In *Azotobacter*, alginate is involved in the encystment process and enables the bacteria to survive unfavorable conditions (8). In *Pseudomonas aeruginosa,* alginate production has a

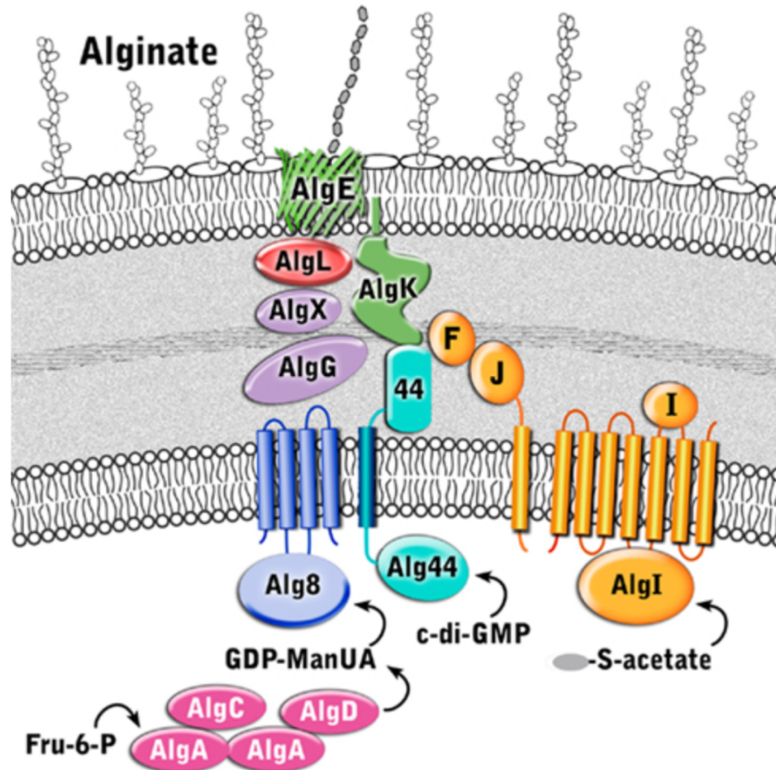
key role in its infection of cystic fibrosis patients. *P. aeruginosa* can switch from a non-mucoid to a mucoid, alginate-producing phenotype as the disease progresses. This is an advantage as the alginate protects the bacterium from antimicrobial agents, the host´s immune system and dehydration (9). The alginate produced by seaweeds have different compositions, depending on the season, growth conditions, species and geographical location. Alginate´s role in seaweed is to confer structure, mechanical strength and flexibility. The amount of G-residues they contain depends on the environment they inhabit. Bacteria on the other hand, can be used to produce alginate with more tailored properties and structure. The bacterial alginates acetylation degree, molecular weight and ratio of M- and G-residues are properties that can be modified (8).

2.2.1 Alginate biosynthesis

In Pseudomonas, the essential genes in alginate biosynthesis are *algC, algD, alg8, alg44, algK, algE, algG, algX, algL, algI, algJ, algF* and *algA* (8). These genes, except for *algC*, comprises the alginate operon, shown in Figure 1. This figure also shows an overview of how the proteins these genes encode, together with regulators like c-di-GMP, are arranged to form the membrane-spanning alginate biosynthesis complex.

The *algC* gene encodes a phosphomannomutase, an enzyme responsible for the conversion of mannose-6-phosphate to mannose-1-phosphate. This is one of the four steps in the precursor synthesis (10). However, the *algC* gene product also have phosphoglucomutase activity, which is related to the production of lipopolysaccharides and rhamnolipids (11). For this reason, AlgC is vital for the alginate production and the gene is transcribed from its own promoter.

1A



1B

Figure 1: Overview of the proteins in the alginate biosynthesis complex (12) and the genes in the alginate operon. *Figure 1A shows the alginate biosynthesis complex which spans the membrane and consists of 13 proteins and regulators. The figure shows the cytoplasmic membrane and the outer membrane. Figure 1B shows the genes in the alginate operon. The genes coloured blue comprises the alginate operon and are under the control of a promoter upstream of algD.*

The first step in alginate production is the synthesis of the precursor GDP-mannuronic acid. AlgC, AlgD and AlgA catalyzes the conversion of fructose-6-phosphate into the precursor. The first and third step is catalyzed by AlgA, the second by AlgC and the fourth and final step by AlgD. These four steps take place in the cytosol (13). Following precursor synthesis, the alginate is polymerized by Alg8 and Alg44. The second messenger c-di-GMP is an important regulator of alginate polymerization. Upon binding of c-di-GMP to the PilZ domain of Alg44, alginate polymerization is activated. MucR is a c-di-GMP synthesizing protein and has been shown to influence alginate production in *P. aeruginosa.* The developing alginate chain is then translocated across the membrane by a multi-protein complex consisting of AlgX, AlgG and AlgK. These proteins also protects the alginate from degradation by AlgL – an alginate lyase. During this transport, the alginate can be modified by epimerization and acetylation. Neither are necessary for alginate production but these modifications can have an effect on

the produced alginate. O-acetylation, unique to bacterial alginates, can increase the water holding capacity and is important for biofilm production in *Pseudomonads*. While it is AlgX that adds O-acetyl ester linkages to the M residues, AlgI, AlgJ and AlgF are also important for O-acetylation (8). In *P. aeruginosa*, O-acetylation is involved in the protection of the bacteria from the hosts immune responses (14). The other way of modifying the alginate chain – epimerization – is catalyzed by AlgG and involves turning M-residues into G-residues. Lastly, the alginate is secreted across the outer membrane through the AlgE protein, which forms a selective channel that transports the alginate (8)

2.2.2 MucR and its regulation of alginate biosynthesis

As mentioned in the previous section, the diguanylate cyclase MucR, is a c-di-GMP synthesizing enzyme and an important regulator in the biosynthesis of alginate. MucR consists of two domains (EAL and GGDEF), as well as an inner membrane sensor domain (MHYT). The two domains are either involved in c-di-GMP synthesis (EAL) or degradation (GGDEF). The sensor part of the protein is thought to bind a copper atom which can then sense nitric oxide (NO), carbon oxide (CO) or oxygen (O_2). Wang et al. (15) showed that MucR can sense nitrate, resulting in a suppressed alginate production. In addition, it was shown that both the EAL and GGDEF domains are necessary for alginate production as inactivation of either of the domains led to a reduced production. A study characterizing MucR showed that the deletion of the *mucR* gene in an alginate-overproducing strain of P. *aeruginosa,* led to a highly reduced alginate production (16). These findings suggested that MucR regulates alginate biosynthesis in a positive manner, by forming a localized c-di-GMP pool near Alg44, which exists in the cytoplasmic membrane. Figure 2 shows the location of MucR in the membrane and its regulation of alginate biosynthesis.

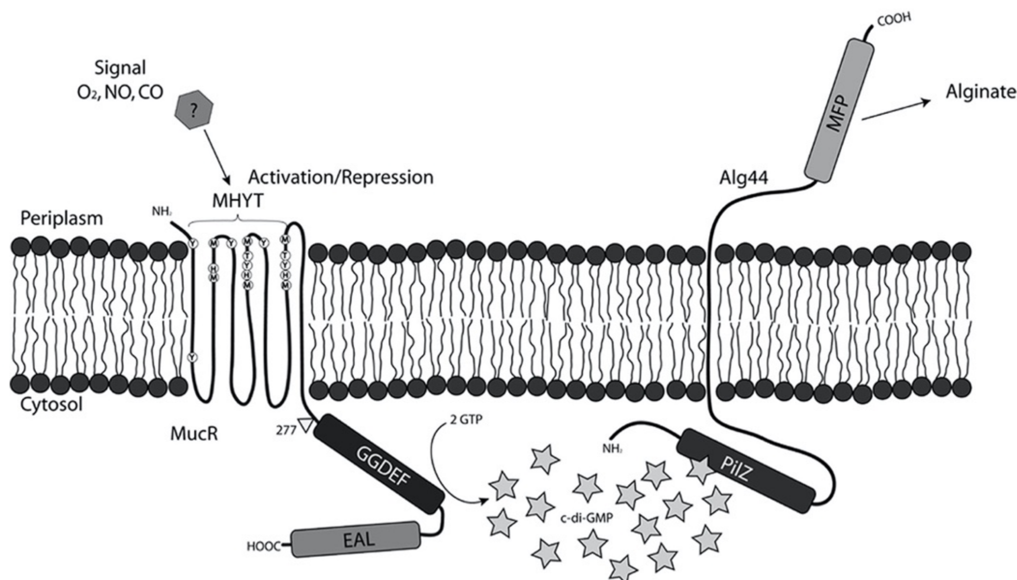


Figure 2: Proposed model showing how MucR regulates the biosynthesis of alginate in P. aeruginosa (16). *MucR consists of an internal membrane domain (MHYT) and to cytosolic domains (GGDEF and EAL). The two cytosolic domains are involved in the degradation and synthesis of c-di-GMP.*

2.2.3 Quantification of c-di-GMP

c-di-GMP is a second messenger involved in processes like biofilm formation, virulence and cell to cell signaling. It is also involved in the regulation in alginate production through binding to Alg44. The measurement of c-di-GMP could therefore provide valuable information about alginate production in *P. fluorescens.* To easily detect c-di-GMP levels, a fluorescent c-di-GMP reporter system has been constructed by Zhou et al. (3). This system is based on c-di-GMP riboswitches, which can bind c-di-GMP and change the expression of downstream genes. Riboswitches exists in the 5´-untranslated region of mRNAs and are able to regulate downstream genes on the same molecule upon binding of a small ligand. Molecules that can act as ligands include metal ions, sugars, anions and nucleotides. Riboswitches are occasionally arranged in tandem. These have been found to require a smaller change in the ligand concentration but still result in a considerably higher gene expression. The system constructed by Zhou et al. (3) consists of a triple-tandem c-di-GMP riboswitch (Bc3-5 RNA) which was found to control expression of the reporter genes more closely. This system was originally discovered in *Bacillus thuringiensis* subsp. *chinensis* CT-43. The triple tandem riboswitch was fused between the fluorescent genes *amcyan* and *turboRFP,* which are in an operon together, to enable measurement of c-di-GMP. When there is no c-di-GMP available, *turboRFP* is not expressed. As the concentration of c-di-GMP

increases, the expression of *turboRPP* is activated. The other gene in the operon, *amcyan*, is used as a normalizer since is it expressed constitutively and can therefore be used to ensure that the non-biological variation is as small as possible. The fluorescence of TurboRFP per OD_{600} divided on the fluorescence of AmCyan represents the relative fluorescence intensity (RFI), and is directly proportional to the c-di-GMP levels in the cell (3). The plasmid containing the reporter system is shown in Figure 3.

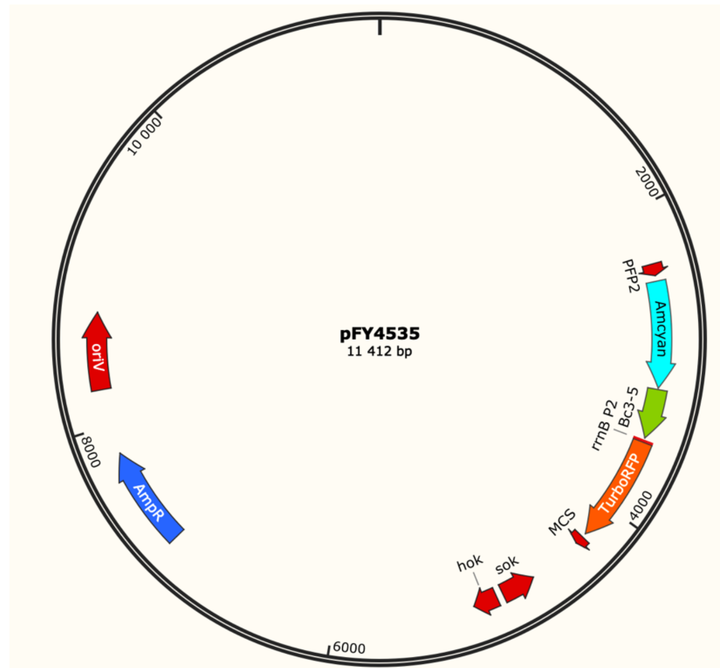


Figure 3: Plasmid map of pFY4535 (17)*. TurboRFP is coloured orange, AmCyan is coloured blue and the c-di-GMP biosensor is coloured green. The PFP2 and rrnB P2 promoters are coloured red.*

2.2.4 Regulation of the alginate operon through AlgU and MucA

The *algU* gene is an important regulator of the *algD* operon and it encodes a sigma factor (σ^{22}) (18). Sigma factors are responsible for guiding the RNA polymerase to the promoter. RNA polymerase core enzyme bound to a sigma factor is called RNA polymerase holoenzyme, and it is this enzyme that can start transcription by binding to a promoter. In bacteria, there is one sigma factor that dominates – the housekeeping sigma factor. This guides the polymerase to the majority of promoters, while alternative sigma factors can guide it to other promoters. However, since the housekeeping sigma factor normally dominates, the concentration of the alternative sigma factor has to be significantly increased for it to be able to compete with the housekeeping sigma factor (19).

The *algU* operon consists of *algU-mucA-mucB-mucC-mucD.* MucA is an anti-sigma factor which can bind to and prevent AlgU from binding to the RNA polymerase and thereby also

from activating several genes necessary for alginate production. When MucA is degraded, AlgU is released from the inner membrane into the cytosol and alginate will be overproduced. Protelysis of MucA is prevented by the binding of MucB to MucA. It is believed that MucB can sense environmental signals and transduce them to MucA (18). When envelope stress is detected, MucB dissociates from MucA and makes it available for degradation by the proteases AlgW and MucP (20). A mutated MucA in *P. aeruginosa* will often result in a conversion to a mucoid phenotype as a result of alginate overproduction. This is beneficial for the bacteria as the biofilms produced are more resistant to phagocytosis as well as antibiotic treatment. As a result, an infection with this mutant phenotype in cystic fibrosis patients is associated with a poor disease prognosis (20). As mentioned earlier, the wild type strain *P. fluorescens* SBW25, cannot produce alginate under laboratory conditions. However, by introducing a mutation in the *mucA* gene, alginate production can be activated

2.3 Gene expression and promoters

A gene is expressed when the RNA polymerase binds to the promoter and initiates transcription. A heterologous host is an organism which expresses a protein that it does not naturally have. With the help of recombinant DNA technology, a gene of interest can be cloned into and expressed in a host organism. This is called heterologous expression. *E. coli* is a commonly used heterologous expression system for producing recombinant proteins as it has a short generation time and simple nutrimental demands (21). Homologous expression refers to the over-expression of a gene naturally found in the host, through a promoter. Overexpression of wild-type genes can lead to mutant phenotypes. By overexpressing genes in a heterologous host, the genes function can be studied.

2.3.1 Regulation of gene expression

Genes can be expressed constitutively, inducible or repressible. Constitutive genes are always expressed and include genes with essential functions. Other genes, whose products are only needed under certain conditions, are inducible, meaning their expression can be turned on and off. Regulator genes encode activators and repressors. These bind to operators, which are specific binding sites on the DNA. Whether or not binding occurs is dependent on inducers and co-repressors, which are called effector molecules. When a gene is repressed, often because its product is in excess or not needed anymore, its expression is turned off. Conversely, when a gene product is needed, the expression of the gene is turned on.

Positive and negative control mechanisms is involved when the expression of a gene is regulated. Negative regulation involves repressors and positive regulation involves activators. In either case, transcription may be increased or decreased depending on which effector molecule is involved. For negative regulation, a repressor bound to a co-repressor enables it to either increase or decrease transcription. This depends on whether the conformational change that occurs upon binding between the two molecules causes the repressor to dissociate from the operator or bind to it. Activators on the other hand can increase transcription by promoting the RNA polymerase to bind to the promoter. Transcription can also be decreased if the activator receives a signal that causes is to dissociate from the polymerase (22).

2.3.2 Promoter systems

Promoter systems can function differently in different bacterial species, possibly because the RNA polymerase does not bind identical in all bacterial species. It could also depend on the expression of the regulator genes. Promoter systems can be tested by constructing a set of plasmids with a reporter gene under the control of different promoters and their regulators. By measuring the fluorescence of the reporter gene, the strength of the promoter may be evaluated. Balzer et.al (23) constructed a set of plasmids with identical vector backbones and the expression cassettes inserted at the same location. This was done to compare expression systems commonly used for recombinant gene expression in *E. coli.* When comparing promoters to each other, having identical vector backbones may be an advantage as vector-related factors that could affect expression are eliminated. These factors include amongst other things different terminators, origins of replication and selection markers (23). The promoters used in this study were P_m, P_{trc}, P_{BAD}, P_m ML1-17 and P_{mG5} and are described in the following paragraphs.

The XylS/P_m expression cassette contains the positive regulator XylS and the inducible P_m-promoter. Benzoate-derived inducers like m-toluate can induce this expression cassette by activating XylS. The inducers are cheap and non-toxic, and are passively taken up by the cell without being metabolized. In addition, it can be used in a variety of Gram-negative bacteria as well as some Gram-positive. Another advantage is that the background expression in the absence of an inducer can be very low. The P_{mG5} promoter is a weaker variant of the P_m promoter and gives a lower background expression in *P. fluorescens* than the original P_m

promoter when there is no inducer present (24). The high level expression mutant P_m ML1-17 is another variant of P_m (23)

The AraC/P_{BAD} expression cassette contains the regulator AraC and the L-arabinose-inducible P_{BAD}-promoter. *araC* is one of the genes necessary for uptake and catabolism of the sugar L-arabinose. It functions as a positive regulator of the *ara*BAD operon. When L-arabinose is added, the P_{BAD}-promoter is induced and the genes are transcribed. If the sugar is not present, gene expression is repressed when AraC, a dimer, generates a DNA loop by binding two nearby DNA sites. This loop prevents access of the RNA polymerase to P_{BAD}. Further repression can happen when glucose is present in the growth media (25).

The Lacl/P_{trc} expression cassette contains the inducible P_{trc}-promoter and the regulator Lacl, a repressor. The promoter is induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). Addition of IPTG lowers the binding affinity of Lacl to the operator. The promoter study performed by Balzer (23) showed that this expression cassette was the leakiest and least inducible of all the cassettes tested in *E. coli*. Other expression cassettes tested by Balzer included Xyls/P_m, Xyls/P_m ML1-17, LacI/P_{T7lac} and AraC/P_{BAD}.

The reporter plasmid pAG032 was used in this study. It contains three promoter/reporter pairs. The three promoters P_m, P_{ibpfxs} and P_{rpsJ} controls the expression of the reporter genes *rfp, cfp* and *yfp*, respectively. P_{ibpfxs} and P_{rpsJ} are both stress-responsive promoters. P_{rpsJ} responds well to optimal nutrient conditions and deficiencies of ribosomal components while P_{ibpfxs} is σ^{32}-dependent and responds to heat shock and misfolded proteins (26). σ^{32} is also called the heat shock sigma factor and when the RNA polymerase holoenzyme contains this factor, it starts transcription at heat shock promoters (27).

Both promoters controlling the expression of *amcyan* and *turboRFP* were developed for *Bacillus anthracis*. AmCyan is under control of the promoter PFP2, which is a strong constitutive promoter. TurboRFP is under control of the same promoter, as well as the *rrnb* P2 promoter, which is a ribosomal protein promotor from *Bacillus subtilis (28).* These promoters were shown to function in *Vibrio cholerae* (17), where the c-di-GMP reporter system was used to evaluate c-di-GMP accumulation.

2.4 Transposons and their use in genetic engineering

Transposons are mobile genetic elements that can insert themselves into bacterial chromosomes and plasmids. These DNA sequences can create or reverse mutations and can have a significant effect on the evolution of genomes. The transposase is the enzyme that catalyzes the transposition by binding to the ends of the transposon. Autonomous transposons encode their own transposase, while nonautonomous transposons are dependent on transposases encoded elsewhere in the genome (29). Transposon mutagenesis is widely used in the laboratory and transposons with antibiotic resistance genes are used to identify which colonies contain the transposon (21). Tn5 is a composite transposon which cuts and pastes the DNA to move in the genome. The transposon is cut from its position through double stranded DNA breaks made by the transposase, and pasted into the target site. The mini Tn5-based transposon vector pHE319 was used in this study (Figure 4). An outer (O) and inner (I) sequence flank the genes to be transposed, and the gene encoding the transposase lies outside the transposable element. This ensures that a second transposition event is impossible.

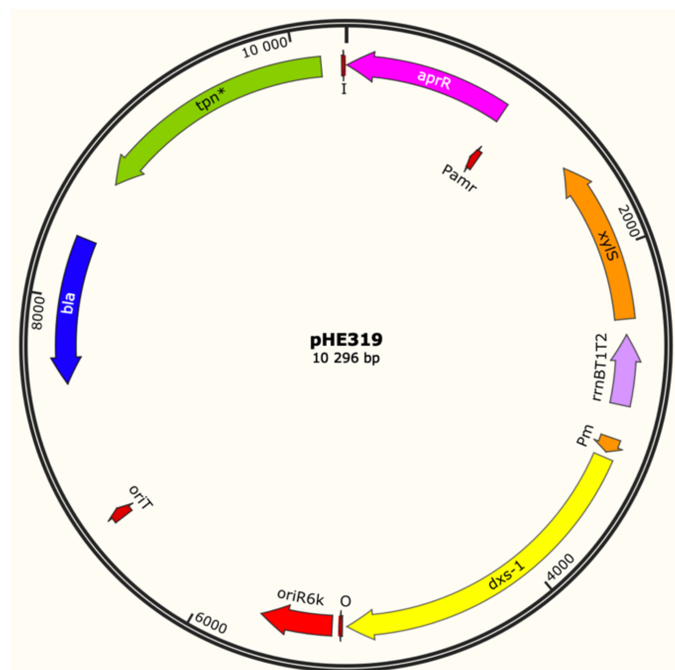


Figure 4: Plasmid map of the mini Tn5-based transposon vector pHE319 used in this study (30)*. The gene encoding the transposase is coloured green.*

2.5 Characterization of reporter genes used in this study

The usage of reporter genes has made it possible to detect and measure gene expression. They make it possible to know whether a gene has been expressed or not. The reporter genes codes for easily measured products, and these genes can be fused together with the regulatory

sequence of genes of interest in order to evaluate their expression. Common reporter genes include green fluorescent protein (GFP) and mCherry. Reporter genes can, amongst other things, be used to evaluate the strength of promoters (31). To do this, the promoter of a gene of interest is fused to a reporter gene which can easily be measured. The reporter genes GFP, YFP, CFP, RFP, AmCyan and TurboRFP were used in this study and are described in the following paragraphs.

The green fluorescent protein from the jellyfish *Aequorea victoria* is widely used as a reporter gene in gene expression studies. When exposed to ultraviolet light, GFP will fluoresce green. It absorbs blue light at 395 nm and emits green light at 509 nm. An advantage with this protein is its ability to fluoresce in the absence of external cofactors. The chromophore forms spontaneously, requiring only oxygen (32). Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are derivatives of GFP which contain amino acid substitutions (33, 34). Another fluorescent protein is AmCyan, which originates from the sea anemone *Anemonia majano.* This fluorescent protein has an excitation and emission maximum of 458nm and 489nm, respectively (35).

The first red fluorescent protein (RFP) discovered was DsRed, which was isolated from the Discosoma sp. coral. It has an excitation maxima at 558nm and emission maxima at 583nm. Improved variants of Dsred include mOrange, mStrawberry and mCherry. The latter is the most widely used of the Dsred derivatives. mCherry also has a high maturation time, which is defined as the time it takes for the newly translated fluorescent protein to produce the chromophore (36). The monomers excitation and emission maximum is 587nm and 610nm, respectively. mCherry displays a good pH resistance and high photostability (37). TurboRFP is an enhanced version of a red fluorescent protein derived from the sea anemone *Entacmaea quadricolor.* It is a dimeric protein which matures rapidly at 37C and is characterized by high brightness (38). It has an excitation and emission maximum of 553nm and 574nm, respectively.

3. Materials and Methods

3.1 Media and solutions

Growth media, stock solutions of antibiotics, buffers and other solutions used were made as described in this section.

PSI-medium

- 5 g/l Yeast extract
- 20 g/l Tryptone
- 5 g/l $MgSO_4$

pH was adjusted to 7.6 with KOH and the medium was autoclaved.

Luria Broth (LB)

- 10 g/l Tryptone
- 5 g/l Yeast extract
- 5 g/l NaCl

The medium was autoclaved.

LA

15 g/l agar was added to the LB medium before autoclaving.

TFB1

- 0.588 g KAc
- 2.42 g RbCl
- 0.294 g $CaCl_2$ x2H_2O
- 2.0 g $MnCl_2$ x4H_2O
- 30 ml Glycerol

The pH was adjusted to 5.8 with dilute acetic acid. Deionized water was added in order to get a total volume of 200 ml. The solution was sterilized by filtration.

TFB2

- 0.21 g MOPS (3-(N-Morpholino)propanesulfonic acid)
- 1.1 g $CaCl_2$ x 2H_2O

- 0.121 g RbCl
- 15 ml Glycerol

The pH was adjusted to 6.5 with diluted NaOH. Deionized water was added in order to get a total volume of 100 ml. The solution was sterilized by filtration.

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 $MgCl_2$

The solution was sterilized by filtration and aliquoted into 1.5 ml tubes. Stored at -20°C.

Pseudomonas Isolation Agar (PIA)

- 45 g/L Difco Pseudomonas Isolation Agar (PIA)
- 20 ml/L glycerol

The agar was autoclaved.

Pseudomonas Isolation Media (PIM)

- 20 g/l peptone
- 5 g/l NaCl
- 1,4 g/l $MgCl_2$
- 10 g/l K_2SO_4
- 20 g/l glycerol

The medium was autoclaved.

0.5M Tris-HCl

- Dissolve 6.057g Tris(hydroxymethyl)aminomethane (TRIS, Trometamol) in 80ml RO-water.
- Adjust pH to 7.5 with concentrated HCl (ca. 7ml).
- Let solution cool to room temperature before making final pH-adjustments.
- Add RO-water to 100ml. Autoclave.

Stock solutions of antibiotics

The concentration of antibiotics used in this study is listed in Table 1. The antibiotics were dissolved in deionized water and the solutions were sterilized by filtration. After aliquoting the solutions into 1.5 ml tubes, they were stored at -20°C.

***Table 1**: Concentration of antibiotics used in P. fluorescens and E. coli.*

Antibiotic	Stock solutions (mg/ml)	Concentration for *P. fluorescens* (µg/ml)	Concentration in *E. coli* (µg/ml)
Ampicillin	200	-	200
Kanamycin	50	-	50
Gentamycin	10 & 20	10	20
Apramycin	50	25	50
Tetracycline	10	10	-

3.2 Growth of bacteria and bacterial strains and plasmids used in this study

E. coli were grown at 37°C in LB or PSI medium. *P. fluorescens* were grown at 30°C in PIM or LB-medium. Antibiotics were added to media or plates in concentrations as described in Table 1. Growth of bacteria was measured at OD_{600}. The bacterial strains used are listed in Table 2. Recombinant DNA was in most cases transformed into competent *E. coli* S17-1. However, *E. coli* S17-1 *λ*pir strain was used as recipient strain when plasmids with an R6K origin of replication were transferred. For initiation of R6K replication, the *π*-protein is needed. The *λ*pir prophage was introduced in *E. coli* S17-1 to enable these vectors to replicate (39). Plasmids used or constructed in this study are shown in Table 3.

Table 2: *Bacterial strains used in this study.*

Strain	Description	Source
E. coli S17-1	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn7	(40)
E. coli S17-1 **λ**pir	*recA thi pro hsdR$^-$ M$^+$* RP4::2-Tc::Mu::Km Tn7 Tp^r Sm^r *λpir*	(41)
P. fluorescens SBW25	Nonmucoid wild type. $Tric^r$	(42)
P. fluorescens SBW25 mucA	Derivative of *P. fluorescens* SBW25. Truncated version of *mucA*. $Tric^r$	(43)

P. fluorescens SBW25 mucA Ptrc algC	Derivative of *P. fluorescens SBW25 mucA*. *algC* under control of the P_{trc} promoter.	(This study)

Table 3: *Plasmids used in this study.*

Plasmid	Description	Source
plB11	RK2-based expression vector. P_m-promoter. Kan^r, Apr^r.	(44)
pHE536	Derivative of pIB11 where a 1.2 kb PCR fragment encoding *algW* replaced *bla*. Kmr.	(30)
pKD24	RK2-based expression vector. P_{mG5} promoter. Kan^r.	(Degnes, unpublished)
pAG032	Reporter plasmid derived from the RK2 replicon. Three promoters (P_m, P*rpsJ,* P*ibpfxs*) controlling expression of CFP, YFP and RFP. Amp^r.	(26)
pSB-M1b	Originally name pTA16. Derivative of pIB11. P_m promoter. Amp^r. Kan^r.	(45)
pSB-M1g	Derivative of pSB-M1b. *bla* gene replaced with GFP. Kan^r.	(23)
pSB-B1g	Derivative of pSB-M1b. *bla* gene replaced with GFP. P_{BAD} promoter. Kan^r.	(23)
pSB-T1g	Derivative of pSB-M1b. *bla* gene replaced with GFP, P_{trc} promoter. Kan^r.	(23)
pSB-M1g-17	Derivative of pSB-M1b. P_m ML1-17 promoter. Kan^r, Amp^r.	(23)
pSV1	Derivative of pAG032 and pHE536. Kan^r. RFP, CFP and YFP.	(This work)
pSV2	Derivative of pSB-M1g and pSV1. P_m promoter. Kan^r. RFP.	(This work)
pSV3	Derivative of pSB-T1g and pSV1. P_{trc} promoter. Kan^r. RFP.	(This work)
pSV4	Derivative of pSB-M1g-17 and pSV1. P_m ML1-17 promoter. Kan^r. RFP.	(This work)

pSV5	Derivative of pSB-B1g and pSV2. P_{BAD} promoter. Kan^r. RFP.	(This work)
pSV6	Derivative of pKD24 and pSV2. P_{mG5} promoter. Kan^r. RFP.	(This work)
pFY4535	Derivative of pFY4357 containing the *hok/sok* region from pXB300. Contains the c-di-GMP biosensor. Gm^r	(17)
pHE319	Mini Tn5-based transposon vector. OriT and OriR6K. P_m promoter. Amp^r, Apr^r.	(30)
pHE228	Derivative of pHE226. P_m promoter. Amp^r.	(Ertesvåg, unpublished)
pHNB4	Derivative of pMG48. Apr^r, Tet^r, Amp^r. *lacZ* for blue-white selection.	(Burud, unpublished)
pSV7	TOPO-vector with *mucR* inserted in a counter-clockwise direction. Kan^r.	(This work)
pSV8	P_{trc} and lacI PCR amplified from pSV3 and inserted into TOPO vector. Kan^r.	(This work)
pSV9	Derivative of pSV8 and pHE319. Amp^r, Apr^r. P_{trc} promoter. Transposon vector.	(This work)
pSV10	Derivative of pHE228 and pSB-T1g. Amp^r. P_{trc} promoter.	(This work)
pSV11	Derivative of pSV10 and pHNB4. Tet^r, Amp^r. P_{trc} promoter. *lacz* gene for blue-white selection.	(This work)

3.3 Analysis of reporter genes

In the reporter gene study, the plate reader Tecan Infinite M200 was used. The wavelengths and gain-values used to measure the four different proteins is listed in Table 4. Pre-cultures with LB and Km were made and incubated at 30°C. Before inoculation, OD_{600} was measured and a volume corresponding to a starting OD_{600} of 0.05 was transferred to the culture flasks. The frequency of sampling for OD_{600} and fluorescence for each experiment is reported in the results. Fluorescence and OD_{600} were measured each time. 200 µl of the culture was used for fluorescence measurement while 100 µl was used for measurement of OD_{600}.

Table 4: Wavelength and values for gain used for the reporter gene study and promoter study on Tecan Infinite M200

Protein	Excitation wavelength (nm)	Emission wavelength (nm)	Gain
GFP	485	520	80
YFP	505	538	80
CFP	433	475	80
RFP	580	615	100
TurboRFP	553	574	50
AmCyan	458	489	50

3.4 Plasmid isolation

The isolation of plasmids was performed using the ZR Plasmid Miniprep kit from Zymo Research. The kit uses the alkaline lysis method and three different buffers to efficiently isolate plasmid DNA. The resuspension buffer (P1 buffer) resuspends the cell pellet and contains EDTA. EDTA binds divalent cations, which leads to a destabilized cell membrane, and inhibits DNases that could degrade the DNA. The lysis buffer (P2 buffer) is an alkaline solution and contains sodium hydroxide (NaOH) and the detergent sodium dodecyl sulfate (SDS). SDS lyses the cell and NaOH releases and denatures the DNA. The neutralization buffer (P3 buffer) contains potassium acetate and leads to renaturation of plasmid DNA and also precipitation of SDS, lipids, proteins and chromosomal DNA. After centrifuging, only the plasmid DNA will remain (46).

For plasmids with low copy number, the method was slightly modified. A low copy number plasmid typically has less than fifty plasmids per cell, whereas a high copy number plasmid can have hundreds of plasmids per cell. To achieve a high enough DNA concentration after isolation, a larger culture volume was needed. (47).

Protocol

1.5 ml of bacterial culture was transferred to an 1.5 ml tube and centrifuged for 2 minutes at full speed. However, for low copy number plasmids 5 ml of bacterial culture was used. 200 µl P1 buffer was added to each tube and dissolved by pipetting. Next, 200 µl P2 buffer was added and the tube was mixed by inverting it 2-4 times in order for the solution to turn purple

and viscous. After 1-2 minutes, 400 µl P3 buffer was added and mixed gently until the solution turned yellow. The lysate was incubated for an additional 1-2 minutes before it was centrifuged for 2 minutes. The supernatant was placed in a Zymo-spin IIN column in a collection tube and centrifuged for 30 seconds to bind the DNA to the membrane in the column. 200 µl Endo-wash buffer was added to the column. After centrifuging for 30 seconds, 400 µl of Plasmid wash buffer was added and the column was centrifuged for 2 minutes. The column was transferred to a clean 1.5 ml tube, 30 µl DNA elution buffer was added and the tube was centrifuged to elute the plasmid DNA from the column to the 1.5 ml tube.

3.5 Measure concentration of DNA with Nanodrop

To measure the concentration of DNA, a Nanodrop One spectrophotometer was used. The instrument is a UV-Visible spectrophotometer that can measure several different sample types, like peptides, isolated DNA, proteins and RNA. The lower detection limit for purified double stranded DNA (dsDNA) is 2.0 ng/µl. The upper detection limit is 27 500 ng/µl. The calculations are based on a modified Beer-Lambert equation (48).

$$A = \varepsilon c b \qquad \text{(eq.1)}$$

$$Factor\ (f) = \frac{1}{\varepsilon * b} \qquad \text{(eq.2)}$$

$$c = A * f \qquad \text{(eq.3)}$$

In Equation 1, the Beer-Lambert equation, A is the absorbance, ε is the molar absorptivity coefficient ($M^{-1}cm^{-1}$), b is the pathlength (cm) and c is the concentration of the analyte (M). This equation relates the absorbance of a solution to its concentration. By solving for concentration, one can calculate the concentration of a solution based on its measured absorbance. The modification of the equation that NanoDrop uses for calculation involves the combination of the molar absorptivity coefficient and the pathlength, which is called factor (ng-cm/µl), shown in Equation 2 and 3. The modified Beer-Lambert equation is shown in Equation 3 (48). The buffer used for DNA elution was in each case used as blank solution in the measurement. The dsDNA program was chosen to measure the concentration of DNA, and 1 µl of the sample was used for measurement.

3.6 Restriction cutting of plasmids

Restriction enzymes cut DNA at specific sites and produce restriction fragments. The biological function of these enzymes is to protect the cell's DNA from entry of foreign DNA,

which it destroys by cleaving. The cell´s own DNA is protected from its own restriction enzymes by methylation. In molecular biology these enzymes are used to make restriction fragments that can be joined by a DNA ligase in order to create recombinant DNA molecules (49).

Protocol

The amount of DNA that was to be cut was usually around 100-150ng and depended on the concentration of the isolated DNA measured on Nanodrop. If the desired gel bands were small in size, more DNA were cut in order to visualize the DNA on the gel. DNase-free water and DNA were added to a total of 17 µl. In addition, 2 µl optimal 10x buffer and 0.5 µl of each restriction enzyme to be used was added and the mixture was incubated at the enzymes' optimal temperature for 1 hour to overnight. The restriction enzymes and buffers used were produced by New England Biolabs.

3.7 Agarose gel electrophoresis

DNA can be efficiently separated by agarose gel electrophoresis by loading the DNA into a gel and applying a current. Since DNA is negatively charged (and have the same charge per unit of mass), it will migrate through the pores in the agarose gel, towards the positive electrode when an electric tension is applied. This is exploited in gel electrophoresis where the DNA is separated based on size – shorter fragments migrate through the gel faster than longer ones. (50). GelRed and GelGreen are ultrasensitive, stable and non-toxic stains. GelGreen was used to make the gel when the resulting DNA fragments were to be cut out of the gel for DNA purification. This is because GelGreen can be used with a blue light transilluminator to prevent ultraviolet radiation from damaging DNA. For other purposes, GelRed was used.

Protocol

The concentration of agarose in the gel was 0.8%. 2 µl Gel Loading Dye Purple 6X (New England Biolabs) was added to the samples prior to loading them into the gel. This was done to visualize the migrating DNA on the gel and to increase the density of the DNA in order for it to sink to the bottom of the gel instead of diffusing into the TAE-buffer. The gel was placed in the electrophoresis chamber containing 1xTAE-buffer, the samples were loaded into the wells and the gel was run at 100V for sufficient time to obtain good separation of the bands.

The DNA Ladders Lambda DNA HindIII and Lambda DNA PstI were made by adding 10 µl Lambda DNA (500 µg/ml), 10 µl 10x cutsmart buffer, 79 µl DNase free water and 1µl of enzyme (HindIII or PstI). These were used in order to estimate the size of the sample fragments run on the gel. The ladders are shown in Appendix B.

3.8 Purification of DNA restriction fragments from gel

Gel-fragments cut out from agarose gel were purified using the Monarch DNA Gel Extraction kit (New England Biolabs). The gel dissolving buffer contains guanidine thiocyanate and is sodium iodide-based. It dissolves the agarose when heat is applied to the sample. The silica membrane in the column binds DNA upon centrifugation. The chaotropic salt guanidine thiocyanate allows DNA to bind to the silica surface, even though both DNA and the silica surface are negatively charged (51). Chaotropic salts disrupts hydrogen bonding, van der Waals forces and hydrophobic interactions. This destabilizes nucleases that can degrade the DNA. In addition, the association of nucleic acids with water is disrupted, enabling them to bind to the silica membrane. The DNA wash buffer removes contaminants like salts, enzymes, DMSO, detergents and short primers. The elution buffer contains 10mM Tris, 0.1mM EDTA with pH 8.5 and results in the release of DNA from the silica membrane.

Protocol

The desired DNA fragments were cut out of the agarose gel and placed in an 1.5 ml tube. 400 µl gel dissolving buffer per 100mg agarose was added, therefore the tube containing the gel piece was weighed beforehand in order to know how much of the buffer to add. The sample was then incubated for 5 to 10 minutes at 50°C and vortexed periodically to completely dissolve the gel piece. Next, the sample was loaded into a column and collection tube and spun for 1 minute at 13 000 rpm. The flow-through was discarded and 200 µl DNA wash buffer was added before the sample was spun once more for 1 minute. The column was transferred to a clean 1.5 ml tube and 15 µl DNA elution buffer was added. After 1 minute the sample was spun for 1 minute to elute the DNA into the 1.5 ml tube.

3.9 Ligation

The function of the T4 DNA ligase (New England Biolabs) is to catalyze the formation of a phosphodiester bond between a 5´-phosphorylated end on one fragment, and a 3´-hydroxyl

end on another fragment. The T4 DNA ligase is ATP-dependent and widely used in the laboratory (52). The T4 DNA ligase can ligate both sticky and blunt ends (53).

Protocol

To ligate two DNA fragments, three times more insert than vector in molar ratio were mixed. To this mixture, 2 µl 10x ligase buffer and 1 µl T4 DNA ligase was also added. The total volume was 20 µl. The ligation mix was incubated at 16°C overnight.

3.10 Making competent cells and transformation

Competent cells are cells with the ability to take up free DNA from their environment. Not all bacterial cells have this ability, only those that are expressing the necessary genes encoding the necessary proteins needed for uptake of DNA (54). Chemically competent *E.coli* cells that has been subjected to $CaCl_2$ can take up DNA from their environment by using the heat-shock method. The exact mechanisms of how this works is still not known, but it is believed that $CaCl_2$ – found in TFB1 and TFB2 - aids in the adsorption of DNA to the cell and the heat-shock step participates in the transportation of the DNA into the competent cells and the depolarization of their membrane (55). $RbCl_2$, also found in TFB1 and TFB2, likely have a similar effect to $CaCl_2$.

Protocol

First, an overnight culture with 10 ml PSI-medium and *E. coli* cells was made and incubated at 37°C and 225 rpm. The following day, 1 ml of the overnight culture was transferred to 100 ml PSI-medium. Optical density (OD) was measured using a spectrophotometer at 600nm, and the culture was incubated until OD_{600}=0.40 was reached. This was controlled by frequent OD measurements. After almost two hours, the culture had reached OD_{600}=0.40 and was placed on ice for 15 minutes together with TFB1 and TFB2. The next step was to centrifuge the culture for five minutes at 4000 rpm and 4°C. The cell pellet was then dissolved in 40 ml cold TFB1 before it was put on ice for 5 minutes and then centrifuged again for five minutes at 4000 rpm and 4°C. After the centrifugation, the cell pellet was dissolved in 3 ml TFB2 and distributed into 1.5 ml tubes. These were frozen using liquid nitrogen and then stored at -80°C.

For the transformation, a tube with 100 µl chemically competent *E.coli* cells and 10 µl DNA ligation mix was incubated on ice for 30 minutes to 1 hour. After incubation, the mixture was placed in a water bath at 37°C for 1 minute and 30 seconds (heat shock) and then put directly on ice for 2 minutes. Next, 900 µl SOC-medium prewarmed to 37°C was added to achieve maximum transformation efficiency. The cells were incubated at 37°C with agitation for 2 hours. Lastly, the sample was plated out on LA-plates containing the appropriate antibiotic to select for the plasmid, and incubated at 37°C.

3.11 PCR

PCR is a method used to amplify a specific sequence of interest. Performing a PCR-reaction requires the DNA template, DNA polymerase, primers and nucleotides. The DNA template is the sequence one wish to amplify and the DNA polymerase adds the nucleotides to the 3´-end of the primers in order to create the PCR-product. The primers are short sequences which are complementary to the DNA-template sequence and will bind to it. One cycle in the PCR-reaction consists of three steps: denaturation, annealing and elongation (56). The PCR program is described in Appendix C and the primers used in this study are listed in Appendix D.

Protocol

The reaction mix were made as described in Appendix C. The samples were placed in the PCR machine and the appropriate program was started. To denature the double-stranded DNA into single strands, the reaction chamber was heated to a temperature of 98°C for 30 seconds. Next, the temperature was lowered to 3-5°C lower than the lowest annealing temperature of the primers (T_m) for 10-30 seconds. Finally, to allow the DNA polymerase to extend the primers by adding nucleotides, the temperature was raised to 72°C. These tree steps were repeated in 25-35 thermal cycles. After the reaction was finished, the temperature in the reaction chamber was lowered to 4-10°C; this was for short-term storage of the PCR-product. Gel electrophoresis was used to confirm whether the correct PCR-product was made.

3.12 Topo cloning

By exploiting topoisomerase I isolated from the *Vaccinia* virus, TOPO-cloning is a technique that is not dependent on restriction enzymes and ligases. The Zero Blunt TOPO PCR Cloning Kit from Invitrogen was used to perform Topo cloning. This technique allows the insertion of

a blunt PCR-product into a plasmid vector. The vector comes linearized and contains two topoisomerase I, each bound to the 3`-end of the DNA-strands. When the compatible ends from the PCR-product is introduced, the ends are ligated and the topoisomerase is released. This way, the enzyme functions as both a restriction enzyme and a ligase (57). In addition, the TOPO vector contains the *ccd*B-gene which codes for a toxic protein leading to cell death. When a PCR-product is inserted into the vector, the *ccd*B-gene will be disrupted. Because of this, only colonies with inserted PCR-product will grow after transformation (58).

Protocol

1 µl of the PCR-product was mixed with 0.5 µl salt solution, 1 µl water and 0.5 µl PCR II-Blunt-Topo vector. The solution was incubated for 5 minutes at room temperature. After incubation, the reaction was placed on ice if transformation was performed immediately, or stored at -20°C overnight. When transforming into *E. coli* S17-1, performed as described in section 2.5, the cells were plated out on LA-plates with kanamycin.

3.13 One step sequence- and ligation- independent cloning (SLIC)

With the one-step SLIC method, linearized vector and PCR-prepared inserts can be mixed together with T4 DNA polymerase and buffer and be directly transformed into competent *E. coli* cells. This can be done without a ligase as SLIC takes advantage of the homologous recombination system in *E. coli*. A key step is the generation of an insert with a 15bp or longer sequence homology with the vector. Through its exonuclease activity, the T4 DNA polymerase creates 5` overhangs on the vector and the PCR-amplified insert that are complementary to each other (59). The original SLIC method was dependent on RecA to catalyze homologous recombination in order to obtain a high cloning efficiency (60). One-step SLIC however, does not require the presence of RecA. The one-step SLIC method has been further optimized to reduce the incubation time and temperature with the T4 polymerase from 2.5 minutes at room temperature to 30 seconds at 50°C. This was to prevent reduced cloning efficiency due to formation of stable secondary structures within the DNA (61).

Protocol

First, the vector was linearized with restriction enzymes. Next, the insert was prepared by PCR using primers with 20-22bp long ends homologous to the linearized vector. The vector and insert was purified using the Monarch nucleic acid purification kit, with the exception of the supplied elution buffer. Instead of using the supplied buffer, NEB 2.1 buffer diluted to 1X

concentration was used. The vector was mixed with the insert at a molar ratio of 1:2 or 1:4 (vector:insert). The amount of ng insert needed was calculated by using the vector and insert size, as well as the vector amount. 1 µl 2.1 1X NEB buffer was added, together with water to get a final reaction volume of 10 µl. Finally, 0.5 µl T4 DNA polymerase was added and the reaction was incubated for 30 seconds at 50°C (61). To stop the reaction, the mixture was put on ice. Following 10 minutes of incubation on ice, 10 µl of the mixture was transformed into competent *E. coli* S17-1 λpir., as described in section 3.5.

3.14 Conjugation

Together with transduction and transformation, conjugation is one of the main methods bacteria employ to transfer genes. Conjugation involves cell-to-cell contact where the donor cell transfers a plasmid to the recipient cell (62). Conjugation is often used in the laboratory to transfer genetic elements from one bacterial strain to another. *E. coli* S17-1 is the strain most often used as donor strain in conjugation. The RP4 plasmid contains transfer genes necessary for conjugation and is integrated into the genome of *E. coli* S17-1, making it an ideal donor strain for conjugation (40).

Protocol

To transfer the donor plasmids to the recipient *P. fluorescens*, precultures were first made. *P. fluorescens* was inoculated in 25 ml LB and incubated at 30°C. *E. coli* S17-1 with the plasmids were inoculated in 10 ml LB with the appropriate antibiotics and incubated at 37°C. The next day, 500 µl of the *P. fluorescens* preculture was transferred to 25 ml LB and incubated at 30°C. Two hours later, 200 µl of the *E. coli*-cultures was transferred to 10 ml LB and incubated at 37°C. After two more hours, exponential phase was reached (OD_{600}=0.40). 3 ml of *P. fluorescens* was mixed with 3 ml of *E. coli* S17-1 in sterile tubes, one for each plasmid. The tubes were centrifuged for 5 minutes at 5000-7000 rpm. The supernatant was discarded, leaving approximately 0.1 ml of liquid to resuspend the cell pellet in. The drops were placed on LA-plates and incubated at 30°C over night. After incubation, a dilution series were made by scraping of the cell growth from the plates and transferring it into 1 ml LB. 100 µl of the dilutions (undiluted, 10^{-2},10^{-4} and 10^{-6}) were plated out on PIA-plates with the appropriate antibiotic and incubated at 30°C for 3-4 days.

3.15 Homologous recombination for gene replacement

Homologous recombination is a process important for repairing double-stranded breaks in DNA which can be the result of stalled or broken replication forks. It involves two nearly identical double-stranded DNA sequences, where the undamaged sequence acts as a template to repair the damaged sequence, without losing or altering nucleotides. The ends of the damaged strand are first digested by nucleases to generate overhanging single-strand 3`-ends which can invade the template DNA and find a homologous sequence. The RecA ATPase in *E. coli* is vital in the strand invasion process. Its role is to catalyze the strand exchange by binding the invading single-stranded DNA and promoting base-pairing between the two strands in order to find sequence homology. Once a longer stretch of homology is found, the invading strand is stabilized and completely exchanged with the damaged strand (63). This process is taken advantage of in genetic engineering, where it is used to introduce and remove mutations in the bacterial chromosome. In this study, homologous recombination was used to replace the *algC* promoter P_{algC} with P_{trc} through a double crossover experiment.

The vector used for homologous recombination is a derivative of pMG48 – a RK2-based suicide gene replacement vector. pMG48 and its derivatives have a gene encoding for tetracycline resistance and *lacZ* (64). The *lacZ*-gene encodes for B-galactosidase, an enzyme that is able to break down 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) to galactose and the blue pigment 4-chloro-4-brom-indigo (54). When the vector is inserted into the genome of *P. fluorescens* through the first recombination, blue tetracycline resistant colonies are formed in the presence of X-Gal. After the colonies have been grown in the absence of tetracycline for some time, a second recombination event occurs and only the gene that was to be inserted will be left in the genome and the rest of the vector will be lost. This is indicated by the formation of white colonies even in the presence of X-Gal (64).

Protocol

The protocol for conjugation was followed as described in 2.13 up until plating out the dilution series. The three dilutions were plated out on PIA-plates with X-gal and tetracycline (Tc) and incubated for 48 hours. Next, two colonies were picked from the plates and inoculated in new LB-medium and the cultures were incubated overnight. Then; 1-2 times per day for 2-4 days, the following was performed:

1. 1% of the cultures were inoculated in new LB-medium and incubated overnight.

2. A dilution series was made and 100 µl of the 10^{-5} and 10^{-7} dilutions were plated out on PIA with X-gal. The plates were incubated for 48 hours.

Step number 2 was done in order to evaluate when enough white colonies appeared, indicating a double recombination event, started to appear. When the plates showed enough white colonies, some of these colonies were transferred to PIA-plates with and without Tc. However, when the plates had been stored in +4°C for some days, the colonies became very mucoid and could not be separated from each other. Therefore, some colonies were transferred to LA plates with and without Tc instead of PIA plates. Colonies that grew on PIA or LA without Tc but not on the plates with Tc, were checked with PCR to determine whether the colonies contained the wild-type-genome or had the P_{trc} promoter inserted in front of *algC*.

3.16 Sequencing

Sanger sequencing is a method that determines the nucleic acid sequence of DNA. To perform Sanger sequencing, a DNA template, primer, DNA polymerase, dNTPs (deoxyribonucleotides), ddNTPs (dideoxyribonucleotides) and a reaction buffer is needed. ddNPTs are modified nucleotides which lack the OH-group at the 3´end, making them unable to form phosphodiester bonds with the next nucleotide. Chain-termination PCR, which is similar to normal PCR except for the addition of ddNTPs, is carried out. Both the dNTPs and the fluorescently labeled ddNTPs are mixed in a single reaction. Once a ddNPT has been randomly incorporated, the extension catalyzed by the DNA polymerases stops because there is no free 3´OH group. This results in multiple sequences of varying lengths being generated, each with a fluorescent label at the 5´end. These sequences are then separated by size with capillary gel electrophoresis, and the fluorescence of the ddNTPs is detected by a laser and converted into a chromatogram. Sequencing was used to control the products after PCR, as well as PCR products cloned into a TOPO vector.

Protocol

A reaction mix with 190ng template DNA and 1.9 µl of 20pmol/µl primers were made. Only one primer was added per reaction, but enough reactions with different primers were used in order to cover the entire DNA template. The sequencing was performed by the GATC Eurofins.

3.17 Alginate assay

A simple enzymatic assay to measure alginate has been developed by Østgaard (65). Alginate lyases cleave the alginate chain, generating an unsaturated unit at the non-reducing end through a ß-elimination reaction. This unit absorbs light at 230nm. An increased absorbance at this wavelength reflects the enzyme activity. When there is enough enzyme, the reaction reaches an endpoint which reflects the initial substrate concentration. This takes about three hours. By constructing and comparing with a standard curve, one can determine the concentration of alginate in the sample. Both M-lyase and G-lyase is used in the assay. However, as bacterial alginates can be O-acetylated, they must be deacetylated. This is because their O-acetyl groups may interfere with the enzyme conversion (65). Also, deacetylated alginate is more susceptible to degradation by alginate lyases, and this degradation is a key point in the quantification of alginate (66).

Protocol

Pre-cultures of the strain-of-interest were made by inoculating the bacteria in PIM-medium. The next day, 0.1ml of the pre-cultures were added to 24.9 ml PIM-medium with proteases (alcalase and neutrase). Addition of proteases prevents lyases from degrading the alginate. The cultures were incubated at 30°C for as long as the sampling was carried out.

Samples was taken out 24, 48 and 72 hours after inoculation. 8 and 24 hours after inoculation the cultures containing *P. fluorescens* SBW25 *mucA Ptrc algC* were induced with 0.2mM IPTG. At each sampling, OD_{600} in the cultures were also measured. 1.00 gram of the culture was measured out and diluted with 4 ml 0.2M NaCl to decrease viscosity. Next, 3 ml of the solution was distributed to two 1.5 ml tubes and centrifuged for 10 minutes at 13 000 rpm. Following centrifugation, 1ml of the supernatant was transferred to two new tubes. Lastly, 33 µl 3M NaOH was added to the tubes before they were frozen at -20°C.

The samples were thawed and deacetylated for 1 hour. Next, they were centrifuged for 10 minutes at 13 000 rpm. 100 µl of the supernatant was transferred to a new tube and 300 µl buffer (0,05M Tris-HCl, 0,2M NaCl, 1mM CaCl2, pH7,5) was added.

For the assay, a 96-well UV-plate was used. The following was added to each well:

- 206.25 µl buffer (0,05M Tris-HCl, 0,2M NaCl, 1mM CaCl2, pH7,5)
- 18.75 µl sample, standard or blank (deionized water)

There were three parallels for each sample, two for the standard and one for the blank. OD was measured at 230nm. Following measurement, 8 µl 1 U/ml M-lyase and 8 µl 1 U/ml G-lyase was added to each well (except for the blank). The plate was incubated at room temperature and OD_{230} was measured after 3 and 4 hours.

The standard curve was made by calculating the increase in OD_{230} from before addition of the lyases and 4 hours after addition. These values were plotted against the known alginate concentrations of the standard solutions. For the samples, the increase in OD_{230} was also calculated. These values for the samples were also corrected for the dilution made with NaCl, NaOH and buffer. By using the equation from the linear trendline in the standard curve, the alginate concentration in the samples was calculated.

3.18 Calculation of standard deviation

The standard deviation is calculated in order to know how well the average value of a number of samples represents the data set. Samples can be nearly identical, or they can be spread around the mean value. If the standard deviation is high, the variation in the data set is high. In order to make error bars for the graphs, the standard deviation of the values for the fluorescence or OD was calculated. To calculate, the STDAV function in Excel was used. This function calculates the standard deviation of a set of data. STDAV uses the “n-1”-method. The following formula is used in the function:

$s = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}}$ where n indicates the sample size and x indicates the sample mean.

3.19 Bioinformatics tools

3.19.1 Programs used to simulate cloning

Benchling contains software tools that enable the user to edit DNA sequences, keep an electronic lab notebook, design primers and align sequences (67). In this project, Benchling has mainly been used in cloning to find restriction enzymes and visualize plasmids.

SnapGene is a molecular biology software tool which specializes in molecular cloning tools (68). The user is able to simulate restriction cloning, Gateway cloning, Gibson assembly, TOPO-cloning, TA cloning and In-fusion cloning in silico. The features of plasmid can be

easily viewed, agarose gel electrophoresis can be simulated, primers can be designed and PCR can be simulated. SnapGene was the main program used for cloning purposes in this study.

Clone Manager is another molecular cloning tool that can simulate cloning, design primers and align sequences (69). In this study, Clone Manager was mainly used when designing primers for PCR.

3.19.2 Programs used to visualize sequencing results

Clustal Omega is a freely available multiple sequence alignment program which can align three or more sequences (70). Input sequences can be DNA or protein in various formats. To convert the results generated by Clustal Omega into a coloured multiple alignment, Mview was used (71). The four bases are coloured differently, thymine bases are blue, cytosine bases are yellow, adenine and guanine bases are green. Bases with no colour in the aligned sequence are bases that does not match the reference sequence. When a base is lacking, this is indicated with a dash symbol. Errors that occur near the ends of the sequencing results are predictable and should not be taken into account. These errors are the result of the gel losing resolution which makes the peaks broader and harder to read accurately.

4. Results

Section 4.1 describes the construction of plasmids with different reporter genes and the measurement of these genes. Section 4.2 describes the evaluation of the strength of different promoters. Section 4.3 describes the construction of a transposon vector with the P_{trc} promoter, as well as an attempt at inserting *mucR* into transposon vectors. In section 4.4, a reporter system for measuring the intracellular concentration of c-di-GMP was evaluated in *P. fluorescens*. Finally, section 4.5 describes the construction of a strain with *algC* under control of the promoter P_{trc}, and the measurement of alginate in this strain.

4.1 Evaluation of reporter genes

An important part of this project was to evaluate the strength and inducibility of different promoters. In order to do this, a reporter gene study was performed to investigate which protein´s fluorescence was easiest to measure in *P. fluorescens* SBW25. In this section, the reporter genes RFP, CFP, GFP and YFP were evaluated. Plasmids with those genes were either constructed or used directly and conjugated into *P. fluorescens* SBW25 (Figure 5)

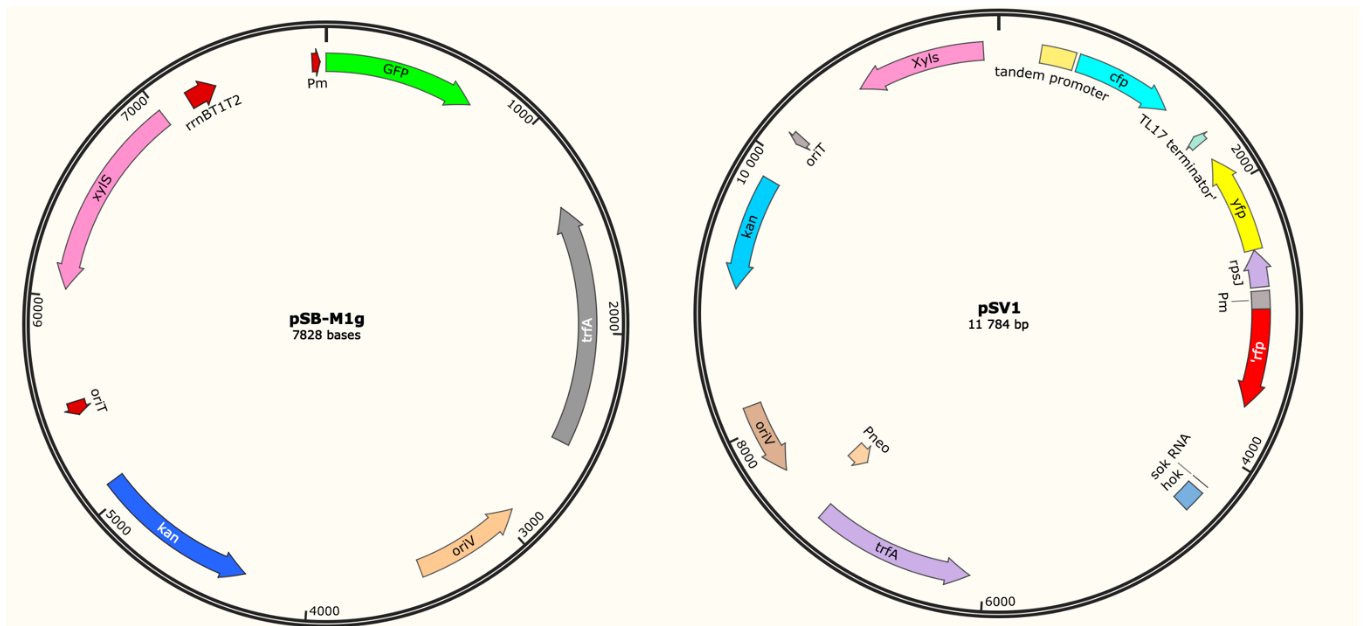


Figure 5: Plasmid maps of pSV1 and pSB-M1g which together contain the reporter genes to be evaluated. *pSV1 contains YFP, RFP and CFP. The expression of RFP is controlled by the* P_m *promoter. The expression of YFP is controlled by the PrpsJ promoter. The expression of CFP is controlled by the Pibpfxs promoter (26). pSB-M1g contains GFP and its expression is controlled by the* P_m *promoter .*

4.1.1. Construction of plasmid pSV1

The antibiotic resistance gene in pAG032 had to be changed from ampicillin to kanamycin as *P. fluorescens* is resistant to ampicillin. pAG032 was digested with ApaLI, Acc65i and BgiII and pHE536 was digested with Acc65i and BgiII. The ligated plasmid pSV1, shown in Figure 5, was transformed into competent *E. coli* S17-1-cells and transformants were selected for by using LA plates with kanamycin. Plasmids from ten transformants were isolated and digested with NotI. Four of the colonies yielded the expected fragments of 9500 and 2300bp (Figure 6). The plasmid from transformant 5 was chosen as pSV1.

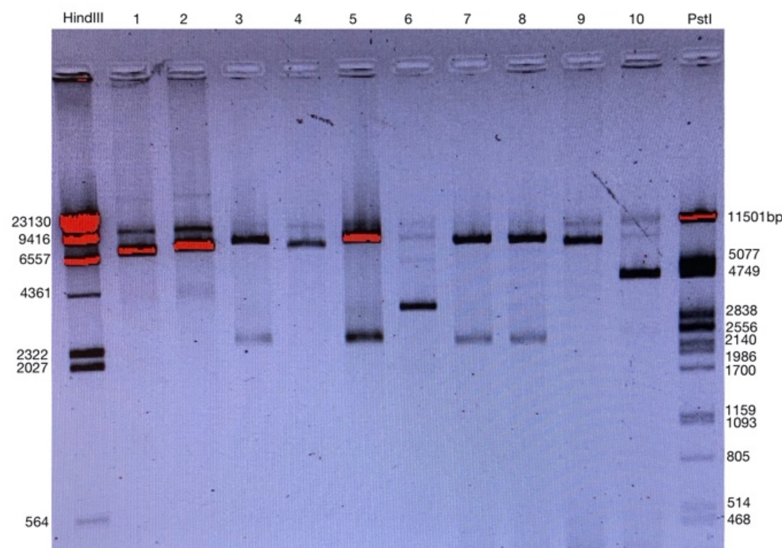


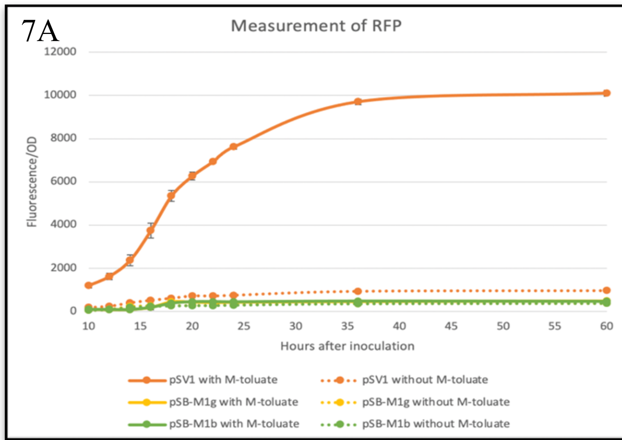
Figure 6: Verification of pSV1 with gel electrophoresis. *Lane 1 and 11 shows the DNA ladders HindIII and PstI with their known molecular sizes, respectively. Lane 2-10 shows the plasmids isolated from ten transformant colonies and digested with the enzyme NotI. The expected fragments of pSV1 digested with NotI was 9500bp and 2300bp. Sample 3, 5, 7 and 8 contain these fragments.*

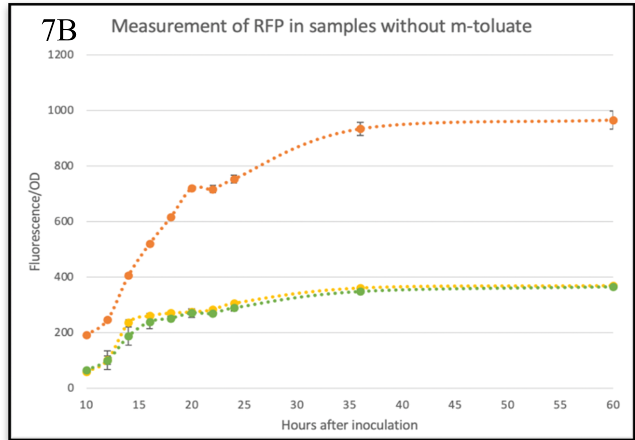
4.1.2 Measurement of reporter genes

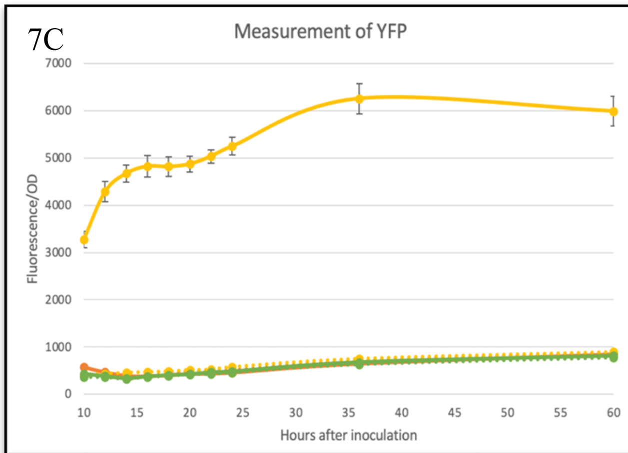
The reporter gene study was performed to find out which protein was easiest to measure in *P. fluorescens* and, if it was possible to use some reporter genes at the same time. By using pSV1, three different reporters could be tested – YFP, RFP and CFP. The fourth reporter to be tested, GFP, was provided by pSB-M1g. pSB-M1b did not encode any fluorescent proteins, and was used to test for any interference by the autofluorescence of *P. fluorescens.*

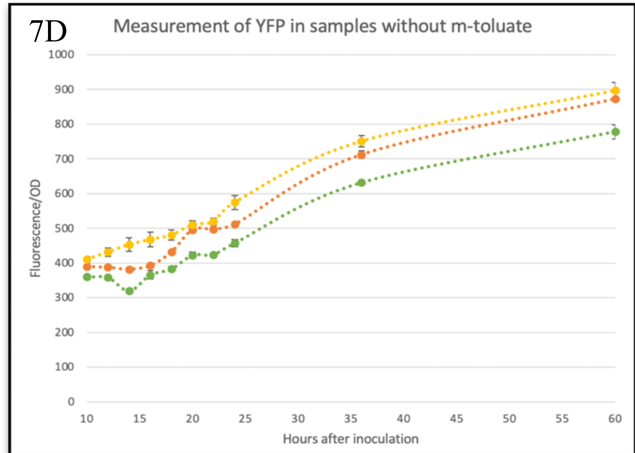
Prior to the measurements, pSV1, pSB-M1g and pSG-M1b were conjugated into *P. fluorescens* SBW25 as described in section 2.11. There were three independent pre-cultures for each plasmid. OD was measured after overnight incubation. For each pre-culture, a certain

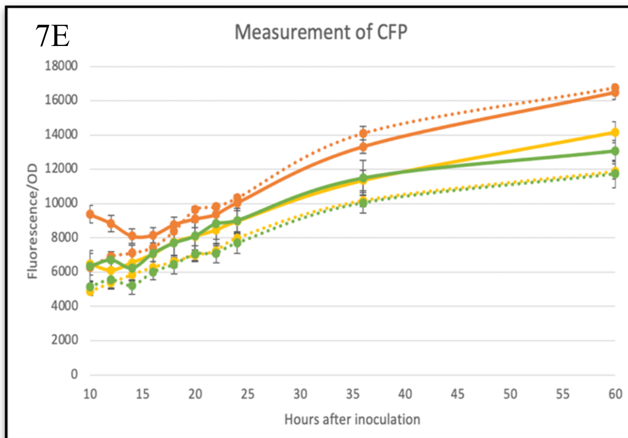
volume was added to new flasks with and without 20 µl of the inducer m-toluate (1mM), in order to obtain an OD-value of 0.05 in the new flasks. M-toluate induces the P_m promoter and all its variants. Each plasmid had 6 samples – 3 without m-toluate and 3 with. Measurements of OD and fluorescence were carried out every second hour from 10 hours until 24 hours after inoculation, and then after 36 and 60 hours. The absorbance and excitation values used for each reporter gene is listed in Table 2 in section 3.2. The results were then plotted as fluorescence per OD (Figure 7).

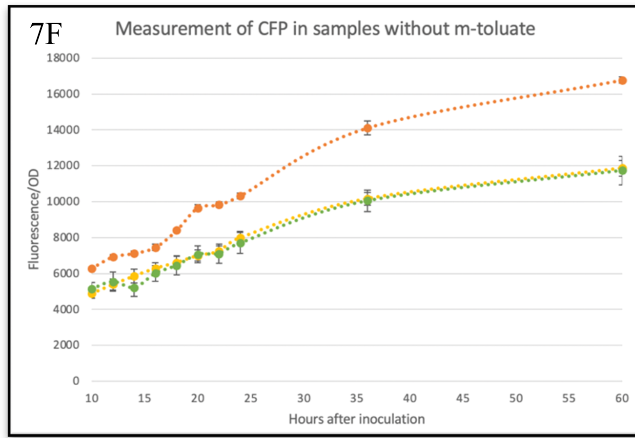

7A
Measurement of RFP
Fluorescence/OD
Hours after inoculation
pSV1 with M-toluate
pSV1 without M-toluate
pSB-M1g with M-toluate
pSB-M1g without M-toluate
pSB-M1b with M-toluate
pSB-M1b without M-toluate

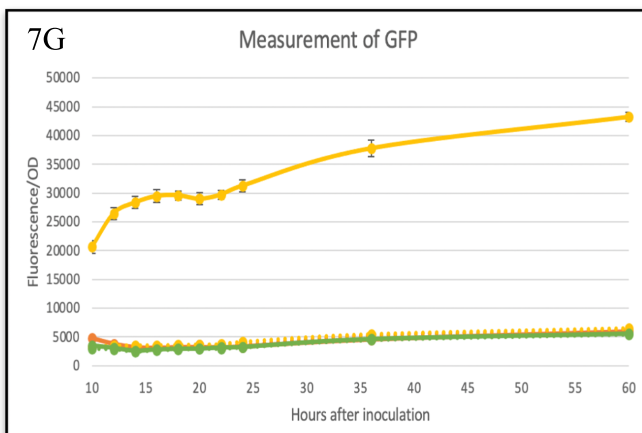

7B
Measurement of RFP in samples without m-toluate
Fluorescence/OD
Hours after inoculation


7C
Measurement of YFP
Fluorescence/OD
Hours after inoculation


7D
Measurement of YFP in samples without m-toluate
Fluorescence/OD
Hours after inoculation


7E
Measurement of CFP
Fluorescence/OD
Hours after inoculation


7F
Measurement of CFP in samples without m-toluate
Fluorescence/OD
Hours after inoculation


7G
Measurement of GFP
Fluorescence/OD
Hours after inoculation

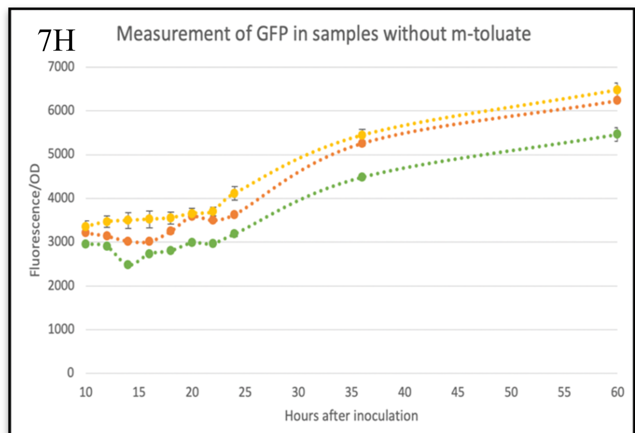

7H
Measurement of GFP in samples without m-toluate
Fluorescence/OD
Hours after inoculation

Figure 7: Results from measuring fluorescence of RFP, YFP, GFP and CFP. *There were 6 samples for each plasmid where 3 samples contained the inducer m-toluate and the other 3 did not. Each point on the graphs show the average of three independent cultures The dotted lines indicates samples without m-toluate, while the solid lines indicate samples with m-toluate. Graph A, C, E and G shows the average fluorescence of RFP, YFP, CFP and GFP, respectively, in the 3 samples with m-toluate calculated for each timepoint. Graph B, D, F and H shows the fluorescence of RFP, YFP, CFP and GFP, respectively, in samples without m-toluate calculated for each timepoint.*

The standard deviation error bars shown on each graph in Figure 7A-7H and 8 are small, indicating that the values of the three independent samples are close to the mean value and that the mean value accurately represents the data set.

The results show that RFP is easily measured in *P. fluorescens* when the promoter was induced with m-toluate. The measured fluorescence of RFP in pSB-M1g and pSB-M1b was low. This was expected as neither plasmid contains RFP and it could mean that the background fluorescence was low at this excitation value. There was a difference in fluorescence intensity between induced and uninduced samples, as can be seen in Figure 7A, indicating that the promoter that controls RFP was, as expected, inducible. The measured fluorescence of RFP in cultures with m-toluate is approximately 10 times higher than in cultures without m-toluate (Figure 7A and B). In addition, RFP displayed a higher fluorescence even when comparing the uninduced samples only (Figure 7B). This relatively high fluorescence in the absence of m-toluate indicated that the promoter had a high background expression. When using the optimal excitation values for YFP (Table 2, section 3.3), it was expected to see elevated fluorescence levels for pSV1 only. However, in this case pSB-M1g displayed the highest fluorescence. The results in Figure 7C and D indicated that m-toluate could have had an effect on the expression of YFP. As expected for GFP, it was pSB-M1g that showed the highest fluorescence. Also, for this reporter the background fluorescence was much lower. The promoter controlling GFP was highly inducible, as can be seen in Figure 7G. However, the difference in fluorescence in the cultures with and without m-toluate was lower than for RFP. The uninduced samples had fairly similar fluorescence levels (Figure 7H). Considering the results for GFP, it could indicate interference between the measurements with YFP- and GFP-settings. When measuring with CFP-settings, all of the samples displayed a relative high fluorescence, even the control pSB-M1b (Figure 7E). The background fluorescence from *P. fluorescens* was high and there were interference of the fluorescence of the other reporters. Inducing the samples with m-toluate had little to no effect.

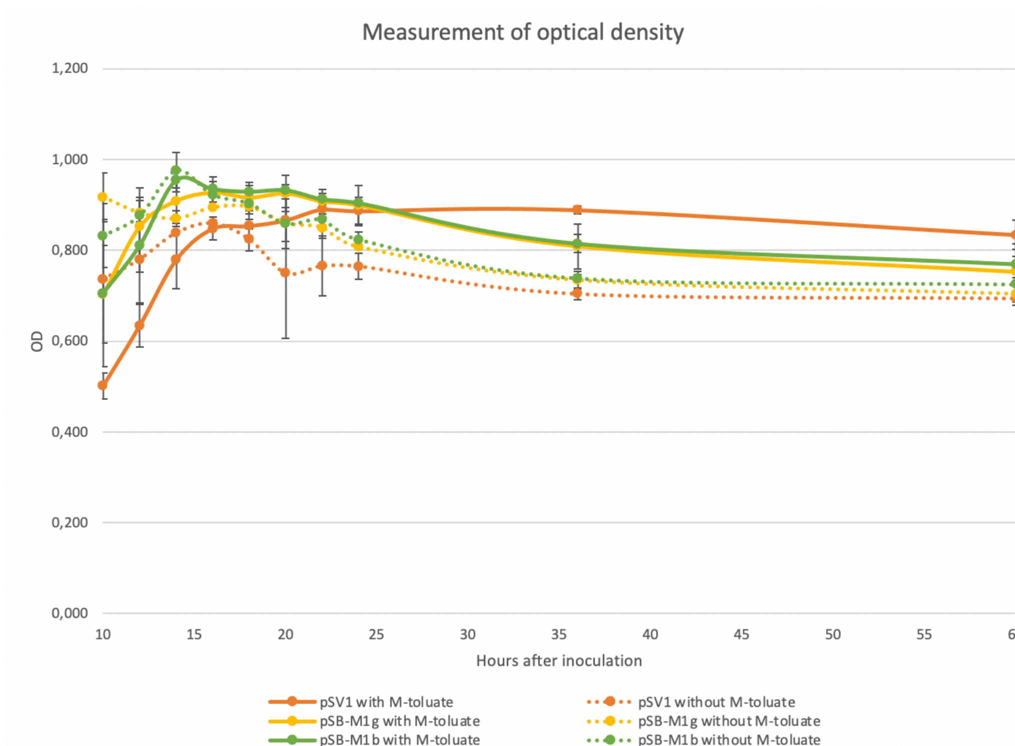


Figure 8: Results from measuring OD_{600} in pSV1, pSB-M1g and pSB-M1b. *There were 6 samples for each plasmid where 3 samples contained the inducer m-toluate and the other 3 did not. Each graph shows the average of three independent cultures The dotted lines indicates samples without m-toluate, while the solid lines indicate samples with m-toluate. Standard deviation error bars are shown on the graph.*

The OD-graph in Figure 8 shows that pSB-M1g and pSB-M1b with m-toluate reached stationary phase about 15 hours after inoculation, whereas pSV1 with m-toluate reached the same phase 20 hours after inoculation.

4.2 Evaluation of the strength of different promoters in *P. fluorescens*

The results in the previous section led to RFP being chosen as a good reporter gene to use in further experiments. The next step was to clone different promoters in front of RFP in order to evaluate their strength. Five different promoters were chosen: P_{BAD}, P_m, P_m ML1-17, P_{mG5} and P_{trc}. The reporter gene RFP was cloned from pSV1. Figure 9 and 11 shows plasmids that were used and which promoter they contained. Maps of the plasmids constructed are shown in Appendix A.

4.2.1 Construction of vectors with promoters and RFP

The construction of the plasmids pSV2, pSV3, pSV4, pSV5 and pSV6 is presented in this section. Figure 9 shows the cloning process to make pSV2, pSV3 and pSV4. The *rfp* gene

was cut from pSV1 by digestion with NdeI and BamHI-HF. The vectors pSB-M1g-1-17, pSB-M1g and pSB-T1g were digested with the same enzymes to replace the *gfp* gene with *rfp* from pSV1. The ligated plasmids were transformed into competent *E. coli* S17-1. Plasmids from two or three of the transformants for each promoter were checked with the enzyme PvuI. Figure 10 shows that all transformants contain the desired plasmids with RFP. The plasmid from the transformant in lane 2 was chosen as pSV2. The plasmid from the transformant in lane 5 was chosen as pSV3. The plasmid from the transformant in lane 9 was chosen as pSV4.

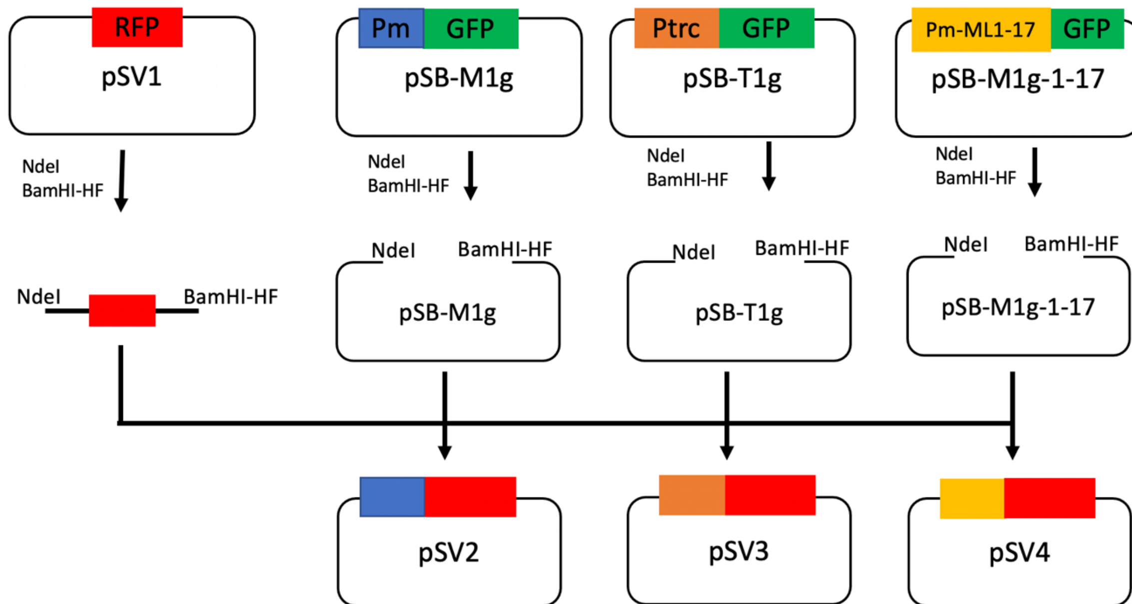


Figure 9: Flow diagram showing the cloning process to construct pSV2, pSV4 and pSV4. *RFP and GFP are shown in red and green colours, respectively.* P_m *is shown in blue,* P_{trc} *in orange and* P_m *ML1-17 in yellow. The restriction enzymes used are displayed in the figure.*

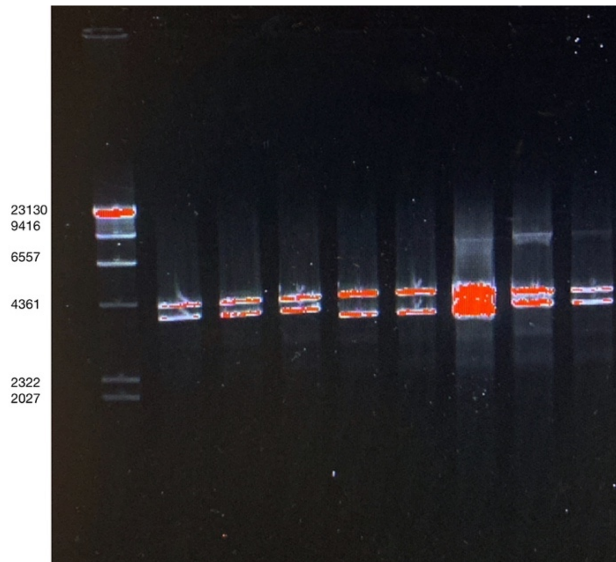


Figure 10: Verification of pSV2, pSV3 and pSV4 with gel electrophoresis. *Lane 1 show the DNA ladder HindIII with its known molecular sizes. Lane 2-8 shows the plasmids isolated from 8 transformant colonies and digested with the enzyme PvuI. Lane 2-4 contains pSV2.. Lane 5-7 contains pSV3. Lane 8 and 9 contains pSV4. The expected fragments of pSV2, and pSV4 digested with PvuI were 4236bp and 3785bp. The expected fragments of pSV3 digested with PvuI were 4248bp and 3515bp. PvuI cuts the vectors only once, so a religated vector would result in only one band.*

The cloning process to construct pSV5 and pSV6 is shown in Figure 11. In order to clone RFP into pSB-B1g to make pSV5, P_{BAD} and the regulator Arac was cut out of pSB-B1g and cloned into pSV2 to replace P_m and Xyls. Both plasmids were digested with NdeI and XhoI.. The ligated plasmid was transformed into *E. coli* S17-1. To check the transformants, EcoRV-HF was used. All four transformants were shown to contain the desired plasmid, hereby denoted as pSV5. The plasmid from transformant 1 was chosen as pSV5 (Figure 12).

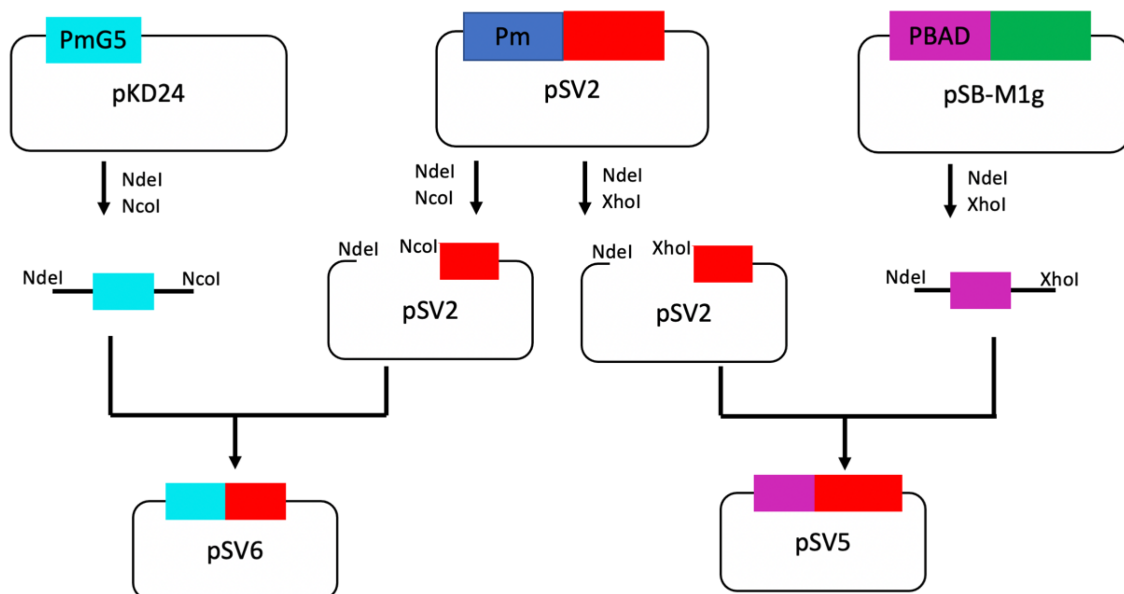


Figure 11: Flow diagram showing the cloning process to construct pSV5 and pSV6. *The P_{mG5} promoter is shown in a light blue colour while P_{BAD} is shown in purple. RFP and GFP are shown in red and green colours, respectively. The restriction enzymes used are displayed in the figure.*

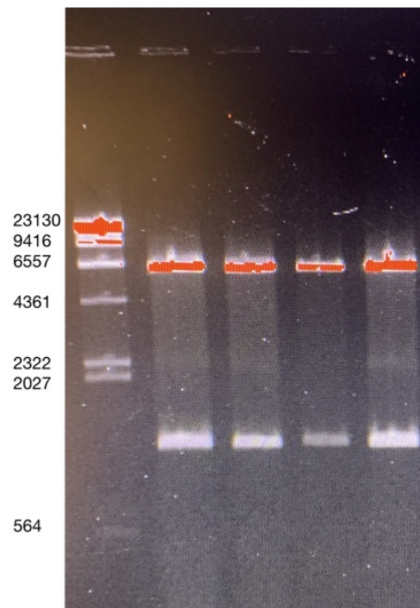


Figure 12: Verification of pSV5 with gel electrophoresis. *Lane 1 show the DNA ladder HindIII with its known molecular sizes. Lane 2-5 shows the plasmids isolated from four transformant colonies and digested with EcoRV. The expected fragments for pSV5 were 6020bp and 1181bp. All samples contain these fragments. In the case of a religated vector, EcoRV would cut only once.*

To make the vector with the P_{mG5}-promoter and RFP (pSV6) the plasmids pKD24 and pSV2 were digested with NdeI and NcoI. To check the transformants, XbaI and XhoI were used in the digestion of the isolated plasmids. The results from the gel electrophoresis are shown in Figure 13, and indicates that the plasmid constructed is either pSV6 or the religated vector pSV2. The plasmid from transformant 1 in Figure 13 was chosen as a possible pSV6. As there were only three base pairs in difference between pSV6 and pSV2, sequencing was performed to distinguish between the two. As the plasmids had a low copy-number, a PCR-product was made prior to the sequencing to make sure there were enough template for the Sanger sequencing protocol. To make the PCR-product, the primers 2900 and 385Bam (Appendix D) was used. The sequencing results (Appendix I) showed that the plasmid had the correct sequence.

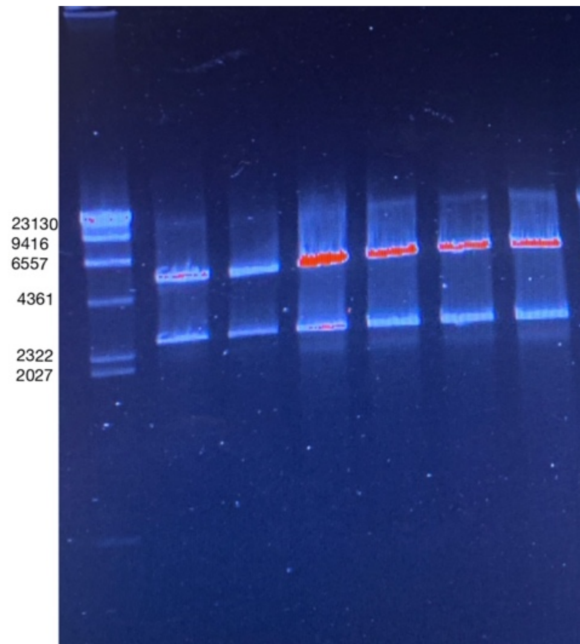


Figure 13: Verification of possible pSV6 with gel electrophoresis. *Lane 1 show the DNA ladder HindIII with its known molecular size. Lane 2 to 7 shows the six isolated plasmids from six transformant colonies. The plasmids were digested with XbaI and XhoI. The expected fragments for pSV2 and pSV6 were 5338bp +2683bp and 5338bp and 2680bp , respectively.*

4.2.2 Measurement of promoter strength using RFP as a reporter

The plasmids made as described in 4.2.1 were conjugated to *P. fluorescens* SBW25. pSB-M1g was used as a control in the fluorescence measurement as this plasmid did not contain RFP and it was shown in section 4.1.2 that RFP and GFP did not affect each other's fluorescence. Cultures with OD_{600}=0.05 were made as described previously. The cultures were induced 8 hours after inoculation. P_m, P_m ML1-17 and P_{mG5} were induced with 1mM m-toluate, P_{BAD} with 0.015% arabinose and P_{trc} with 0.2mM IPTG. Fluorescence and OD was measured 8, 12, 24 and 48 hours after inoculation. Raw data from the promoter study is shown in Appendix F.

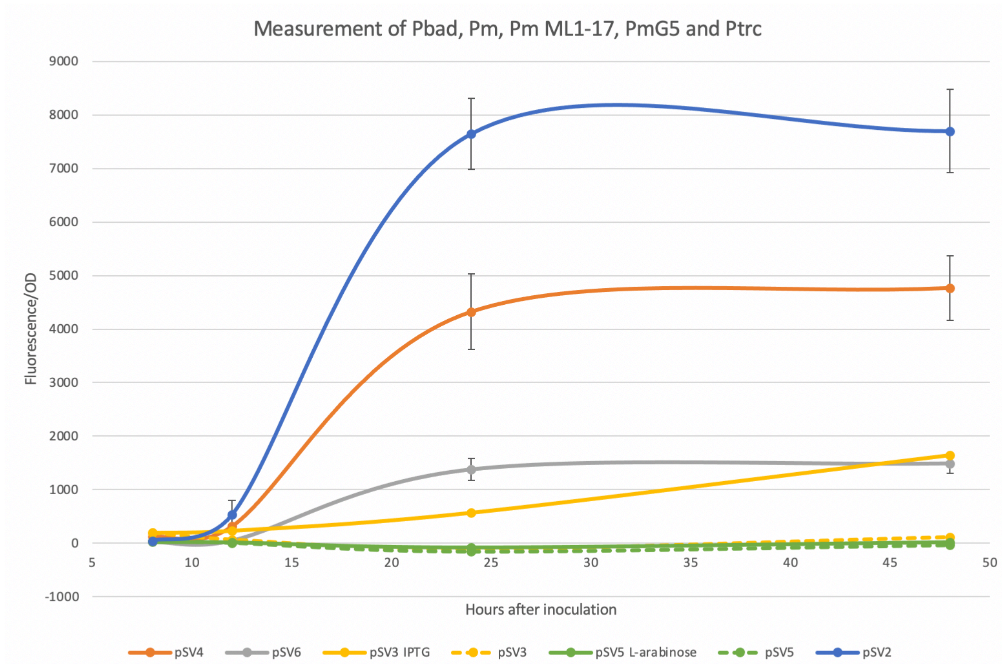


Figure 14: Results from measuring fluorescence of RFP in five different plasmids. *Each plasmid had three parallels, and each point on this graph show the average of three independent cultures. Standard deviation error bars are shown on the graph. For pSV2, only two of the three parallels have been used in calculations in order to avoid a high standard deviation.*

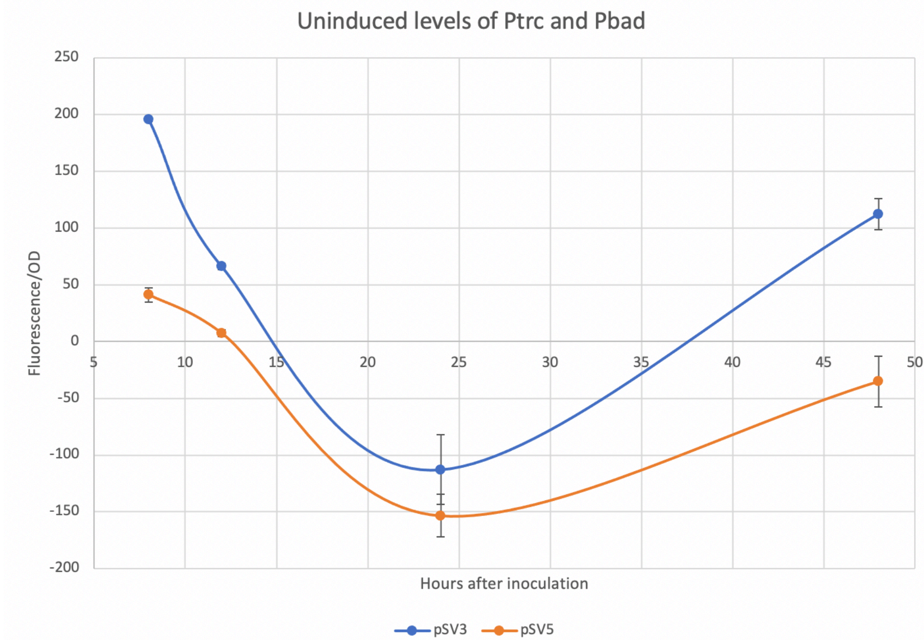


Figure 15**: **Results from measuring fluorescence of RFP in cultures with pSV3 and pSV5 in which the promoters P_{trc} and P_{BAD} were not induced. *Each plasmid had three parallels, and each point on this graph show the average of three independent cultures. Standard deviation error bars are shown on the graph.*

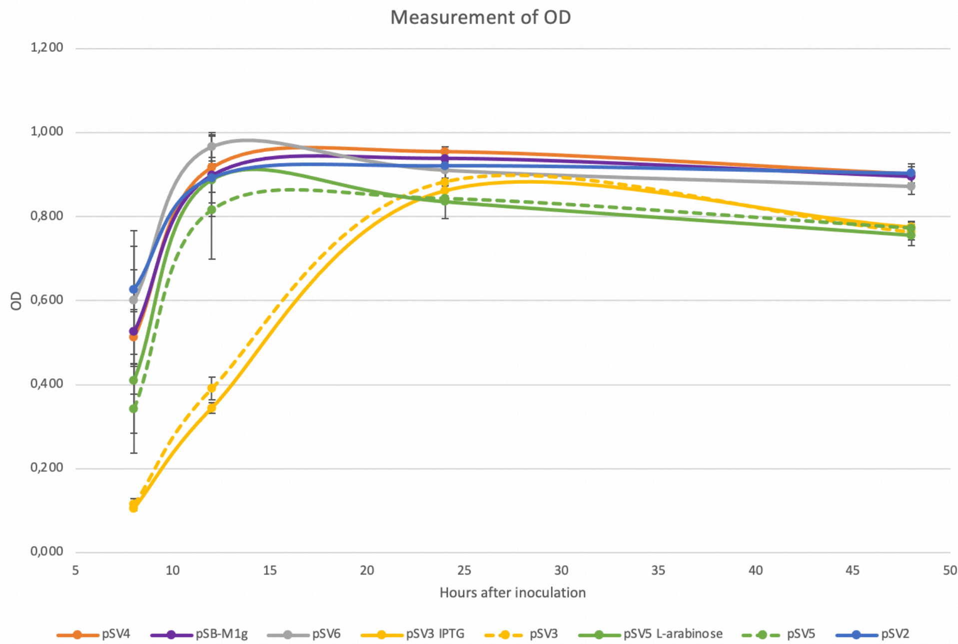


Figure 16: Results from measuring OD in cultures of five different plasmids. *Each plasmid had three parallels and each point on this graph show the average of the three independent cultures. Standard deviation error bars are shown on the graph. For pSV2, only two of the three parallels have been used in calculations in order to avoid a high standard deviation.*

It is clear that the P_m promoter was the strongest, and that its variants displayed different levels of strength. The original P_m promoter was the strongest, P_m ML1-17 was slightly weaker while P_{mG5} was, as expected, the weakest. For pSV2, which contained P_m, one of the parallels was very different from the other two results, creating a large standard deviation. For this reason, only two of the parallels were used in calculations when constructing the graphs shown in Figure 14 and 16. For P_{trc}, there was some effect when induced with IPTG. When comparing the OD_{600} and fluorescence of RFP when P_{trc} were induced with IPTG in Figure 14 and 16, the fluorescence increased even though the cells had reached stationary phase. This could indicate that P_{trc} could be induced in the stationary phase. However, further experiments were necessary in order to confirm this. Figure 15 shows that the fluorescence produced by RFP when the promoters P_{trc} and P_{BAD} were not induced, was lower than the fluorescence produced by *P. fluorescens* itself. These results indicated that P_{trc} and P_{BAD} could only be used in *P. fluorescens* if they were induced. The OD_{600} measurements in Figure 16 showed that all cultures, expect the one that contained pSV3, reached stationary phase 12 hours after inoculation. The culture with pSV3, both induced and uninduced, reached this phase around 25 hours after inoculation. The pre-cultures of pSV3 had to be incubated for 48 hours to reach OD_{600}=0.05 because there was little growth after overnight incubation. When inoculating the pre-cultures, plates with colonies containing pSV3 that had been stored in +4°C for some time

were used. Most likely these bacteria were dead or in a resting stage, which could explain the long lag phase observed in these cultures. To verify this hypothesis and that the plasmid itself did not cause the problem, a pre-culture was made by inoculation from a bacterial glycerol stock with pSV3. After overnight incubation the culture was clearly turbid.

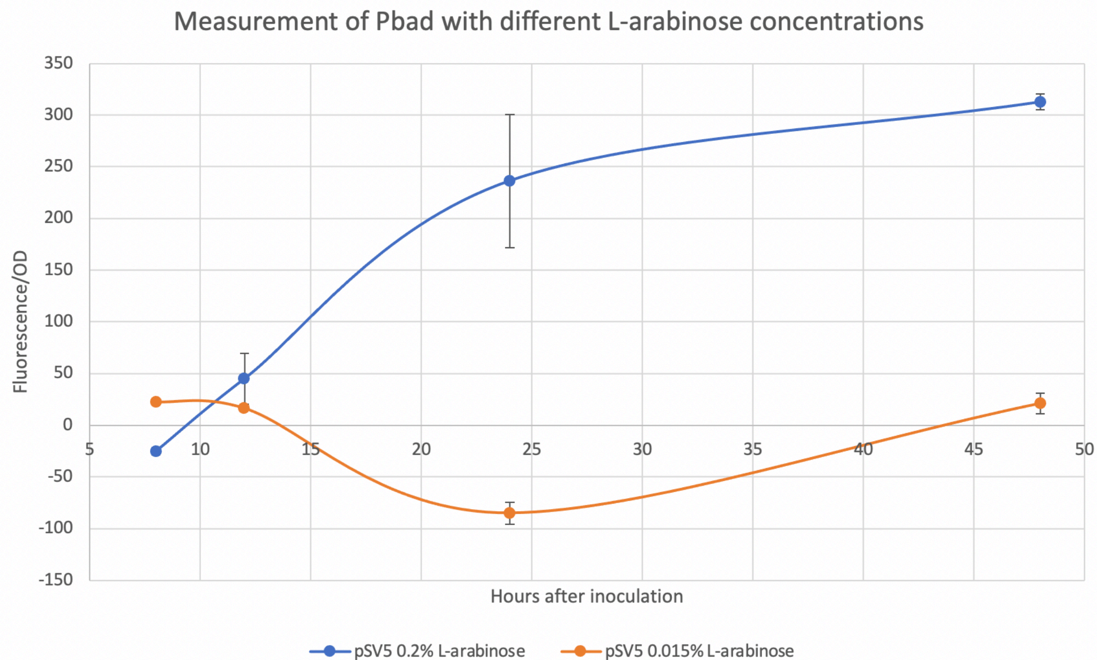


Figure 17: Fluorescence of RFP under control of P_{BAD} induced with 0.015% and 0.2% L-arabinose. *Each plasmid had three parallels and each point on this graph show the average of the three independent cultures. Standard deviation error bars are shown on the graph. The orange line indicates P_{BAD} induced with 0.015% L-arabinose while the blue line indicates P_{BAD} induced with 0.2% L-arabinose.*

The results displayed in Figure 14 shows that P_{BAD} did not function in *P. fluorescens* and that induced and uninduced levels were similar and low. A new experiment was performed where P_{BAD} was induced with more L-arabinose; a final concentration of 0.2% instead of 0.015%. This was done to find out whether P_{BAD} required a higher concentration of L-arabinose to be induced. Figure 17 shows results from both measurements for comparison reasons. The results indicates that increasing the L-arabinose concentration had a small effect on P_{BAD}, but the fluorescence values were still low.

4.2.3 New measurement to investigate a possible stationary phase promoter

A new measurement of the P_{trc} promoter, but with some altered conditions, was performed in order to evaluate the hypothesis from the first measurement that P_{trc} could be turned on in the stationary phase. In the previous measurement, the effect of inducing the promoters were observed. The samples were induced and measured 24 hours after inoculation. An additional measurement was performed 48 hours after inoculation. Figure 18 show the results of the

measurements, as well as the fluorescence-values from the first measurements for comparison. The fluorescence in the second measurements is lower than in the first, however, the results indicate the same thing – that P_{trc} was in fact inducible in the stationary phase. As seen in Figure 18, the cells were in the stationary phase when P_{trc} was induced, 24 hours after inoculation.

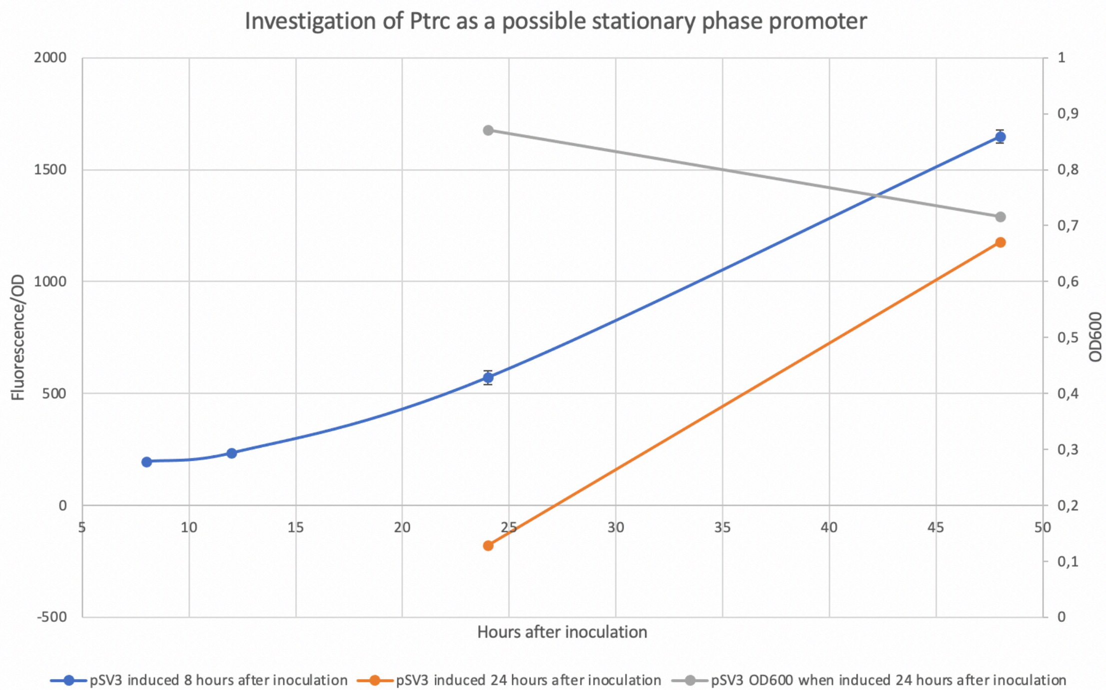


Figure 18: Investigation of P_{trc} as a possible stationary phase promoter. *Each plasmid had three parallels and each point on this graphs show the average of the three independent cultures. Standard deviation error bars are shown on the graph. The primary Y-axis shows fluorescence/OD and the secondary y-axis show the OD_{600}-values for pSV3 in the second measurement. The blue line indicates the first measurement of pSV3 and the orange line indicates the second measurement.*

4.4 Test of a c-di-GMP reporter system in *P. fluorescens*

As described in section 2.2.3, a method for easy quantification of c-di-GMP is interesting because it could help investigate whether there is a measurable difference in c-di-GMP in *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA*. The fluorescent c-di-GMP reporter system was discovered in *Bacillus thuringiensis* sups. *chinensis* CT-43 and characterized by Zhou et al. (3). The fluorescence produced by the reporter genes *amcyan* and *turboRFP*, gave an indication of the c-di-GMP levels inside the cell in *B. thuringiensis.* In this section, the reporter system was tested in *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA*. The plasmid pFY4535 containing the reporter system is shown in Figure 3 in section 2.3.3.

pFY4535 was conjugated into *P. fluorescens* SBW25 and into the alginate producing strain *P. fluorescens* SBW25 *mucA*. The wild type strain *P. fluorescens* SBW25 without the plasmid was used as a blank in the fluorescence measurements. Optical density and the fluorescence of AmCyan and TurboRFP was measured 10, 12, 24, 34, 48 and 64 hours after inoculation of the cultures from pre-cultures.

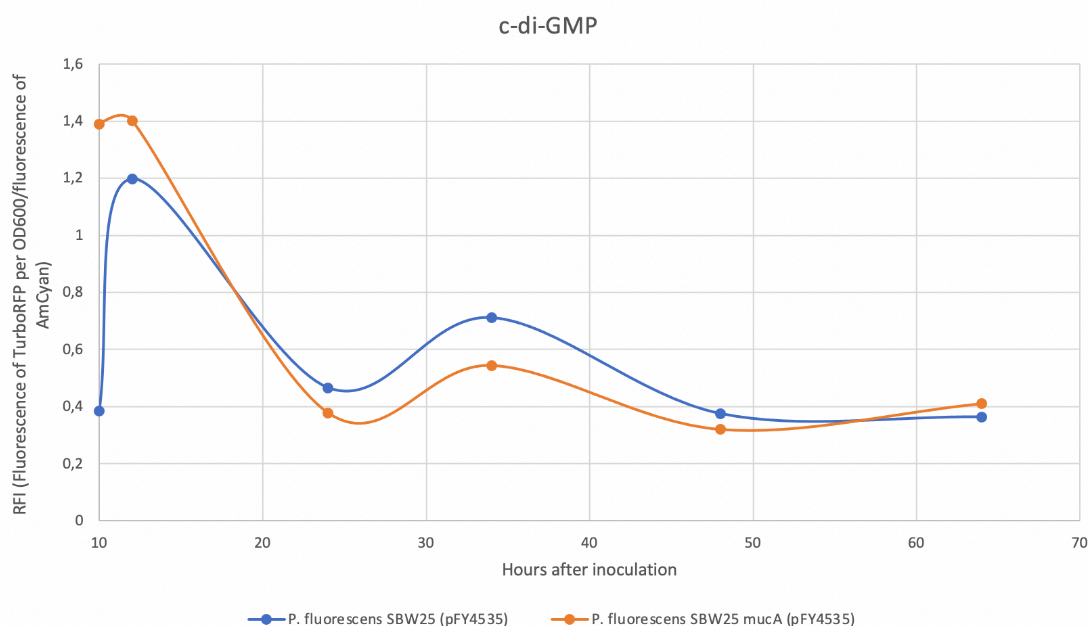


Figure 19: Test of c-di-GMP reporter system in P. fluorescens SBW25 and P. fluorescens SBW25 mucA. *The Bc3-5 dual-fluorescence c-di-GMP reporter in the plasmid pFY4535 were used to quantify intracellular c-di-GMP concentrations. RFI represents the relative fluorescence intensity of TurboRFP and AmCyan, and is directly proportional to c-di-GMP concentrations. Each point on this graphs show the average of the three independent cultures.*

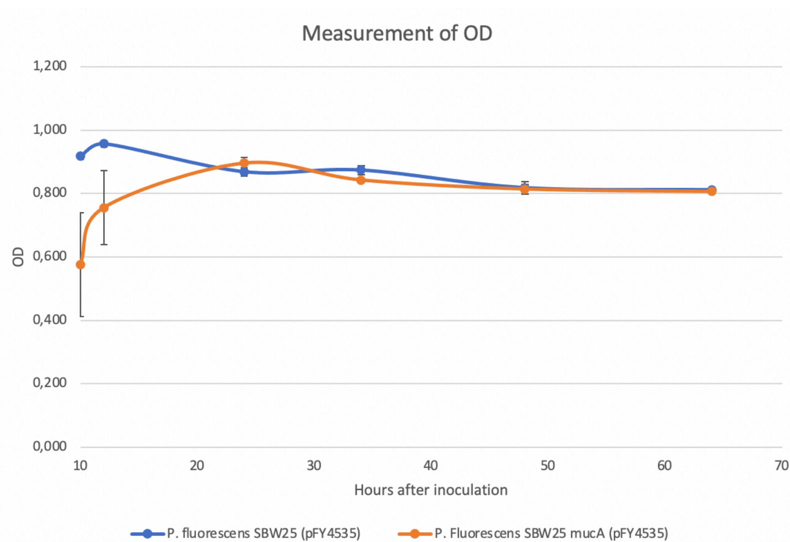


Figure 20: Measurement of OD in P. fluorescens SBW25 and P. fluorescens SBW25 mucA. *Each point on this graphs show the average of the three independent cultures. OD was measured 10, 12, 24, 34, 48 and 64 hours after inoculation. Standard deviation error bars are shown on the graph.*

Figure 19 gives an indication that c-di-GMP could be measured in *P. fluorescens* by using the c-di-GMP reporter system from Zhou et al. (3). It also shows that there is a correlation between RFI in *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA*. The second measurement, 12 hours after inoculation, indicates that both strains already had reached a peak in RFI-values. However, to know if that was the actual peak, earlier measurements would have been necessary. According to Figure 20, *P. fluorescens* SBW25 reached stationary phase before *P. fluorescens* SBW25 *mucA*.

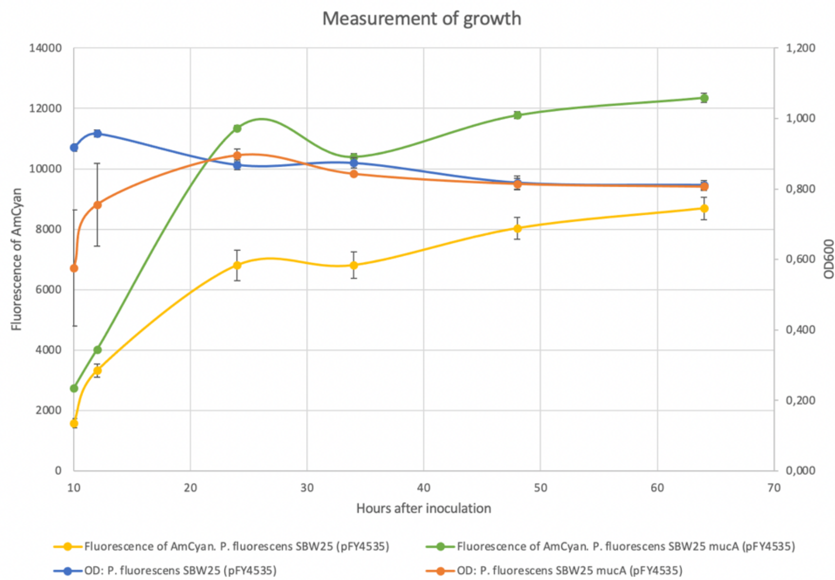


Figure 21: Measurement of growth in P. fluorescens SBW25 and P. fluorescens SBW25 mucA. *Each point on this graphs show the average of the three independent cultures. The green and yellow lines indicate the fluorescence of AmCyan measured in P. fluorescens SBW25 mucA (pFY4535) and P. fluorescens SBW25 (pFY4535), respectively. The blue and orange lines indicate the OD_{600} measured in P. fluorescens SBW25 (pFY4535) and P. fluorescens SBW25 mucA (pFY4535), respectively. The primary Y-axis show the fluorescence and the secondary Y-axis show the OD_{600}. For the fluorescence of AmCyan measured in P. fluorescens SBW25 mucA (pFY4535), only two of the three parallels have been used in calculations in order to avoid a high standard deviation. Error bars are shown on the graph.*

In Figure 21 the correlation between AmCyan and OD_{600} is shown. As can be seen in the graph, there was not any obvious correlation between the fluorescence of AmCyan and the OD_{600}, indicating that the fluorescence of AmCyan could not be used as a measurement of cell numbers.

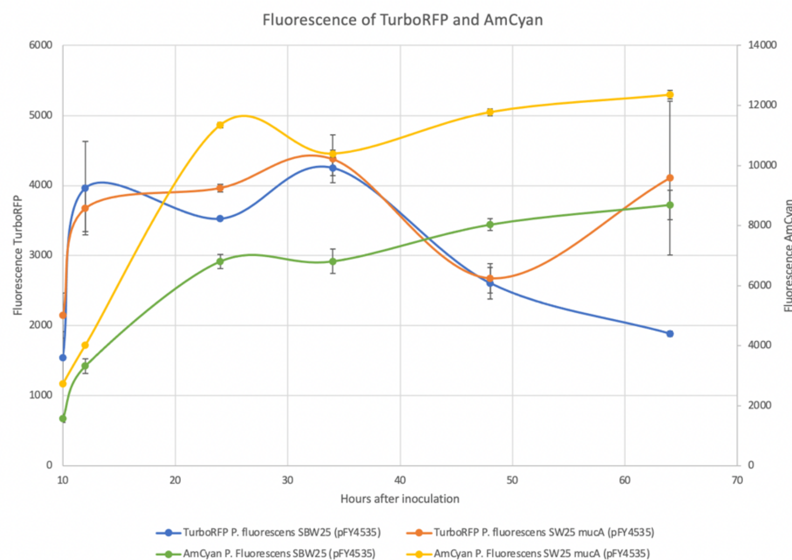


Figure 22: Fluorescence of TurboRFP and AmCyan in P. fluorescens SBW25 and P. fluorescens SBW25 mucA, both containing the reporter plasmid pFY4535. *The primary Y-axis show the fluorescence of TurboRFP while the secondary Y-axis show the fluorescence of AmCyan. For all measurements, except the ones for AmCyan in P. fluorescence SBW25 mucA (pFY4535), only two parallels have been used in calculations.*

The fluorescence of both proteins in both strains is shown in the graph in Figure 22. For some of the points, the standard deviation was high even though only 2 of the parallels were used in calculations to avoid this. However, the graph clearly shows that there was a difference between the amount of fluorescence produced by these proteins, which could be compared as the genes encoding the fluorescent proteins were controlled by the same promoter. As mentioned in section 2.3.2 , PFP2 is a strong constitutive promoter, and when the observed increase in fluorescence started to slow down it could be an indication of dead or inactive cells. Overall, the fluorescence of AmCyan was higher than the fluorescence of TurboRFP, but for both proteins it was the *mucA* strain that displayed the highest fluorescence. The raw data is shown in Appendix G.

4.3 Cloning of MucR

The c-di-GMP producing enzyme MucR is an important regulator of alginate biosynthesis in *P. aeruginosa*. Constructing strains where this protein is overproduced could give insights on how the alginate production is affected by this overproduction, as well as serve as a positive control when evaluating the c-di-GMP reporter system in *P. fluorescens.* To construct the overproducing MucR-strains, transposon vectors with the two promoters P_m and P_{trc} controlling MucR were to be used. Transposons were chosen since plasmids with homologous sequences are often recombined in *P. fluorescens,* and homologous recombination would not be ideal as it is more time-demanding than transposon mutagenesis. Another alternative would be to insert it as a self-replicating plasmid, however this was not done as experiences using this method often have been negative. Maps of the plasmids constructed are shown in Appendix A.

The promoters P_{trc} and P_m were chosen to regulate expression of *mucR.* pHE319 was chosen as a transposon vector as it already contained the P_m promoter. To make a transposon vector with P_{trc}, this promoter had to be inserted into pHE319. Following plasmid construction, the plan was to insert *mucR* into these transposon vectors, integrate the transposons into the genome of. *P. fluorescens* SBW25 *mucA* and measure alginate production in these two new strains. Partial digestion was first attempted in order to insert *mucR* into the two transposon vectors. When this failed, SLIC was performed. After several attempts , the experiment was terminated due to limited time left.

4.3.1 Clone mucR into TOPO-vector and partial digestion to insert mucR

As *mucR* contained an internal NdeI-site, partial digestion was performed. However, before attempting partial digestion, the gene had to be cloned into a TOPO vector. The *mucR* gene was amplified through PCR from *P. fluorescens* SBW25 with the primer pair MucRR and MucRF (Appendix D). The PCR-product (2155bp) was cloned into a TOPO vector and transformed into competent *E. coli* S17-1. Plasmids from colonies of the transformants were tested with the restriction enzymes BamHI-HF and SacII. Two of the isolated plasmids from the transformants were sequenced with primers mucrsekv1, mucrsekv2, M13 forward (-20) primer and M13 reverse primer (Appendix D).

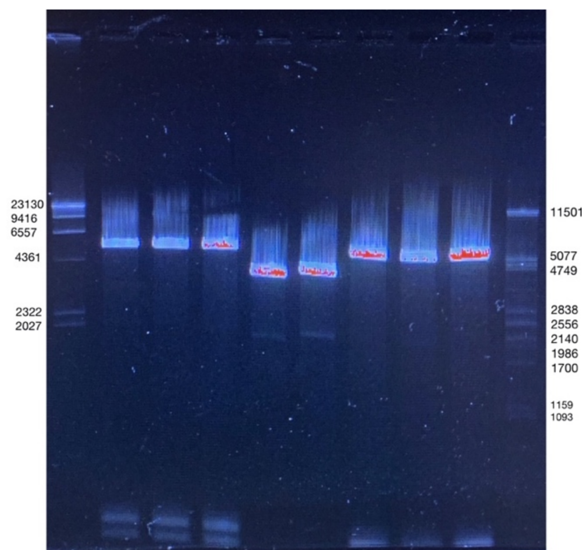


Figure 23: Investigate orientation of mucR gene in TOPO-vector. *Lane 1 and 10 shows the DNA ladders HindIII and pstI, respectively, with their known molecular sizes. Expected fragment size for the TOPO-vector with mucR inserted in the clockwise direction digested with BamHI-HF and SacII were 3517bp + 1682bp + 267bp + 208bp. Expected fragment size for the TOPO-vector with mucR inserted in the counter-clockwise direction digested with BamHI-HF and SacII were 5115bp + 273bp + 208bp + 78bp.*

The gel image in Figure 23 shows that lane 2, 3, 4, 7, 8 and 9 contain plasmids with *mucR* inserted in a counter-clockwise orientation while lane 5 and 6 likely contain the TOPO-vector without *mucR* inserted. The sequencing results in Appendix J shows that the plasmid in lane 2 has the *mucR* gene inserted in the counter-clockwise direction. This plasmid was therefore denoted pSV7. Before receiving the sequencing results, it was believed that the two plasmids in lane 5 and 6 contained the TOPO-vector with *mucR* inserted in the clockwise direction and that the reason for the two smallest bands not showing on the gel was that it was likely run for too long. Multiple attempts at partial digestion with NdeI and NotI on one of these plasmids were therefore carried out, but without success. The sequencing results, confirmed that *mucR* was not inserted into the TOPO-vector in a clockwise direction, explaining why partial

digestion did not give the expected results. Instead of partial digestion, one-step SLIC was carried out to get *mucR* inserted into the transposon vectors.

4.3.2 Construction of transposon vector with the P_{trc} promoter

Before attempting one-step SLIC, the transposon vector with P_{trc} had to be made. The P_{trc} and *lacI* genes were amplified through PCR with the primers ReverseNdeI and ForwardBsiWI to generate NdeI and BsiWI restriction sites. The amplified PCR product of 1518bp were then cloned into a TOPO vector to generate the plasmid denoted pSV8. Next, pSV8 and pHE319 were digested with NdeI and BsiWI to replace P_m and XylS from pHE319 with P_{trc} and lacI from pSV8. The ligated plasmid was transformed into *E. coli* S17-1 λpir. Plasmids isolated from transformants were checked with AflIII, and all the transformants were found to contain the plasmid now denoted as pSV9. The transformant in lane 2 in Figure 24 was used as pSV9 in further experiments.

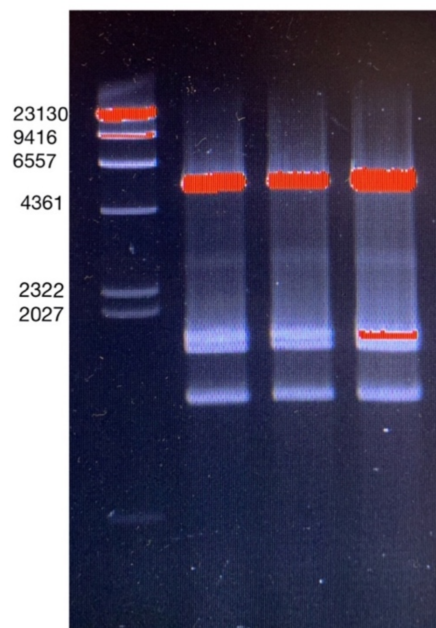


***Figure 24: Verification of pSV9 with gel electrophoresis**. Lane 1 shows the DNA standard HindIII. Plasmids from three transformants were digested with AflIII. Expected fragments of pSV9 digested with AflIII were 5183, 1708, 1605 and 1156bp. Expected fragments of pHE319 digested with AflIII were 5183, 2706, 1708, 569 and 103bp.*

4.3.3 Construction of transposons with mucR through one-step SLIC

To insert the *mucR* gene into the transposon vectors pHE319 and pSV9, one-step sequence- and ligation-independent cloning (SLIC) was performed as shown in Figure 25. The vectors pSV9 and pHE319 were linearized by digestion with NotI and NdeI. The insert was amplified from pSV7 or from an ON-culture of *P. fluorescens* SBW25 by PCR using the primers

slicreverseny and either slicFptrc0903 for pSV9 or slicFpm0903 for pHE319, creating a fragment of 2139 bp. The cloning procedure was performed a number of times, but without success. Due to a limited time left, the experiment was terminated.

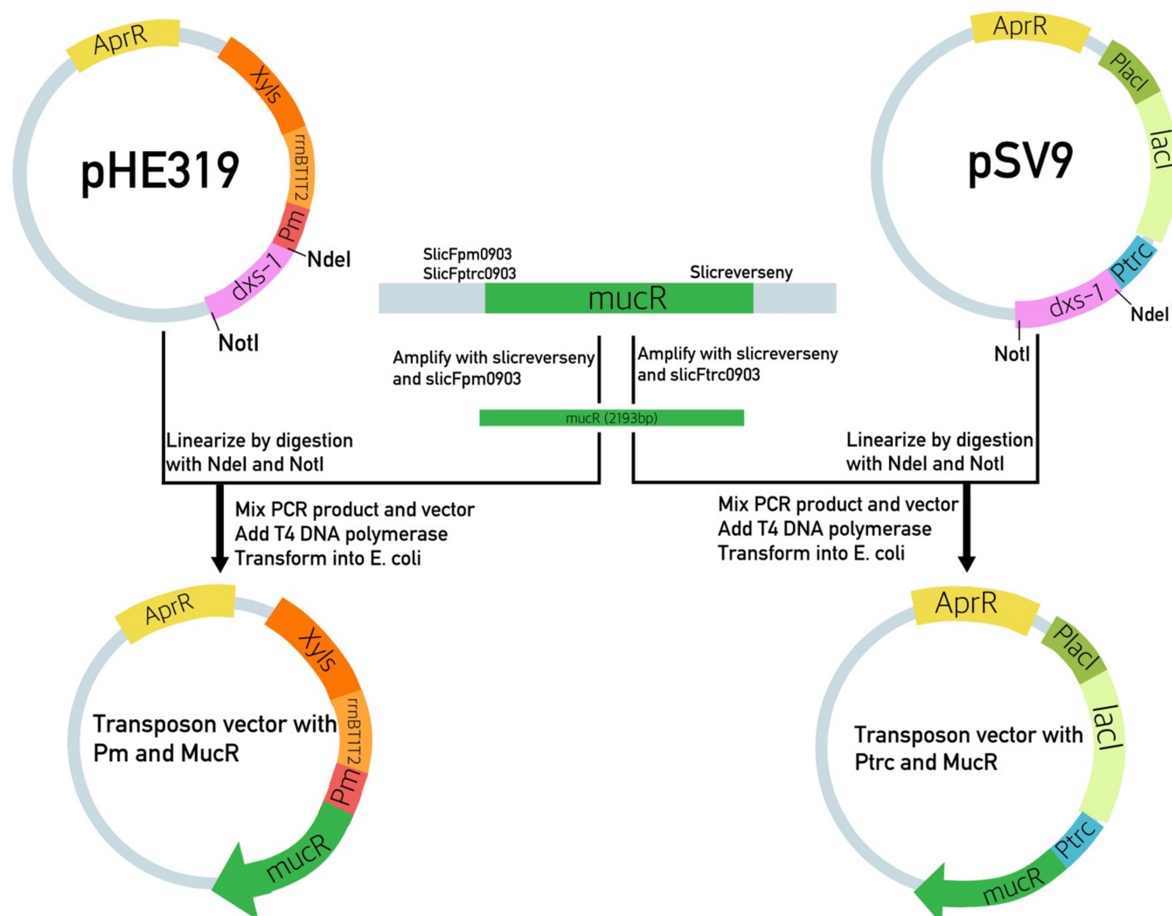


Figure 25: Cloning strategy for SLIC. *The primer slicreverseny was used in combination with either slicFpm0903 or slicFtrc0903 to create transposon vectors with the mucR gene, which was amplified from P. fluorescens SBW25 or pSV7. The cloning was performed as described in section 3.17.*

4.5 Studies on the effect of a stationary phase promoter on *algC* and this system´s effect on alginate production

In section 3.2.2 it was shown that the transcription of the P_{trc} promoter could be induced in the stationary phase. This was to be exploited to study the *algC* gene and its influence on alginate production when regulated by a stationary phase promoter. Maps of the plasmids constructed are shown in Appendix A.

4.5.1 Construction of vector with algC under control of the P_{trc} promoter

A two-step cloning process was carried out to construct a plasmid with the P_{trc} promoter in front of *algC*. The cloning steps are displayed in Figure 26. Previously, the plasmid pHE228 have been used to put *algC* under control of P_m instead of P_{algc} (Ertesvåg, personal communication). First, pHE228 and pSB-T1g was digested with NdeI and SphI to replace P_m and *Xyls* in pHE228 with P_{trc} and *lacI* from pSB-T1g. Next, the resulting plasmid pSV10 and pHNB4 were digested with AvrII and NotI to replace the topocloning site in pHNB4 with P_{trc}, *algC, lacI*, *placI and oppalgC* from pSV10 to construct pSV11.

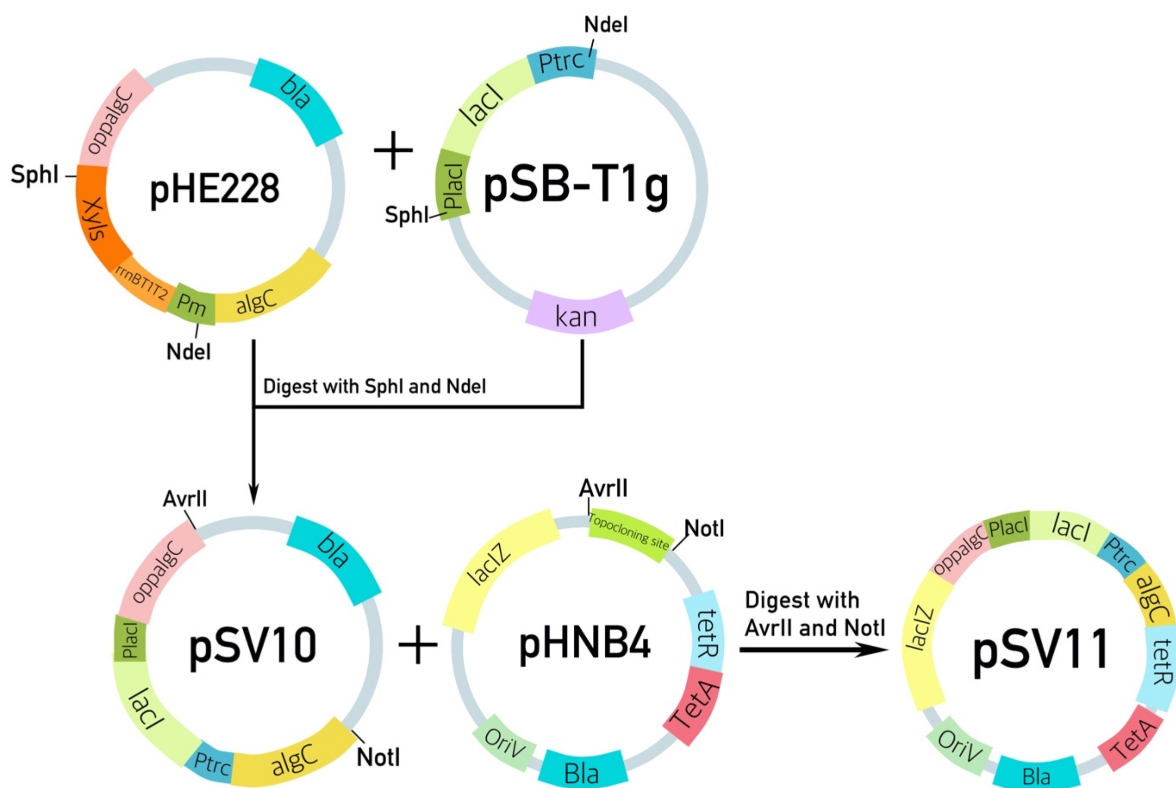


Figure 26: Diagram showing the cloning process to produce pSV10 and pSV11. *Restriction enzymes are shown in the figure. pHE228 and pSB-T1g were used to construct pSV10. pSV10 and pHNB4 were used to construct pSV11. Restriction enzymes are shown in the figure.*

When pSB-T1g was digested with NdeI and SphI, the resulting fragments were not of expected sizes. It was hypothesized that pSB-T1g contained an extra SphI-seat. This was confirmed by digesting the plasmid with NdeI, SphI and MluI as well as with SphI only and with NdeI, SphI and NotI (Figure 27).

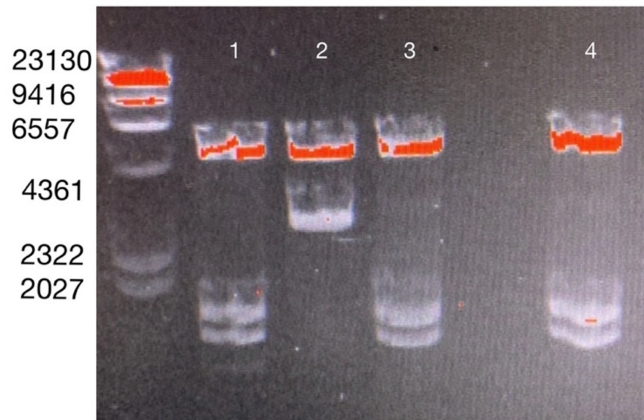


Figure 27: Diagnostic restriction digestion of pSB-T1g, as well as digestion with NdeI and SphI. *1: pSB-T1g digested with NdeI, SphI and MluI. Expected bands: 6093, 1038 and 439bp. 2: pSB-T1g digested with SphI. Expected band: 7570bp. 3: pSB-T1g digested with NdeI, SphI and NotI. Expected bands: 6093 + 1477. 4: pSB-T1g digested with NdeI and SphI: Expected bands: 6094 + 1477bp.*

With this information, the bottom two bands of pSB-T1g digested with NdeI and SphI were cut out. Both bands were ligated with the fragment from pHE228. A diagnostic restriction digestion with SnaBI and BspHI was performed on plasmids from transformants to see if one of the two fragments produced the expected fragments of pSV10 (Figure 28A). Three transformants with the upper band inserted into pHE228 were shown to be correct. An additional digestion of transformant number 3 from Figure 28A with EcoRV proved that it contained the expected product and not the religated vector pHE228 (Figure 28B).

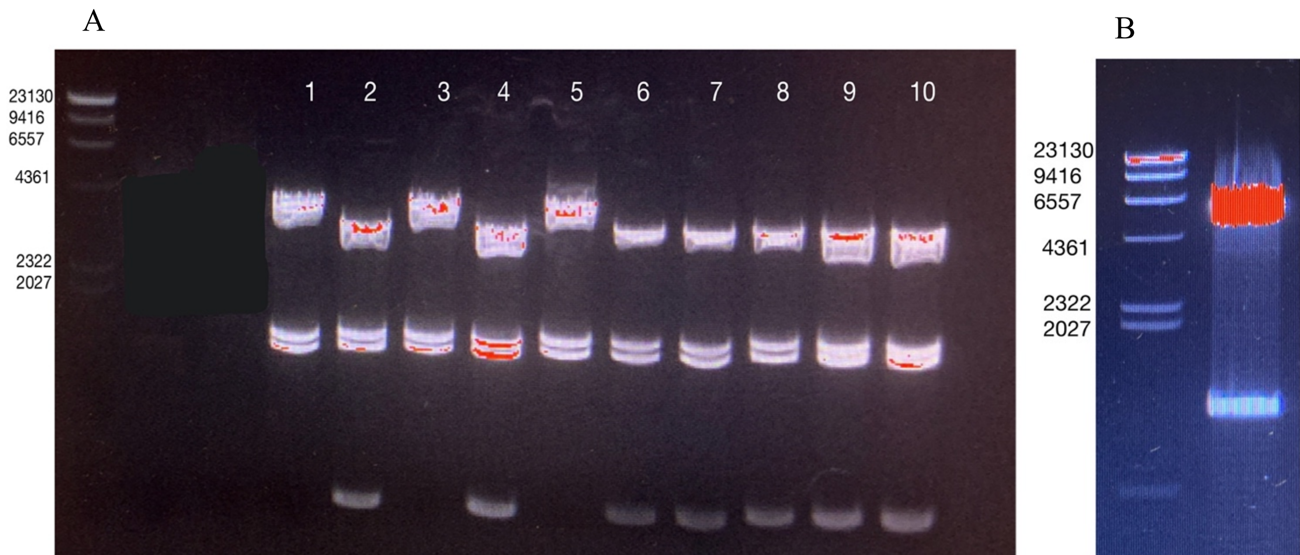


Figure 28A: Transformants of pHE228 with either the top or bottom band of pSB-T1g inserted digested with SnaBI and BspHI. *Lane 1 show the DNA standard HindIII. 1-5 are plasmids with the top band and 6-10 are plasmids with the bottom band. Expected fragment sizes for pSV10 digested with SnaBI and BspHI were 3900, 1499 and 1342bp. Expected fragment sizes for pHE228 digested with SnaBI and BspHI were 2303, 1933, 1499 and 1342bp.*

Figure 28B**: **Transformant number 3 digested with EcoRV to distinguish between pSV10 and pHE228. *Expected fragment sizes for pHE228 digested with EcoRV were 7077bp. Expected fragment sizes for pSV10 digested with EcoRV were 5684 and 1057bp.*

The resulting plasmid, denoted pSV10, and pHNB4 were then digested with NotI and AvrII. This replaced the insert in pHNB4 with the *oppalgC, lacI,* P_{trc} and *algC* genes from pSV10. Isolated plasmids from transformants were digested with SnaBI and BspHI, and two transformants were shown to contain the desired plasmid (Figure 29). The plasmid in lane 3 was denoted pSV11. pSV11 could then be used to replace the P_{algC} in *P. fluorescens* with P_{trc}.

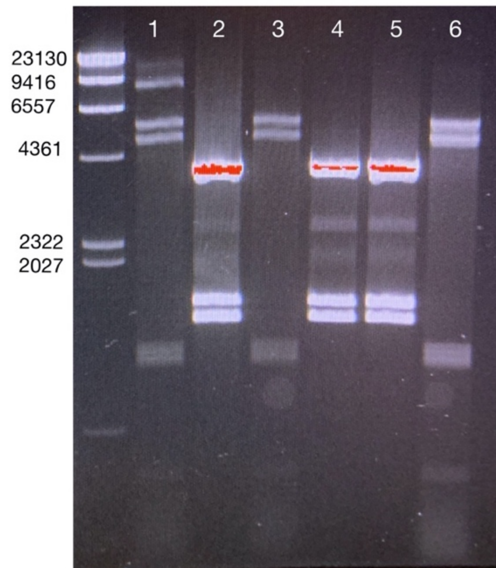


Figure 29: Verification of pSV11 with gel electrophoresis. 6 transformants were digested with SnaBI and BspHI*. Lane 1 show the DNA standard HindIII with its expected sizes. Expected fragment sizes for pSV10 digested with SnaBI and BspHI were 3900, 1499 and 1342bp. Expected fragment sizes for pSV11 digested with SnaBI and BspHI were 5618, 4945, 1066, 1008, 967 and 366bp. Expected fragment sizes for pHNB4 digested with SnaBI and BspHI were 7912, 1066, 1008, 967 and 366bp.*

4.5.2 Construction of P. fluorescens SBW25 mucA strain with algc under control of P_{trc}.

First, pSV11 was conjugated to *P. fluorescens* SBW25 *mucA* and transconjugants were plated out on PIA-medium with tetracycline and X-Gal. The process of homologous recombination is shown in Figure 30. Most of the colonies were blue and mucoid, indicating that the plasmid had been integrated into the genome. Two blue transconjugant colonies were grown in LB-medium without tetracycline and re-inoculated twice a day over several days. White mucoid colonies that formed when dilutions from these cultures were plated out on PIA-plates, were then further plated out on PIA-plates with and without tetracycline. The produced alginate led to larger colonies. Therefore, some colonies were later plated out on LA-plates. 66 colonies that grew on PIA or LA without Tc but not on plates with Tc, were tested with PCR with the primer pair PmalgCF and PmalgCR (Appendix D). PCR with these primers would result in a 152bp band in the wild-type and a 1606bp band in the mutant.

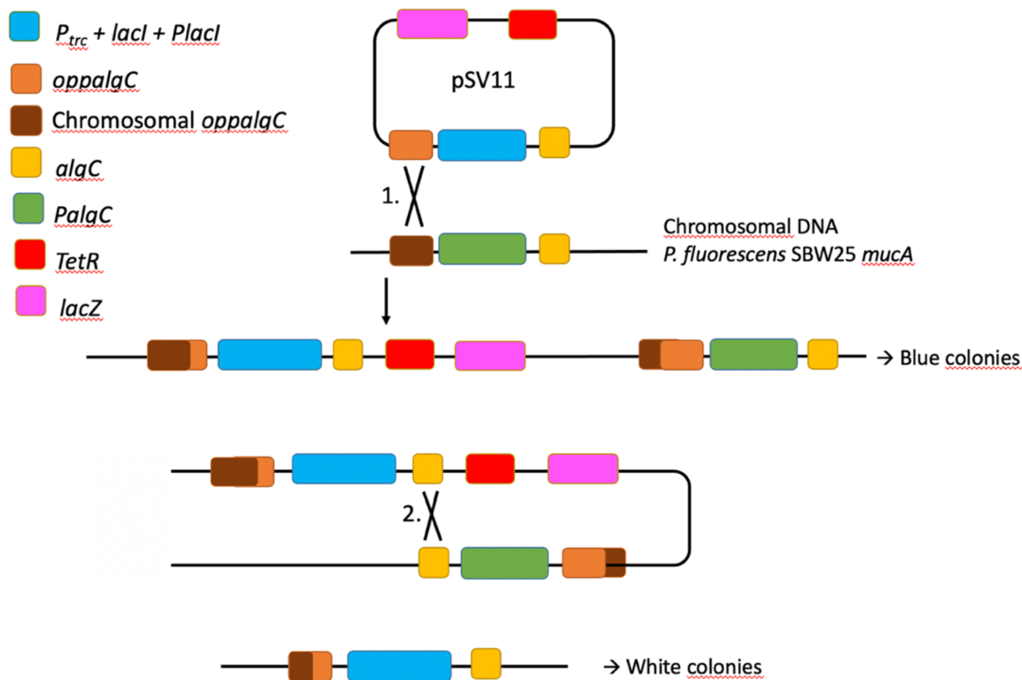


Figure 30: Replacement of P_{algC} with P_{trc} through homologous recombination in P. fluorescens SBW25 mucA. *The genes are colour-coded, and their explanation is shown in the upper left corner. In this figure, it is shown that the first recombination occurs at the oppalgC gene. However, it could also occur at algC. In addition, the second recombination could also occur at oppalgC, in which case the wild-type would be restored.*

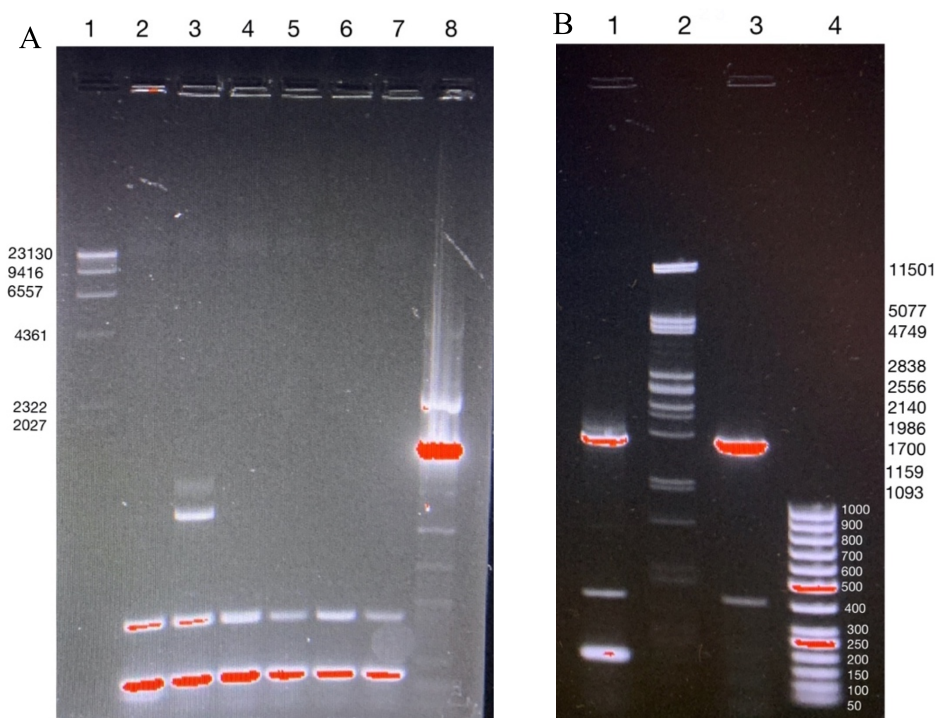


Figure 31: Gel electrophoresis of PCR-products to verify the insertion of the P_{trc} promoter in P. fluorescens SBW25 mucA. *Figure 32A shows the DNA standard HindII with its expected sizes in lane 1. Lane 2-7 contains samples that were checked, while lane 8 contains pSV11, which was used as a positive control. Figure 32B shows two of the 67 PCR-products that were checked. Lane 1 and 3 contains the two samples. Lane 2 contains the PstI-standard with its expected sizes shown in black writing. Lane 4 contains the GeneRuler 50bp DNA ladder with its expected sizes shown in white writing.*

Of the 66 colonies checked, one colony contained the mutant which resulted in only a fragment of 1606bp after PCR amplification (Figure 31B). Figure 31A shows the gel image containing pSV11, which was used as a positive control. This plasmid gave a band at 1606 bp, as expected. The sample in lane 3 in Figure 31B also had this band, and not the 152bp band of the wild-type which indicated that this colony contained the mutant strain, hereby denoted as *P. fluorescens* SBW25 *mucA Ptrc algC.*

If the second recombination were to alco occur at *oppalgC* instead of *algC*, wild-type would be restored but white colonies would still be observed, because the entire plasmid including the tetracycline resistance gene, would be recombined out. This is what most likely happened in the 65 other colonies that did not contain the mutant as these colonies were white and tetracycline sensitive. The gel images for when these colonies were run on an agarose gel is shown in Appendix E.

4.5.3 Measurement of alginate production by P. fluorescens SBW25 mucA Ptrc algC

In this experiment, the alginate production of the new strain with that of the parent strain was to be compared. Moreover, the effect of adding IPTG after 8 and 24 hours were tested. Samples were taken out 12, 24, 48 and 72 hours after inoculation. The alginate assay was carried out as described in section 3.17. The raw data is presented in Appendix H.

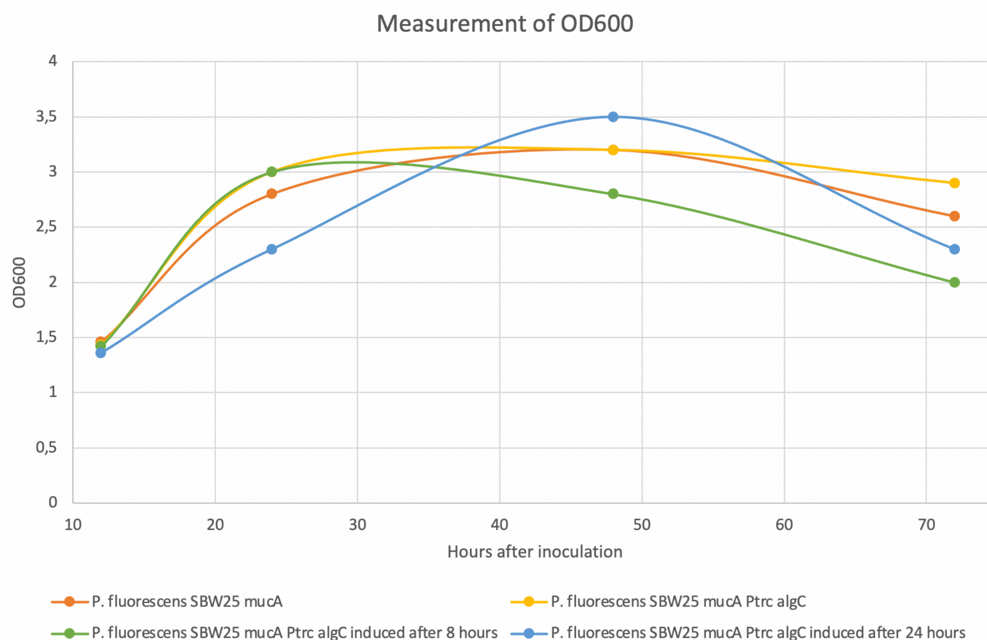


Figure 32: OD_{600} measured in P. fluorescens SBW25 mucA and P. fluorescens SBW25 mucA Ptrc algC when samples were taken out. *Samples were taken out 12, 24, 48 and 72 hours after inoculation and OD_{600} was measured each time. For the culture with the strain marked green, IPTG was added 8 hours after inoculation. For the culture with the strain marked blue, IPTG was added 24 hours after inoculation.*

The OD_{600}-measurements shown in Figure 32 shows that all cultures exhibited similar growth. In one of the cultures, P_{trc} was induced immediately after the sample outtake at 24 hours after inoculation had been carried out. For this culture, OD_{600} was considerably lower than the rest, even though P_{trc} had yet to be induced with IPTG and were therefore expected to be more similar to the other cultures. The figure also shows that the strain where P_{trc} was induced 8 hours after inoculation reached stationary phase at the same time as the strain with P_{trc} not induced. However, after around 30 hours after inoculation, OD_{600} started to decrease for the culture in which IPTG was added after 8 hours, whereas the culture without IPTG remained in stationary phase. For the strain in which P_{trc} was induced 24 hours after inoculation, stationary phase was reached considerably later, at around 50 hours after inoculation. A decrease in OD_{600} was observed once stationary phase was reached.

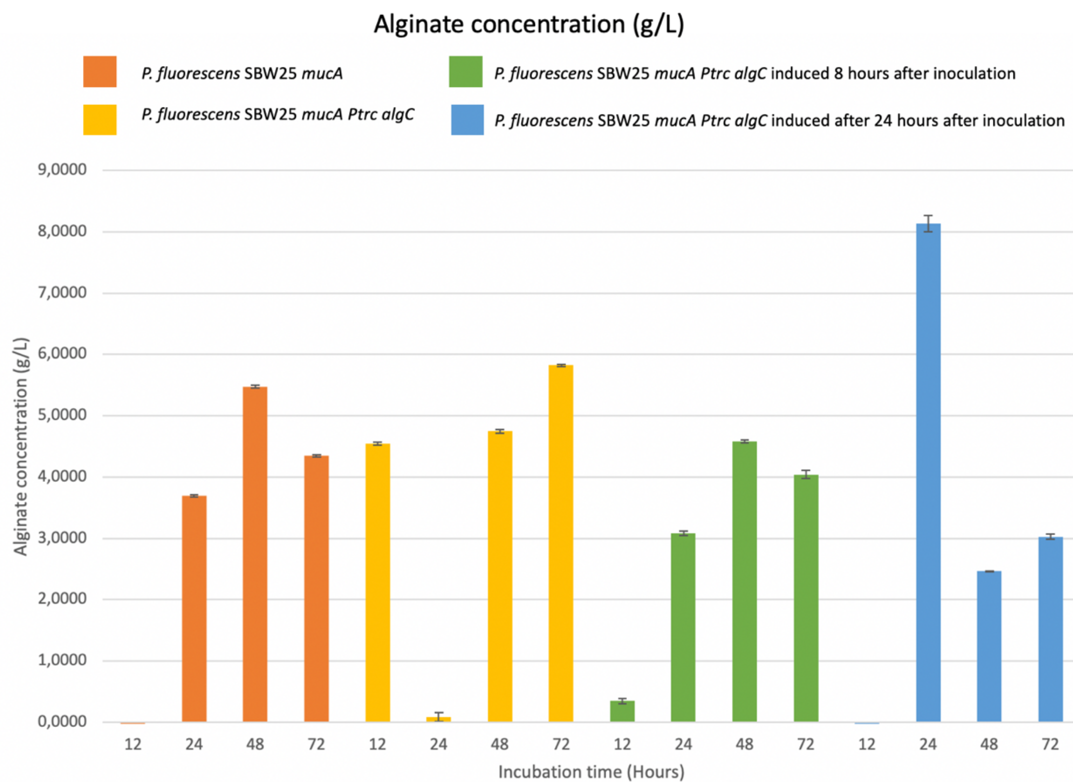


***Figure 33**: Concentration of alginate (g/L) in P. fluorescens SBW25 mucA and P. fluorescens SBW25 mucA Ptrc algC.* *The concentration of alginate in g/L is shown for the 16 samples. The x-axis show the incubation time, which was 12, 24, 48 and 72 hours. Error bars are shown on the graph. The increase in OD_{230} from before and after addition of lyases was used in calculations for each bar. For each OD_{230}-measurement, 3 parallels were measured.*

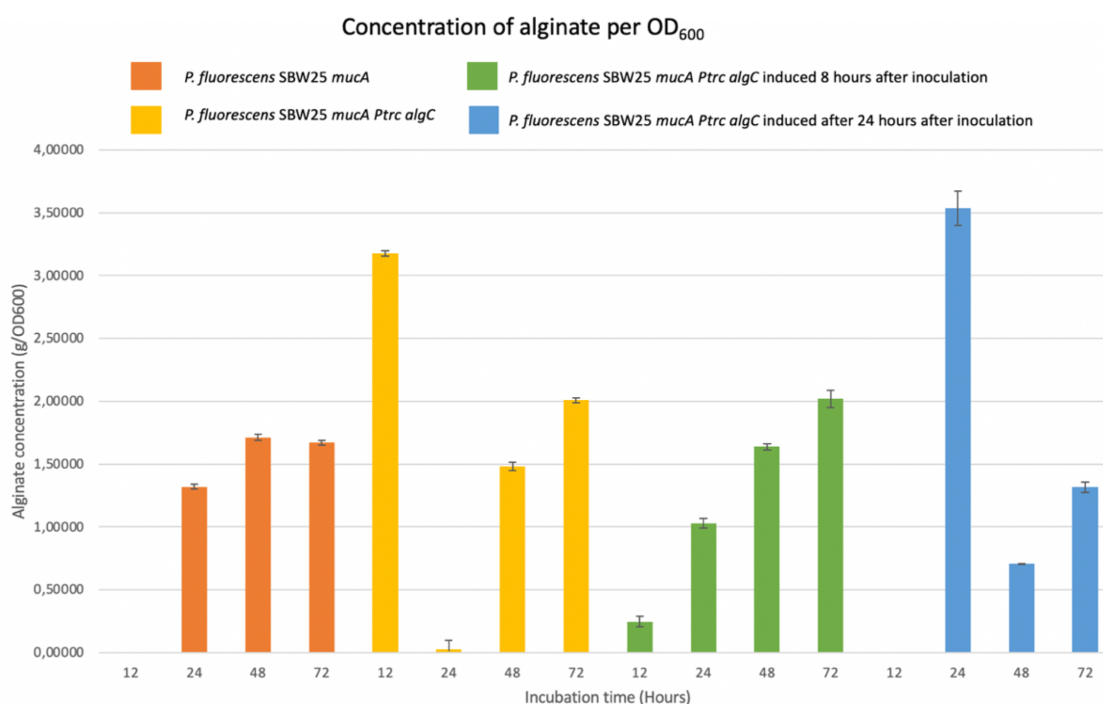


Figure 34*: *Alginate per OD_{600} in P. fluorescens SBW25 mucA and P. fluorescens SBW25 mucA Ptrc algC.
The concentration of alginate per OD_{600} is shown for the 16 samples. The x-axis show the incubation time, which was 12, 24, 48 and 72 hours. Error bars are shown on the graph. The increase in OD_{230} from before and after addition of lyases was used in calculations for each bar. For each OD_{230}-measurement, 3 parallels were measured.

The standard deviation error bars shown on the graphs in Figure 34 and 35 are small, indicating that the parallels were close to the average value and that it accurately represented the data set.

The results from the alginate assay is shown in Figure 33 and 34. Figure 33 show the total amount of alginate (g/L) produced in the two different strains at different time points as well as with and without IPTG. However, alginate produced per cell (Figure 34) should be used when comparing alginate production. For *P. fluorescens SBW25 mucA* alginate was, as expected, not measurable 12 hours after inoculation. This was also the case for the culture with *P. fluorescens SBW25 mucA Ptrc algC*, in which IPTG was not added until 24 hours after inoculation. Surprisingly, for the culture in which P_{trc} was uninduced, the amount of alginate was high 12 hours after inoculation and considerably lower 24 hours after inoculation. For *P. fluorescens SBW25 mucA Ptrc algC* in which P_{trc} was induced with IPTG 8 hours after inoculation, the alginate production steadily increased from the first to the last sample outtake, even though the OD_{600}-measurements in Figure 32 showed that the cells reached stationary phase around 30 hours after inoculation. For *P. fluorescens SBW25 mucA*

Ptrc algC in which P_{trc} was induced with IPTG 24 hours after inoculation, the amount of alginate was not measurable after 12 hours. Only 12 hours later however, an unexpectedly large amount of alginate was produced. This culture was expected to produce a similar amount of alginate as the culture in which P_{trc} was not induced. After addition of IPTG, the production of alginate dropped considerably in this culture.

5. Discussion

5.1 Reporter gene study

A reporter gene study was carried out in order to know which reporter, RFP, CFP, YFP and GFP, was easiest to measure in *P. fluorescens.* The chosen gene would later be used in the evaluation of different promoters strength and inducibility. The criteria used for evaluation was the assay´s sensitivity; how high the fluorescence was compared to the background, as well as the amount of background fluorescence and interference of other reporters. Since the genes were regulated by different promoters, the amount of fluorescence of each reporter could not be compared.

The fluorescence of RFP in samples with m-toluate was ten times higher than in the samples without m-toluate (Figure 7A). However, the fluorescence when P_m was not induced was still measurable. Therefore, RFP when controlled by P_m may be used to test weak promoters. The uninduced fluorescence levels were about 2.5 times higher than the background fluorescence. Even though the fluorescence of RFP when P_m was not induced with m-toluate was relative high compared to when it was induced, there was a significant difference between induced and uninduced levels.

As seen in Figure 7C, the fluorescence measured with YFP-settings was high in pSB-M1g which contained GFP, but not in pSV1 which contained YFP. The reason for this could be the close proximity in the wavelengths of GFP and YFP, and as mentioned in theory, YFP is a derivative of GFP. Why pSV1 did not show fluorescence when measuring with YFP-settings is unknown, but it could be related to the promoter, which is discussed together with the CFP-measurements. GFP in the cultures with pSB-M1g and m-toluate had a high fluorescence when measured with GFP-settings and was also easy to distinguish from the control pSB-M1b. This indicates that GFP would be a good reporter gene. However, owing to the overlap between the fluorescence measured for GFP and YFP, these two would not be ideal to use in combination.

The fluorescence measured with CFP-settings was fairly similar in all of the cultures, which indicated that this would not have been a good reporter gene as its fluorescence was difficult to distinguish from the background. CFP is under the control of the heat-shock responsive

promoter P*ibpfxs* (26). Inducing this promoter with m-toluate did not, as expected, have a distinct effect.

The promoters controlling YFP and CFP may not function well in *P. fluorescens.* Inducing the YFP-promoter P_{rpsJ} with m-toluate was tested by Gawin et al. (26) in *Pseudomonas putida*, and was found to be upregulated after 4 hour incubation with the inducer present. The same was done for the CFP-promoter P*ibpfxs*, which was found to not be affected by m-toluate. The same test was performed in *Azotobacter vinelandii,* where the expression of CFP was slightly increased in the presence of m-toluate. The expression of YFP was not significantly affected by m-toluate. These results show that the promoters behave differently in different strains. Therefore, the results in this study could most likely be explained by the different bacterial strain that was used. In this experiment, all the cultures were induced with m-toluate as the goal was to evaluate reporter genes in *P. fluorescens*, and not promoters

Considering the results from the reporter gene study, RFP was chosen as a good reporter gene for further use in testing promoters in this study.

5.2 Promoter study

Plasmids with the reporter gene RFP and five different promoters were constructed. The promoters evaluated were P_m, P_{mG5}, P_m ML1-17, P_{trc} and P_{BAD}. As the promoter P_m ML1-17 is a high-level expression variant of P_m, it was expected that RFP controlled by this promoter would give the highest fluorescence. However, the results shown in Figure 14 indicates that P_m was the strongest promoter. The reason for this is unknown, but it could be strain-dependent.

The relatively high fluorescence of RFP in samples without m-toluate indicates that P_m has a high background expression (Figure 7B). This has been observed in a study performed by Gawin et al. (24), where the uninduced expression levels of P_m were found to be higher than desired. However, in this study the uninduced levels were not high enough to be considered a disadvantage.

The goal of the promoter study was to find a promoter that was also transcribed in the stationary phase. Figure 18 in section 4.3.2 shows that the fluorescence RFP per OD_{600} when P_{trc} was induced 24 hours after inoculation increased as OD_{600} decreased. This measurement confirmed that P_{trc} was transcribed in the stationary phase. The fluorescence was slightly lower when the IPTG was added after 24 hours compared to when it was added 8 hours after inoculation.

The fluorescence of RFP when controlled by P_{trc} which was not induced with IPTG, was lower than the autofluorescence of *P. fluorescens* (Figure 15). The vector pSV3 may therefore be used to turn off the alginate biosynthesis. As mentioned in the aims-section, a long-term goal would be to regulate alginate production in such a way that it is not activated until optimal growth rate is achieved. This is because alginate production reduces cell growth. A promoter that can regulate gene expression tightly is ideal, and it could seem like P_{trc} had this ability. However, the regulation of the expression of *algC* by P_{trc}, was not as tight considering that the from the alginate assay was variable. This will be discussed later in this chapter. Overall, these results could suggest that the regulation pattern for P_{trc} was gene-specific, though this should be verified in further experiments.

As seen in Figure 17, inducing P_{BAD} with L-arabinose had little effect. It was then discovered that Pseudomonads, including *P. fluorescens* , have the ability to use L-arabinose as a carbon source. The two *P. fluorescens* strains WCS365 and PCL1444 were shown to oxidize L-arabinose (72). L-arabinose is sugar abundant in plants and *P. fluorescens* can, as mentioned in section 1.1, live on the roots of plants in a symbiotic relationship with them. This could explain why inducing P_{BAD} in *P. fluorescens* SBW25 had little effect, as the sugar was most likely metabolized by the bacteria. An even higher concentrations of L-arabinose could have been used to see if that would result in any effect. However, it was not tested in this experiment as there were other promoters that were shown to work in *P. fluorescens*.

5.3 Cloning of *mucR*

It was attempted to place *mucR* under control of the P_m and P_{trc} promoter to see if this would affect alginate production, specifically if the production would start earlier if MucR was overproduced. In addition, if successful, an overproducing MucR-strain would be used as a positive control in the evaluation of the c-di-GMP reporter system in *P. fluorescens*.

However, constructing the transposon vectors with the promoters and *mucR* took longer time than anticipated and the experiment had to be terminated.

The cloning efficiency when using one-step SLIC can sometimes be poor because of stable secondary structures that can form when the DNA is treated with T4 DNA polymerase. Different treatment durations have previously been tested (61), and the most optimal duration and temperature was found to be 30 seconds at 50°C. This method is good for cloning short fragments with strong secondary structures and was therefore used in this project. Despite using this optimized method, formation of stable secondary structures could still have been a problem in this study. It could also be that the desired mutation was a disadvantage in *E. coli*, making the *mucR* gene especially difficult to clone.

5.4 Testing a c-di-GMP reporter system in *P. fluorescens*

A c-di-GMP reporter system where a triple-tandem riboswitch is fused between two reporter genes – *amcyan* and *turboRFP* – was tested in *P. fluorescens* to see if the system could be used to measure internal concentrations of c-di-GMP in *P. fluorescens* strains. The plasmid pFY4535 containing the reporter system was transferred to *P. fluorescens* SBW25 and *P. fluorescens* SBW25 *mucA*. To completely verify that the reporter system can be used to measure c-di-GMP, a MucR-overproducing strain is required. The values shown in this study are most likely low compared to such a strain. It would therefore be easier to evaluate the biosensor´s effectivity in *P. fluorescens* if a MucR-overproducing strain was used because the difference would be more pronounced. However, this experiment indicated that the reporter system could be used in *P. fluorescens* despite its autofluorescence. The fact that there is little difference between c-di-GMP levels in *P. fluorescens SBW25 and P. fluorescens SBW25* may be because they actually produce a similar amount of c-di-GMP, or it could be because the c-di-GMP biosensor does not function optimally in *P. fluorescens*.

It was thought that the wild-type strain, *P. fluorescens* SBW25, might produce more c-di-GMP than *P. fluorescens* SBW25 *mucA*. From Figure 19 it could look like the levels are similar, but to confirm this a MucR-overproducing strain would be necessary to use as a positive control. However, the results are promising for the usage of the reporter system in *P. fluorescens*.

In Figure 21, a possible correlation between OD_{600} and the fluorescence of AmCyan was investigated. A constitutively expressed GFP has previously been used to predict growth in *P. aeruginosa* (73). Therefore, the question was if AmCyan could be used as a measurement of growth because the gene is expressed constitutively by PFP2, and the amount of fluorescence produced by AmCyan could therefore possibly represent cell numbers. This correlation was shown to be weak as the measurements of fluorescence and OD_{600} were not similar. The reason for this could be that OD_{600} could not distinguish between viable and non-viable cells, whereas only viable cells would produce fluorescence. The fluorescence also increases even though OD_{600} indicates that the cells are in stationary phase. This could be explained by the constitutive promoter PFP2 which controls expression of AmCyan, and that this promoter may also be transcribed in the stationary phase. The weak correlation could also be explained by AmCyan being a stable protein. An unstable protein would be degraded faster. If the transcription rate per cell was the same independent of growth phase, AmCyan would be a better measurement for cell number because then the fluorescence measured would more accurately represent the cell number at the time of measurement. In addition, if the maturation time of the fluorescence protein is long, it could take some time before the fluorescence signal is detected. This could explain why growth curves based on OD_{600} and fluorescence are not identical. Since P. fluorescence has strong autofluorescence, this must also be taken into account to accurately measure growth in such a bacterium based on fluorescent proteins.

5.5 Study effect of stationary phase promoter-regulated *algCs* effect on alginate production

Alginate production was measured in the newly constructed strain *P. fluorescens* SBW25 *mucA Ptrc algC* as well as in *P. fluorescens* SBW25 *mucA.* When P_{trc} was not induced, a high amount of alginate produced per OD_{600} was observed (Figure 34) after only 12 hours. This was not expected, and the results does not correlate with the results for the 12-hour measurement of *P. fluorescens* SBW25 *mucA Ptrc algC* in which P_{trc} was induced 24 hours after inoculation. The promoter was not induced in either culture at this timepoint and similar values were therefore expected, and not completely opposite. In addition, for the cultures in which P_{trc} was not induced, a large concentration of alginate was as mentioned previously observed at 12 hours after inoculation, whereas the alginate concentration 24 hours after inoculation was barely measurable. To be able to explain and verify these results, a new measurement is necessary.

When considering the effect of inducing P_{trc} with IPTG, it could be the case that addition of IPTG was not optimal. A question that was raised was whether too much AlgC may not be ideal for the cells. However, in the culture in which P_{trc} was induced 8 hours after inoculation, the amount of alginate steadily increased (Figure 34). After 72 hours, more alginate was produced per cell than for *P. fluorescens* SBW25 *mucA.* This could indicate that around 8 hours after inoculation was the best time to induce the promoter. However, the assay should be repeated before drawing any conclusions. Previously in this study it was shown that P_{trc} was also transcribed in stationary phase and could be induced in this phase. The effect of inducing the promotor in stationary phase did not appear to have a positive effect on alginate production as the amount of alginate significantly decreased 24 hour after induction. The reason for this is unclear, but to verify these results and to draw any conclusions, it would be necessary to repeat the alginate assay.

Maleki et al. (1) tested the amount of alginate produced by *P. fluorescens* SBW25 *mucA,* and the results showed a concentration of around 1.2 g/L and 3 g/L after 24 and 48 hours, respectively. In this study, the corresponding values were around 3.7 g/L and 5.5 g/L. However, as mentioned in section 4.5.3, total amounts of produced alginate should not be compared as differences in growth could be an influence.

5.6 Further work

If successful, the c-di-GMP reporter system could be used to assess other gene´s influence on the c-di-GMP concentration in *P. fluorescens* and gain further insights on the role of c-di-GMP in alginate polymerization. Further work would involve inserting the *mucR* gene into the transposon vectors pHE319 and pSV9, integrate the transposons into the genome of *P. fluorescens* SBW25 *mucA* and measure alginate production in these two strains. These strains should also be used as positive controls in the evaluation of the c-di-GMP reporter system in *P. fluorescens* to get a confirmation of whether it functions or not.

An interesting question is whether or not a MucR-overproducing strain, such as the ones attempted at making in section 4.3, would result in more c-di-GMP and thus more alginate produced. The hypothesis around a MucR-overproducing strain was whether or not an excess of MucR would lead to more c-di-GMP and together lead to an early start of the alginate production seeing as c-di-GMP regulates alginate polymerization through Alg44. To clone the

mucR gene, TOPO-cloning could be attempted once more to obtain a vector with the gene inserted in a clockwise direction. This vector could then be subjected to partial digestion with NdeI and NotI. If unsuccessful, another cloning strategy should be used.

As shown in Figure 15, the stationary phase promoter P_{trc} could not be used in *P. fluorescens* unless it was induced with IPTG. This indicates that the promoter could be used to turn off alginate production in the strain and should be investigated in further studies. As previously mentioned, the graph in Figure 21 indicates that the constitutive promoter for AmCyan, PFP2, is also transcribed in the stationary phase. In further studies, this promoter could be compared to the P_{trc} promoter and see the effect on alginate production when central genes are controlled by either of these promoters.

The alginate assay should be repeated as the results obtained from the assay in this study were unexpected and difficult to explain. It would be recommended to have more than three sample parallels in order for the results to more accurately represent the data set in case one or more parallel deviates from the others. More frequent sample outtakes would possibly be beneficial to be able to more closely study the alginate production around the time of induction of P_{trc}.

6. Conclusion

The topic of this thesis was to use reporter genes to measure promoter strength and c-di-GMP in *P. fluorescens*, as well as constructing a mutant which could be used to control alginate production tightly. An important finding in this study, was that the P_{trc} promoter could be induced also in stationary phase cells. A new strain was constructed with *algC* under control of P_{trc}. The results from the alginate assay in this strain were not as expected and difficult to explain. Therefore it was recommended that this assay should be repeated in further studies. However, an important observation was made; that induction of P_{trc} with IPTG did not seem to have an as large effect as anticipated. Due to little time left, the assay could not be repeated in this study. Another finding from the promoter study was that that P_{BAD} could not be used in *P. fluorescens* as the inducer L-arabinose were metabolized by the bacterium.

A promising result was the evaluation of the c-di-GMP reporter system in *P. fluorescens.* This has previously not been tested in *P. fluorescens*, and it was unknown whether or not the bacterium's autofluorescence would interfere with the reporter system or not. An important part of the research on this method for measuring c-di-GMP, was the construction of a MucR-overproducing strain, as MucR is an important synthesizer of c-di-GMP. This was attempted in this study, but was unsuccessful due to challenges in the cloning process. The initial findings suggests that the system functions well in *P. fluorescens*. Still, a strain that overproduces MucR will be necessary to use as a positive control to confirm that the reporter system works in *P. fluorescens.* Investigation of this reporter system also lead to the unexpected finding of another possible stationary phase promoter; the PFP2 promoter.

In order to be able to tailor and modify the bacterial alginate to fit the current and future demands of alginate in an expanding industry, a deep understanding of the biosynthesis genes is necessary. This study has contributed to this this mainly by studying promoters, evaluating a c-di-GMP reporter system and constructing a mutant which possibly could be used to control alginate production tightly.

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Appendix

Appendix A – Map of plasmids constructed in this study

Map of plasmids that were constructed in this study are shown in Figure A1 and A2. Map of pSV1 is shown in section 4.1. The plasmid pSV4 is the same as pSV2, only with P_m ML1-17 instead of P_m.

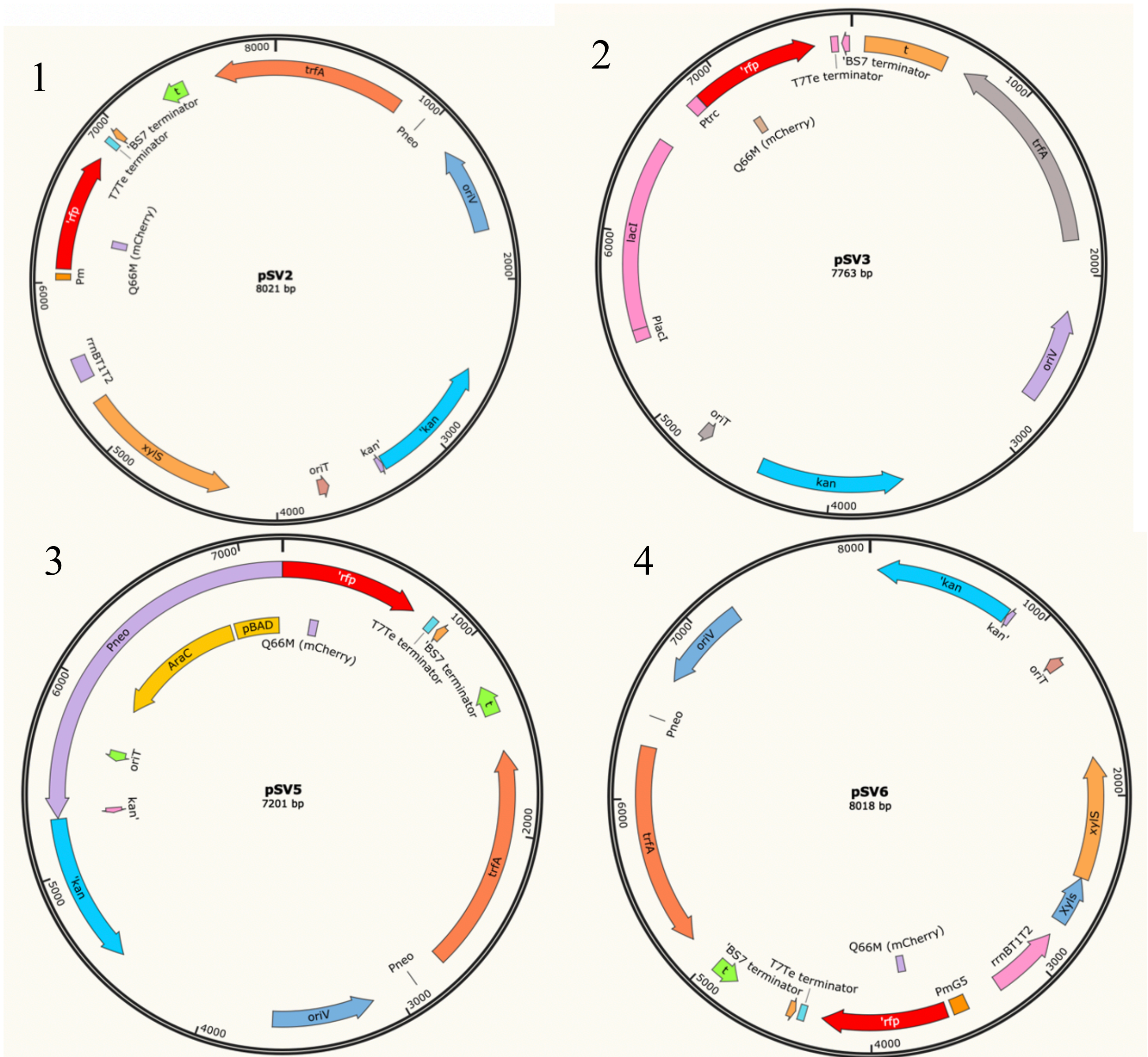

***Figure A1: Plasmids constructed in this study**. Number 1 shows pSV2. Number 2 shows pSV3. Number 3 shows pSV5. Number 4 shows pSV6.*

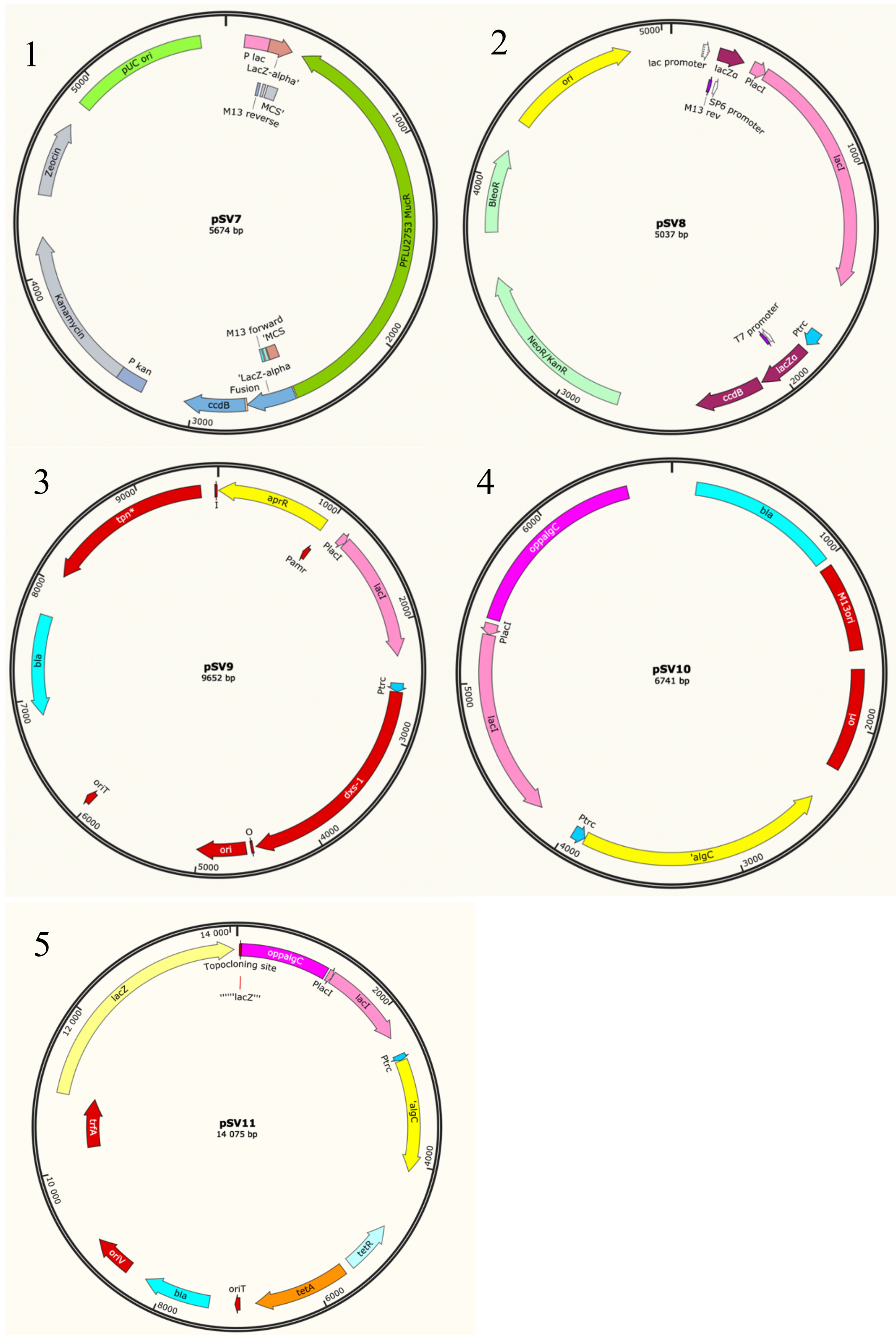

Figure A2: Plasmids constructed in this study. *Number 1 shows pSV7. Number 2 shows pSV8. Number 3 shows pSV9. Number 4 shows pSV10. Number 5 shows pSV11.*

Appendix B – DNA ladders

DNA ladders were used in gel electrophoresis in order to compare the sizes of unknown fragments to the known sizes of the fragments of the DNA ladders. The λ HindIII standard, λ PstI DNA standard and the GeneRuler 50bp ladder were used in this study and are shown in Figure B1.

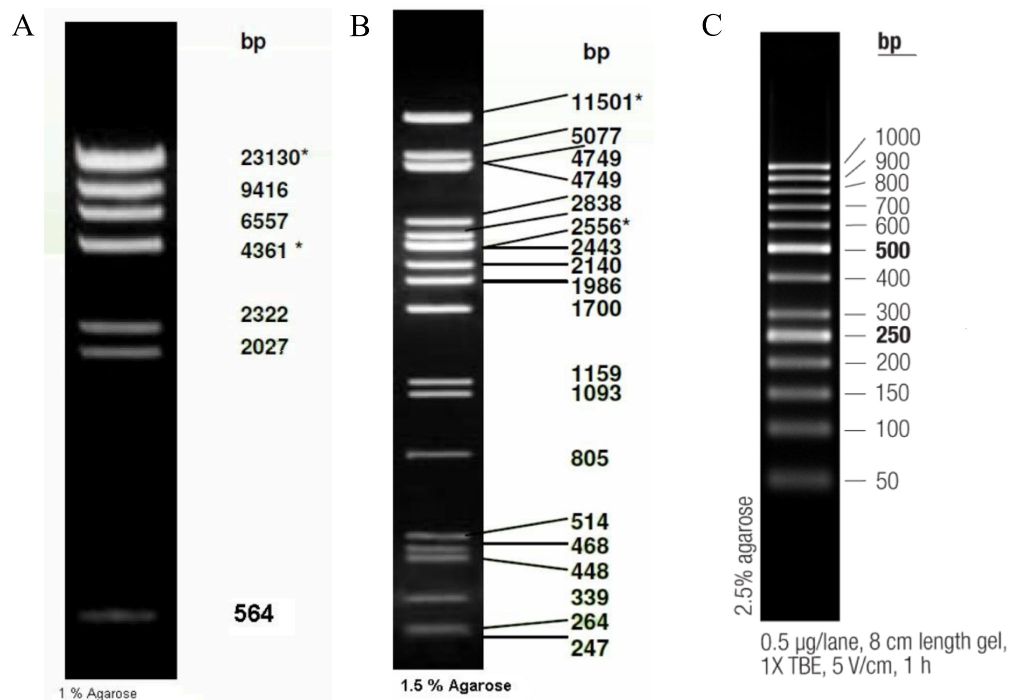

Figure B1: ***The DNA ladders HindIII (GeneOn), PstI (GeneON) and GeneRuler are shown in figure with their respective sizes.*** *The figures show the phage λ DNA digested by either of the restriction enzymes HindIII (A) or PstI (B). Figure C shows the GeneRuler 50bp DNA ladder (Thermo Fisher) which consists of 13 DNA fragments. Fragment sizes are shown in basepairs for all three standards.*

Appendix C – PCR program

Components of the PCR reaction-mix and the PCR-program used are described in this appendix.

The following components were mixed to a final volume of 50ul:

- 33 µl MQ-water
- 10 µl 5X Q5 Reaction Buffer
- 2.5 µl 20uM forward primer
- 2.5 µl 20uM reverse primer
- 1 µl 10mM dNTPs
- 0.5 µl Template DNA
- 0.5 µl Q5 High-Fidelity DNA Polymerase

The program was as described below:

1. Initial denaturation at 98°C for 30 seconds
2. 98°C for 10 seconds
3. 3°C lower than T_m for 30 seconds
4. 72°C for 30 seconds/kb template DNA

Step 2 to 4 were repeated for 25 to 35 cycles. Final extension was at 72°C for 2 minutes. Finally, the temperature was held at 4°C.

Appendix D – Primers

Primers used for PCR and sequencing are listed in table D1.

Table D1: *Primers used in this study.*

Primer	Sequence (from 5`-3`)	Source
Sekvens 2900	CAGCAGGTACATCAGAACAG	Sigma-Aldrich
385Bam	GGCGAAGTAATCGCAACATC	Sigma-Aldrich
MucRR	GTGCGGTCTCCTGATTCAAC	Invitrogen
MucRF	AGTCATATGCTCATCGGTAGTTATTCAC	Invitrogen
MucRsekv1	TAGCCGGTGCCAAAGTCATC	Invitrogen
MucRsekv2	TAGCCGGTGCCAAAGTCATC	Invitrogen
M13 Forward (-20) primer	GTAAAACGACGGCCAG	Invitrogen
M13 Reverse primer	CAGGAAACAGCTATGAC	Invitrogen
slicFpm0903	TAATAATGAAGTCATGAGTCATATGCTCATCG GTAGTTATTCAC	Invitrogen
slicFtrc0903	TTCACACAGGAAACAGAGTCATATGCTCATCG GTAGTTATTCAC	Invitrogen
slicreverseny	TCTGACTCTTATACACAAGTGCGTGCGGTCTC CTGATTCAAC	Invitrogen
ReverseNdeI	CTCCTCGCCCTTGGAAACC	Invitrogen
ForwardBsiWI	TAAGCACGTACGCGGCATGCATTTACGTTGAC	Invitrogen
PmalgCF	GCCGGTCAGCCACATTAAGG	Invitrogen
PmalgCR	TCGGCAGTCAGCGTCTTAGG	Invitrogen

Appendix E – Gel image of colonies after homologous recombination

After homologous recombination, 67 white mucoid colonies were tested with PCR to check whether they contained the mutant or not. Gel images of the positive control and the one colony that contained the mutant are presented in section 4.5.2. The rest is presented in Figure E1 and E2.

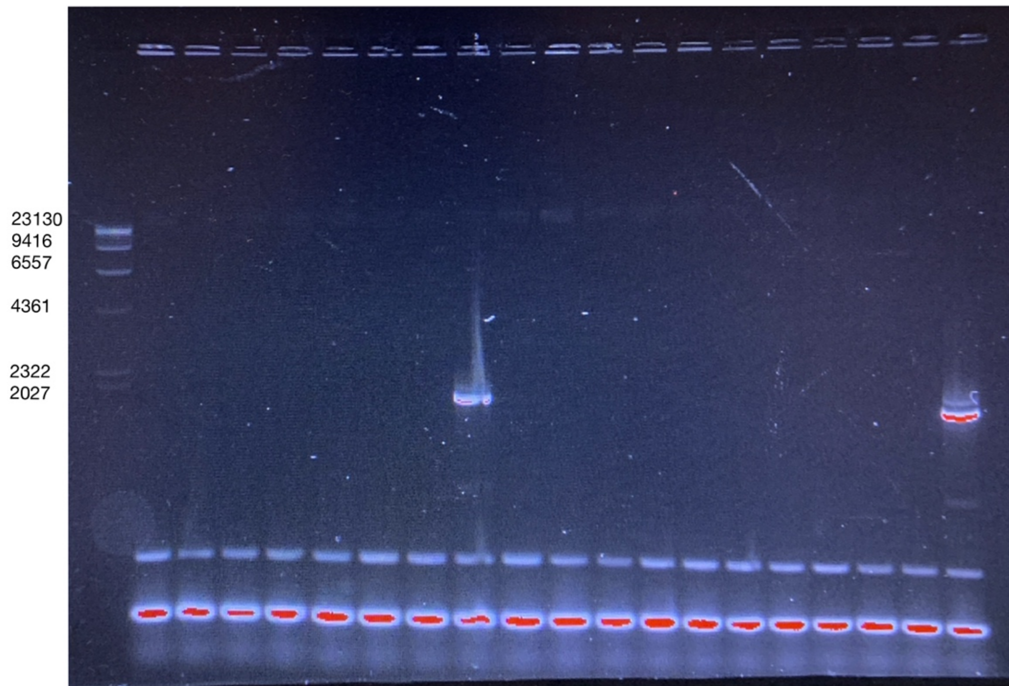

Figure E1: ***Gel electrophoresis after PCR on possible mutant colonies.*** *The wild-type were expected to give a band of 152bp while the mutant were expected to result in a band of 1606bp. None of the colonies in this figure contained the mutation.*

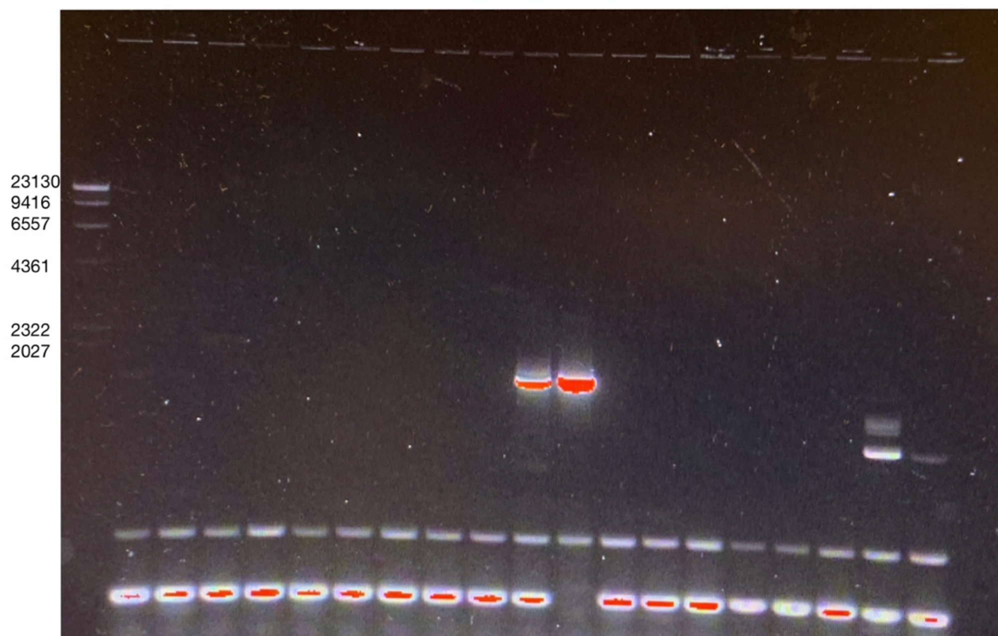

Figure E2*:* ***Gel electrophoresis after PCR on possible mutant colonies.*** *The wild-type were expected to give a band of 152bp while the mutant were expected to result in a band of 1606bp. The PCR-products in lane 11 and 12 were investigated closer and are included in section 4.5.2.*

Appendix F – Raw data from the promoter study

The raw data from measuring fluorescence and O_{600} in the promoter study are shown in Table F1, F2, F3 and F4.

Table F1: ***Raw data from the fluorescence measurements used in section 4.2.2.*** *The colour coding used in this Table and Table F2 is shown in the upper left corner.*

pSV2 M-toluate	pSV6 M-toluate	pSV5 L-Arabinose
pSV4 M-toluate	pSV3 IPTG	pSV5
pSB-M1g M-toluate	pSV3	

Fluorescence 8 hours

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	50	75	107	86	120	96	94	36	44	50	60	63
B	68	26	31	31	33	30	33	42	45	42	49	42
C	36											

Fluorescence 12 hours

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	53	343	704	730	373	355	320	61	61	65	91	113
B	135	106	107	101	56	51	52	77	75	76	62	60
C	66											

Fluorescence 24 hours

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	7	7001	10744	7940	5362	4031	4307	395	456	449	1469	1679
B	1879	870	926	876	275	313	336	317	303	324	276	265
C	239											

Fluorescence 48 hours

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	29	6774	11152	7877	5364	4324	4320	345	390	375	1462	1700
B	1817	1626	1598	1565	386	413	404	338	328	318	279	318
C	279											

Table F2: ***Raw data from the OD_{600}-measurements used in section 4.2.2.*** *The colour coding used is shown in Table F1.*

OD 8 hours

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,0427	0,5931	0,588	0,6602	0,5816	0,4635	0,4921	0,2489	0,639	0,69	0,4626	0,7189
B	0,6211	0,1072	0,1099	0,0985	0,1304	0,1066	0,1082	0,3798	0,4453	0,4062	0,3931	0,4141
C	0,2209											

OD 12 hours

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,0414	0,8688	0,9512	0,9167	0,9629	0,8195	0,9676	0,7995	0,8996	0,9954	0,9661	0,9924
B	0,9415	0,353	0,3497	0,3301	0,4212	0,3825	0,3688	0,8854	0,8882	0,8899	0,8773	0,8898
C	0,6804											

OD 24 hours

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,0401	0,9305	0,905	0,9118	0,9537	0,9419	0,9673	0,9466	0,9446	0,9242	0,919	0,9253
B	0,8891	0,8593	0,8745	0,8505	0,8494	0,9194	0,8794	0,8762	0,7964	0,8337	0,8408	0,8474
C	0,8406											

OD 48 hours

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,0411	0,9198	0,8954	0,8865	0,9032	0,895	0,9058	0,8709	0,9192	0,8968	0,8847	0,8825
B	0,8504	0,7814	0,7846	0,7575	0,7526	0,7843	0,7523	0,7734	0,728	0,7655	0,7567	0,7828
C	0,7785											

***Table F3**: **Raw data from the fluorescence measurements used Figure 18 in section 4.2.2 and Figure 19 in section 4.2.3**. Values for pSB-M1g are marked orange. Values for pSV5 are marked blue. Values for pSV3 are marked pink.*

Fluorescence 8 hours

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	54	52	44	45	40	38						
B												

Fluorescence 12 hours

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	57	61	55	77	112	85						
B												

Fluorescence 24 hours

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	427	644	483	805	714	508	377	232	479			
B												

Fluorescence 48 hours

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	370	553	419	739	728	528	1077	1251	1543			
B												

***Table F4**: **Raw data from the OD_{600}- measurements used in section 4.2.3**. Values for the blank is marked yellow. Values for pSB-M1g are marked orange. Values for pSV5 are marked blue. Values for pSV3 are marked pink.*

OD 8 hours after inoculation

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,0437	0,0559	0,6321	0,5635	0,3667	0,3136	0,1834					
B												

OD 12 hours after inoculation

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,0409	0,9414	0,9821	1,0201	0,9064	0,8193	0,5172					
B												

OD 24 hours after inoculation

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,0424	1,0761	1,0392	0,9979	1,0632	1,0655	1,0063					
B		0,9158	0,9116	0,9138								

OD 48 hours after inoculation

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,0422	0,9743	0,9813	0,9058	0,9386	0,9765	0,8857					
B		0,7504	0,759	0,7673								

Appendix G – Raw data from the measurement of AmCyan and TurboRFP

The raw data from measuring fluorescence of AmCyan and TurboRFP are shown in Table G1, G2 and G3.

Table G1: ***Raw data from measurement of the fluorescence of TurboRFP.*** *Values marked green are cultures with P. fluorescens SBW25 mucA (pfY4535). Values marked blue are P. fluorescens SBW25 (pfY4535). Values marked yellow are P. fluorescens SBW25, which was used as a blank.*

TurboRFP							
Fluorescence 10 hours							
	1	2	3	4	5	6	7
A	-1414	1275	1806	919	1920	2373	21129
Fluorescence 12 hours							
	1	2	3	4	5	6	7
A	3498	4438	3492	3397	3442	3907	19764
Fluorescence 24 hours							
	1	2	3	4	5	6	7
A	1216	3516	3537	3460	3927	4004	21132
Fluorescence 34 hours							
	1	2	3	4	5	6	7
A	4201	4335	4172	5430	4620	4137	20342
Fluorescence 48 hours							
	1	2	3	4	5	6	7
A	2185	2448	2767	3658	2826	2526	19984
Fluorescence 64 hours							
	1	2	3	4	5	6	7
A	3921	1914	1856	3886	3334	4884	20739

Table G2: ***Raw data from measurement of the fluorescence of AmCyan.*** *Values marked green are cultures with P. fluorescens SBW25 mucA (pfY4535). Values marked blue are P. fluorescens SBW25 (pfY4535). Values marked yellow are P. fluorescens SBW25, which was used as a blank.*

AmCyan							
Fluorescence 10 hours							
	1	2	3	4	5	6	7
A	1709	1412	1604	1044	2722	2744	50
Fluorescence 12 hours							
	1	2	3	4	5	6	7
A	3459	3075	3431	2099	4001	4038	61
Fluorescence 24 hours							
	1	2	3	4	5	6	7
A	7359	6369	6700	10930	11289	11412	70
Fluorescence 34 hours							
	1	2	3	4	5	6	7
A	7184	6337	6913	10151	10317	10476	74
Fluorescence 48 hours							
	1	2	3	4	5	6	7
A	8423	7696	7986	11033	11861	11695	80
Fluorescence 64 hours							
	1	2	3	4	5	6	7
A	8856	8267	8955	11878	12252	12462	106

Table G3: ***Raw data from measurement of OD_{600}.*** *Values marked green are cultures with P. fluorescens SBW25 mucA (pfY4535). Values marked blue are P. fluorescens SBW25 (pfY4535). Values marked grey are P. fluorescens SBW25. Values marked yellow are LB-medium.*

OD

OD 10 hours

	1	2	3	4	5	6	7	8 (LB)
A	0,9244	0,9227	0,9062	0,3863	0,6652	0,676	0,7247	0,0386

OD 12 hours

	1	2	3	4	5	6	7	8 (LB)
A	0,958	0,947	0,9671	0,6206	0,8162	0,8309	0,9783	0,0388

OD 24 hours

	1	2	3	4	5	6	7	8 (LB)
A	0,8825	0,8675	0,8558	0,9073	0,9055	0,8753	0,8545	0,0383

OD 34 hours

	1	2	3	4	5	6	7	8 (LB)
A	0,8749	0,8882	0,859	0,8442	0,843	0,8426	0,9347	0,0365

OD 48 hours

	1	2	3	4	5	6	7	8 (LB)
A	0,8365	0,8193	0,7977	0,8162	0,8292	0,799	0,8395	0,0378

OD 64 hours

	1	2	3	4	5	6	7	8 (LB)
A	0,7991	0,8104	0,8244	0,7941	0,8105	0,8168	0,8203	0,037

Appendix H – Alginate assay data and calculations

Alginate was measured in P. fluorescens SBW25 *mucA* and *P. fluorescens* SBW25 *mucA Ptrc algC.* OD_{230}-measurements of the standard solutions with known alginate concentrations are presented in Table H1. This was used to make the standard curve presented in Figure H1. OD_{230}-measurements of the unknown samples are shown in Table H2.

Table H1: *OD_{230} before and after addition of M- and G-lyase for two parallels of standard solutions with known alginate concentration.*

Alginate concentration (g/L)	Parallels	OD230 before lyases	OD230 after lyases (4 hours)	Increase in OD230	Average		Average	Standard deviation
Blank		0,146	0,145	-0,001				
	I	0,139	0,132	-0,007				
0,0	II	0,135	0,134	-0,001	-0,004			
	I	0,133	0,161	0,028		0,032		
0,1	II	0,129	0,155	0,026		0,03	0,031	0,0014
	I	0,12	0,18	0,06		0,064		
0,2	II	0,131	0,181	0,05		0,054	0,059	0,0071
	I	0,132	0,245	0,113		0,117		
0,3	II	0,128	0,222	0,094		0,098	0,1075	0,0134
	I	0,133	0,268	0,135		0,139		
0,4	II	0,127	0,244	0,117		0,121	0,13	0,0127
	I	0,137	0,291	0,154		0,158		
0,5	II	0,147	0,307	0,16		0,164	0,161	0,0042

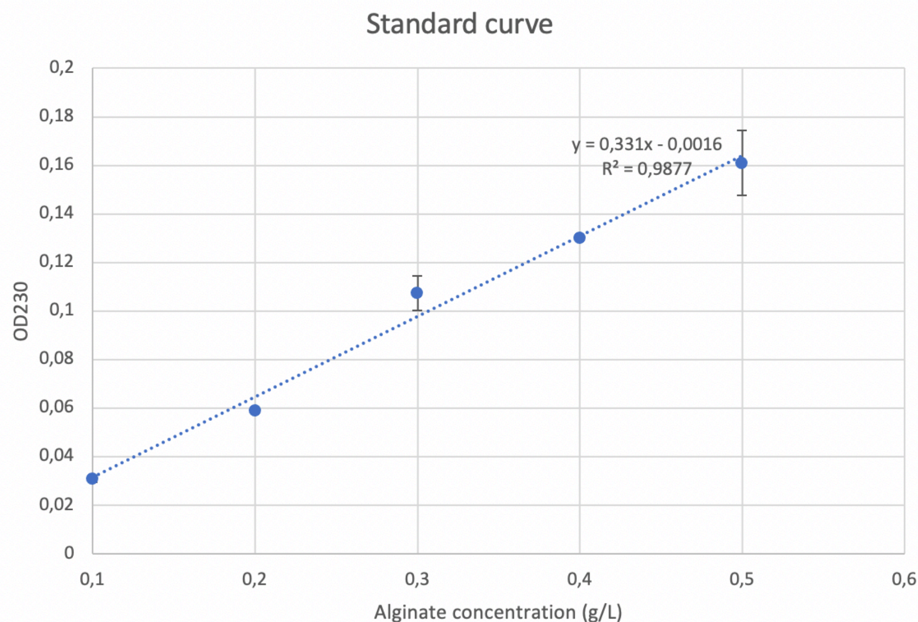

Figure H1: *Standard curve used in calculation of alginate concentrations in unknown samples. The curve shows the correlation between the average increase in OD_{230} and alginate concentration (g/L). A linear trendline resulted in the equation $y=0.331x – 0.0016$.*

Table H2: *OD_{230} before addition of lyases, as well as 3 and 4 hours after addition of lyases. Parallels marked red were not used in calculations to avoid a large standard deviation. .*

		OD230 before addition of lyases			OD230 3 hours after addition of lyases			OD230 4 hours after addition of lyases		
	Incubation time (hours)	Parallel 1	Parallel 2	Parallel 3	Parallel 1	Parallel 2	Parallel 3	Parallel 1	Parallel 2	Parallel 3
P.	12	0,628	0,729	0,712	0,686	0,706	0,678	0,678	0,694	0,67
fluorescens	24	0,668	0,653	0,683	0,726	0,737	0,736	0,719	0,735	0,732
SBW25	48	1,096	0,675	0,688	0,935	0,757	0,816	0,924	0,747	0,796
mucA	72	0,681	0,781	0,722	0,767	0,856	0,827	0,75	0,835	0,813
P.	12	0,64	0,618	0,637	0,724	0,689	0,755	0,709	0,674	0,736
fluorescens	24	0,796	0,723	0,665	0,751	0,735	0,761	0,732	0,717	0,739
SBW25	48	0,727	0,709	0,679	0,791	0,815	0,806	0,77	0,793	0,786
mucA Ptrc	72	0,731	0,87	0,756	0,866	0,922	0,847	0,845	0,966	0,833
P.	12	0,661	0,722	0,766	0,72	0,751	0,743	0,705	0,735	0,726
fluorescens	24	0,755	0,631	0,736	0,791	0,742	0,792	0,774	0,726	0,774
SBW25	48	0,65	0,707	0,704	0,741	0,778	0,822	0,723	0,76	0,804
mucA Ptrc	72	0,762	0,707	0,666	0,779	0,795	0,811	0,76	0,776	0,798
P.	12	0,689	0,687	0,669	0,675	1,001	0,682	0,66	0,979	0,667
fluorescens	24	0,696	0,687	0,702	0,81	0,981	0,724	0,818	0,96	0,708
SBW25	48	0,732	0,747	2,274	0,79	0,804	1,123	0,773	0,787	1,097
mucA Ptrc	72	0,689	0,713	0,7	0,714	0,82	0,762	0,697	0,804	0,75

Appendix I – Sequencing of pSV6

The result from sequencing pSV6 is presented in this appendix. The first line show the template DNA, which was the PCR-product of pSV6 created with the two primers Sekvens2900 and 385Bam. Plasmid from four transformant colonies were isolated and sequenced. The four next lines show the alignment of primer Sekvens2900 of the four different plasmids.

```
Reference sequence (1): pSV6
Identities normalised by aligned length.
Colored by: identity

                                    1                                                                                 80
1 pSV6                              CAGCAGGTACATCAGAACAGTACCATGACTGAAGAACAAATAGTTTTTTCCTGATCCATAAAGCAGAACGGCCTGCTCCA
2 sekvens2900-possible-pSV6.4       -------------------------------CGCCGAATTTGGTTGTTCCTGAATCCATAAAGCAGAACGGCCTGCTCCA
3 sekvens2900-possible-pSV6.1       -------------------------------------AAATAGTTTTTTCCTGATCCATAAAGCAGAACGGCCTGCTCCA
4 sekvens2900-possible-pSV6.2       ------------------------------GGGGGACGACCGGTTCTTTCCTGATCCATAAAGCAGAACGGCCTGCTCCA
5 sekvens2900-possible-pSV6.3       -----------------------------GGGGCGAAAAATAGTTTTTTCCTGATCCATAAAGCAGAACGGCCTGCTCCA

                                    81                                                                               160
1 pSV6                              TGACAAATCTGGCTCCCCAACTAATGCCCCATGCAGCCAGCATAACCAGCATAAACGTGTCCGGTTTGATAGGGATAAGT
2 sekvens2900-possible-pSV6.4       TGACAAATCTGGCTCCCCAACTAATGCCCCATGCAGCCAGCATAACCAGCATAAACGTGTCCGGTTTGATAGGGATAAGT
3 sekvens2900-possible-pSV6.1       TGACAAATCTGGCTCCCCAACTAATGCCCCATGCAGCCAGCATAACCAGCATAAACGTGTCCGGTTTGATAGGGATAAGT
4 sekvens2900-possible-pSV6.2       TGACAAATCTGGCTCCCCAACTAATGCCCCATGCAGCCAGCATAACCAGCATAAACGTGTCCGGTTTGATAGGGATAAGT
5 sekvens2900-possible-pSV6.3       TGACAAATCTGGCTCCCCAACTAATGCCCCATGCAGCCAGCATAACCAGCATAAACGTGTCCGGTTTGATAGGGATAAGT

                                    161                                                                              240
1 pSV6                              CCAGCCTTGCAAGAAGCGGATACAGGAGTGCAAAAAATGGCTATCTCTAGAAAGACCTACCCCTGAGGCTCTATGCAACA
2 sekvens2900-possible-pSV6.4       CCAGCCTTGCAAGAAGCGGATACAGGAGTGCAAAAAATGGCTATCTCTAGAAAGACCTACCCCTGAGGCTCTATGCAACA
3 sekvens2900-possible-pSV6.1       CCAGCCTTGCAAGAAGCGGATACAGGAGTGCAAAAAATGGCTATCTCTAGAAAGACCTACCCCTGAGGCTCTATGCAACA
4 sekvens2900-possible-pSV6.2       CCAGCCTTGCAAGAAGCGGATACAGGAGTGCAAAAAATGGCTATCTCTAGAAAGACCTACCCCTGAGGCTCTATGCAACA
5 sekvens2900-possible-pSV6.3       CCAGCCTTGCAAGAAGCGGATACAGGAGTGCAAAAAATGGCTATCTCTAGAAAGACCTACCCCTGAGGCTCTATGCAACA

                                    241                                                                              320
1 pSV6                              TGTACAATAATAATGAAGTCATGAACATATGGTTTCCAAGGGCGAGGAGGATAACATGGCTATCATTAAAGAGTTCATGC
2 sekvens2900-possible-pSV6.4       TGTACAATAATAATGGAGTCATGAACATATGGTTTCCAAGGGCGAGGAGGATAACATGGCTATCATTAAAGAGTTCATGC
3 sekvens2900-possible-pSV6.1       TGTACAATAATAATGGAGTCATGAACATATGGTTTCCAAGGGCGAGGAGGATAACATGGCTATCATTAAAGAGTTCATGC
4 sekvens2900-possible-pSV6.2       TGTACAATAATAATGGAGTCATGAACATATGGTTTCCAAGGGCGAGGAGGATAACATGGCTATCATTAAAGAGTTCATGC
5 sekvens2900-possible-pSV6.3       TGTACAATAATAATGGAGTCATGAACATATGGTTTCCAAGGGCGAGGAGGATAACATGGCTATCATTAAAGAGTTCATGC

                                    321                                                                              400
1 pSV6                              GCTTCAAAGTTCACATGGAGGGTTCTGTTAACGGTCACGAGTTCGAGATCGAAGGCGAAGGCGAGGGCCGTCCGTATGAA
2 sekvens2900-possible-pSV6.4       GCTTCAAAGTTCACATGGAGGGTTCTGTTAACGGTCACGAGTTCGAGATCGAAGGCGAAGGCGAGGGCCGTCCGTATGAA
3 sekvens2900-possible-pSV6.1       GCTTCAAAGTTCACATGGAGGGTTCTGTTAACGGTCACGAGTTCGAGATCGAAGGCGAAGGCGAGGGCCGTCCGTATGAA
4 sekvens2900-possible-pSV6.2       GCTTCAAAGTTCACATGGAGGGTTCTGTTAACGGTCACGAGTTCGAGATCGAAGGCGAAGGCGAGGGCCGTCCGTATGAA
5 sekvens2900-possible-pSV6.3       GCTTCAAAGTTCACATGGAGGGTTCTGTTAACGGTCACGAGTTCGAGATCGAAGGCGAAGGCGAGGGCCGTCCGTATGAA

                                    401                                                                              480
1 pSV6                              GGCACCCAGACCGCCAAACTGAAAGTGACTAAAGGCGGCCCGCTGCCTTTTGCGTGGGACATCCTGAGCCCGCAATTTAT
2 sekvens2900-possible-pSV6.4       GGCACCCAGACCGCCAAACTGAAAGTGACTAAAGGCGGCCCGCTGCCTTTTGCGTGGGACATCCTGAGCCCGCAATTTAT
3 sekvens2900-possible-pSV6.1       GGCACCCAGACCGCCAAACTGAAAGTGACTAAAGGCGGCCCGCTGCCTTTTGCGTGGGACATCCTGAGCCCGCAATTTAT
4 sekvens2900-possible-pSV6.2       GGCACCCAGACCGCCAAACTGAAAGTGACTAAAGGCGGCCCGCTGCCTTTTGCGTGGGACATCCTGAGCCCGCAATTTAT
5 sekvens2900-possible-pSV6.3       GGCACCCAGACCGCCAAACTGAAAGTGACTAAAGGCGGCCCGCTGCCTTTTGCGTGGGACATCCTGAGCCCGCAATTTAT

                                    481                                                                              560
1 pSV6                              GTACGGTTCTAAAGCGTATGTTAAACACCCAGCGGATATCCCGGACTATCTGAAGCTGTCTTTTCCGGAAGGTTTCAAGT
2 sekvens2900-possible-pSV6.4       GTACGGTTCTAAAGCGTATGTTAAACACCCAGCGGATATCCCGGACTATCTGAAGCTGTCTTTTCCGGAAGGTTTCAAGT
3 sekvens2900-possible-pSV6.1       GTACGGTTCTAAAGCGTATGTTAAACACCCAGCGGATATCCCGGACTATCTGAAGCTGTCTTTTCCGGAAGGTTTCAAGT
4 sekvens2900-possible-pSV6.2       GTACGGTTCTAAAGCGTATGTTAAACACCCAGCGGATATCCCGGACTATCTGAAGCTGTCTTTTCCGGAAGGTTTCAAGT
5 sekvens2900-possible-pSV6.3       GTACGGTTCTAAAGCGTATGTTAAACACCCAGCGGATATCCCGGACTATCTGAAGCTGTCTTTTCCGGAAGGTTTCAAGT

                                    561                                                                              640
1 pSV6                              GGGAACGCGTAATGAATTTTGAAGATGGTGGTGTCGTGACCGTCACTCAGGACTCCTCCCTGCAGGATGGCGAGTTCATC
2 sekvens2900-possible-pSV6.4       GGGAACGCGTAATGAATTTTGAAGATGGTGGTGTCGTGACCGTCACTCAGGACTCCTCCCTGCAGGATGGCGAGTTCATC
3 sekvens2900-possible-pSV6.1       GGGAACGCGTAATGAATTTTGAAGATGGTGGTGTCGTGACCGTCACTCAGGACTCCTCCCTGCAGGATGGCGAGTTCATC
4 sekvens2900-possible-pSV6.2       GGGAACGCGTAATGAATTTTGAAGATGGTGGTGTCGTGACCGTCACTCAGGACTCCTCCCTGCAGGATGGCGAGTTCATC
5 sekvens2900-possible-pSV6.3       GGGAACGCGTAATGAATTTTGAAGATGGTGGTGTCGTGACCGTCACTCAGGACTCCTCCCTGCAGGATGGCGAGTTCATC

                                    641                                                                              720
1 pSV6                              TATAAAGTTAAACTGCGTGGTACTAATTTTCCATCTGATGGCCCGGTGATGCAGAAAAAGACGATGGGTTGGGAGGCGTC
2 sekvens2900-possible-pSV6.4       TATAAAGTTAAACTGCGTGGTACTAATTTTCCATCTGATGGCCCGGTGATGCAGAAAAAGACGATGGGTTGGGAGGCGTC
3 sekvens2900-possible-pSV6.1       TATAAAGTTAAACTGCGTGGTACTAATTTTCCATCTGATGGCCCGGTGATGCAGAAAAAGACGATGGGTTGGGAGGCGTC
4 sekvens2900-possible-pSV6.2       TATAAAGTTAAACTGCGTGGTACTAATTTTCCATCTGATGGCCCGGTGATGCAGAAAAAGACGATGGGTTGGGAGGCGTC
5 sekvens2900-possible-pSV6.3       TATAAAGTTAAACTGCGTGGTACTAATTTTCCATCTGATGGCCCGGTGATGCAGAAAAAGACGATGGGTTGGGAGGCGTC
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641                                                                                        720
1 pSV6                                    TATAAAGTTAAACTGCGTGGTACTAATTTTCCATCTGATGGCCCGGTGATGCAGAAAAAGACGATGGGTTGGGAGGCGTC
2 sekvens2900-possible-pSV6.4             TATAAAGTTAAACTGCGTGGTACTAATTTTCCATCTGATGGCCCGGTGATGCAGAAAAAGACGATGGGTTGGGAGGCGTC
3 sekvens2900-possible-pSV6.1             TATAAAGTTAAACTGCGTGGTACTAATTTTCCATCTGATGGCCCGGTGATGCAGAAAAAGACGATGGGTTGGGAGGCGTC
4 sekvens2900-possible-pSV6.2             TATAAAGTTAAACTGCGTGGTACTAATTTTCCATCTGATGGCCCGGTGATGCAGAAAAAGACGATGGGTTGGGAGGCGTC
5 sekvens2900-possible-pSV6.3             TATAAAGTTAAACTGCGTGGTACTAATTTTCCATCTGATGGCCCGGTGATGCAGAAAAAGACGATGGGTTGGGAGGCGTC

721                                                                                        800
1 pSV6                                    TAGCGAACGCATGTATCCGGAAGATGGTGCGCTGAAAGGCGAAATTAAACAGCGCCTGAAACTGAAAGATGGCGGCCATT
2 sekvens2900-possible-pSV6.4             TAGCGAACGCATGTATCCGGAAGATGGTGCGCTGAAAGGCGAAATTAAACAGCGCCTGAAACTGAAAGATGGCGGCCATT
3 sekvens2900-possible-pSV6.1             TAGCGAACGCATGTATCCGGAAGATGGTGCGCTGAAAGGCGAAATTAAACAGCGCCTGAAACTGAAAGATGGCGGCCATT
4 sekvens2900-possible-pSV6.2             TAGCGAACGCATGTATCCGGAAGATGGTGCGCTGAAAGGCGAAATTAAACAGCGCCTGAAACTGAAAGATGGCGGCCATT
5 sekvens2900-possible-pSV6.3             TAGCGAACGCATGTATCCGGAAGATGGTGCGCTGAAAGGCGAAATTAAACAGCGCCTGAAACTGAAAGATGGCGGCCATT

801                                                                                        880
1 pSV6                                    ATGACGCTGAAGTGAAAACCACGTACAAAGCCAAGAAACCTGTGCAGCTGCCTGGCGCGTACAATGTGAATATTAAACTG
2 sekvens2900-possible-pSV6.4             ATGACGCTGAAGTGAAAACCACGTACAAAGCCAAGAAACCTGTGCAGCTGCCTGGCGCGTACAATGTGAATATTAAACTG
3 sekvens2900-possible-pSV6.1             ATGACGCTGAAGTGAAAACCACGTACAAAGCCAAGAAACCTGTGCAGCTGCCTGGCGCGTACAATGTGAATATTAAACTG
4 sekvens2900-possible-pSV6.2             ATGACGCTGAAGTGAAAACCACGTACAAAGCCAAGAAACCTGTGCAGCTGCCTGGCGCGTACAATGTGAATATTAAACTG
5 sekvens2900-possible-pSV6.3             ATGACGCTGAAGTGAAAACCACGTACAAAGCCAAGAAACCTGTGCAGCTGCCTGGCGCGTACAATGTGAATATTAAACTG

881                                                                                        960
1 pSV6                                    GACATCACCTCTCATAATGAAGATTATACGATCGTAGAGCAATATGAGCGCGCGGAGGGTCGTCATTCTACCGGTGGCAT
2 sekvens2900-possible-pSV6.4             GACATCACCTCTCATAATGAAGATTATACGATCGTAGAGCAATATGAGCGCGCGGAGGGTCGTCATTCTACCGGTGGCAT
3 sekvens2900-possible-pSV6.1             GACATCACCTCTCATAATGAAGATTATACGATCGTAGAGCAATATGAGCGCGCGGAGGGTCGTCATTCTACCGGTGGCAT
4 sekvens2900-possible-pSV6.2             GACATCACCTCTCATAATGAAGATTATACGATCGTAGAGCAATATGAGCGCGCGGAGGGTCGTCATTCTACCGGTGGCAT
5 sekvens2900-possible-pSV6.3             GACATCACCTCTCATAATGAAGATTATACGATCGTAGAGCAATATGAGCGCGCGGAGGGTCGTCATTCTACCGGTGGCAT

961                                                                                        1040
1 pSV6                                    GGATGAGCTGTACAAATAATAATAACCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTAT
2 sekvens2900-possible-pSV6.4             GGATGAGCTGTACAAATAATAATAACCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTAT
3 sekvens2900-possible-pSV6.1             GGATGAGCTGTACAAATAATAATAACCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTAT
4 sekvens2900-possible-pSV6.2             GGATGAGCTGTACAAATAATAATAACCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTAT
5 sekvens2900-possible-pSV6.3             GGATGAGCTGTACAAATAATAATAACCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTAT

1041                                                                                       1120
1 pSV6                                    CTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTAACTGCTCC
2 sekvens2900-possible-pSV6.4             CTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTAACTGCTCC
3 sekvens2900-possible-pSV6.1             CTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTAACTGCTCC
4 sekvens2900-possible-pSV6.2             CTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTAACTGCTCC
5 sekvens2900-possible-pSV6.3             CTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTAACTGCTCC

1121                                                                                       1200
1 pSV6                                    CGGTCTCACTAGAAAATAAAAACAGCCGTGCCTCTTTCTGGCAACGGCTG-TTTTTGCGGCCGCTGGATCCTCTAGCTAG
2 sekvens2900-possible-pSV6.4             CGGTCTCACTAGAAAATAAAAACAGCCGTGCCTCTTTCTGGGAACGGCTGGTTTTTGCGGCCGCTGGATCCTCTAGCTAG
3 sekvens2900-possible-pSV6.1             CGGTCTCACTAGAAAATAAAAACAGCCGTGCCTCTTTCTGGGAACGGCTG-TTTTTGCGGCCGCTGGATCCTCTAGCTAG
4 sekvens2900-possible-pSV6.2             CGGTCTCACTAGAAAATAAAAACAGCCGTGCCTCTTTCTGGCAACGGCTG-TTTTTGCGGCCGCTGGATCCTCTAGCTAG
5 sekvens2900-possible-pSV6.3             CGGTCTCACTAGAAAATAAAAACAGCCGTGCCTCTTTCTGGCAACGGCTG-TTTTTGCGGCCGCTGGATCCTCTAGCTAG

1201                                                                                       1280
1 pSV6                                    AGTCAGCTTTATGCTTGTAAACCGTTTTGTGAAAAAATTTTTAAAATAAAAAAGGGGACCTCTAGGGTCCCCAATTAATT
2 sekvens2900-possible-pSV6.4             AGTCAGCTTTATGCTTGTAAACCGTTTTGGGAAAAATTTTTAAATAAAAAA--GGGGACCTCTAGGGCCCCATTATAATA
3 sekvens2900-possible-pSV6.1             AGTCAGCTTTATGCTTGTAAACCGTTTTGGGAAAAATTTTTAAATAAAAAA--GGGGACCTCTAGGTCCCCATTAATTAT
4 sekvens2900-possible-pSV6.2             AGTCAGCTTTATGCTTGTAAACCGTTTTGGGAAAAATTTTTAAATAAAAAA--GGGGACCTCTAGGGCCCCAATTAATTA
5 sekvens2900-possible-pSV6.3             AGTCAGCTTTATGCTTGTAAACCGTTTTGGGAAAAATTTTTAAAATAAAAA--GGGGACCTCTAGGGCCCCAATTATTAG

1281                                                                                       1360
1 pSV6                                    AGTAATATAATCTATTAAAGGTCATTCAAAAGGTCATCCACCGGATCAGCTTAGTAAAGCCCTCGCTAGATTTTAATGCG
2 sekvens2900-possible-pSV6.4             AAAAA--ATCCA--TTAA-GGGCTTTCAAAGGCT-CCCCGGACGCTAAAGCCCA--------TTAGTTGGTGGGGGGGGT
3 sekvens2900-possible-pSV6.1             AAAAT--AATCTATTA-A-AGGCTTCAAAGGCTCCCCGAA---CCTAAAGCCTA--------GT-TGGGG-----GGGGG
4 sekvens2900-possible-pSV6.2             GAAAA--AAATCCATTAA-GGTCATTCAAAGGCCTCCCCGGACACTATAAGCTC--------CA-TTAGTTGTGGGTGGT
5 sekvens2900-possible-pSV6.3             AAAAT--AAT-CTATTAA-GGGCTTC--AAAGGCTCCCCGGACACTATAAACTC--------CA-TTCAGTGTTGTGTTG

1361                                                    ] 1409
1 pSV6                                    GA-TGTTGCGATTAC---TTCGCC-------------------------
2 sekvens2900-possible-pSV6.4             GGTGGTTTCCCGCCCCCCTCCGCCCCCCCCCCCTCCCCCCA-------
3 sekvens2900-possible-pSV6.1             TGTGTTTTTAAAATAAAAAGAAGAAAGAATAAATTATTTTATTTAA---
4 sekvens2900-possible-pSV6.2             TGTTTTGTTTTCCTCA--ACC--AAAAACAAAAATTTTGGGTTGAAAAA
5 sekvens2900-possible-pSV6.3             TGTTGTTTCCCTCCCTCCCCC--CCCCACACCAA---------------
```

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Appendix J – Sequencing of pSV7

The result from sequencing pSV7 is presented in this appendix. The first line show the template DNA, which was the plasmid DNA. The next four lines show the alignment of the primers M13F, M13R, MucRsekv2 and MucRsekv1 to the template.

```
           641                                                                              720
1 pSV7        AACCGCGGTCGATCTTCAGCTCATTGGCCGGAAGACGCTTGAGGTACATCAGGCTGGAGTAGCCGGTGCCAAAGTCATCG
2 M13forward  --------------------------------------------------------------------------------
3 M13reverse  AACCGCGGTCGATCTTCAGCTCATTGGCCGGAAGACGCTTGAGGTACATCAGGCTGGAGTAGCCGGTGCCAAAGTCATCG
4 MucRsekv2   --------------------------------------------------------------------------------
5 MucRsekv1   --------------------------------------------------------------------------------

           721                                                                              800
1 pSV7        ATGGACAAATCGACACCCATGTCCGACAGCTGCTGCAACACGGTCATGCTCGCATCGGCATCGCTCATGGCCGTGGTTTC
2 M13forward  --------------------------------------------------------------------------------
3 M13reverse  ATGGACAAATCGACACCCATGTCCGACAGCTGCTGCAACACGGTCATGCTCGCATCGGCATCGCTCATGGCCGTGGTTTC
4 MucRsekv2   --------------------------------------------------------------------------------
5 MucRsekv1   -------GTGAATGGACCATGTCCGAACAGCTGCTGCACACGGTCATGCTCGCATCGGCATCGCTCATGGCCGTGGTTTC

           801                                                                              880
1 pSV7        GGTGATTTCCAGGGTCAGGCTGTTGGCCGGCAACTGGTGACGTTCCAGGGCCGCGGCCACGCTTTTGACCAGCCCGACGT
2 M13forward  --------------------------------------------------------------------------------
3 M13reverse  GGTGATTTCCAGGGTCAGGCTGTTGGCCGGCAACTGGTGACGTTCCAGGGCCGCGGCCACGCTTTTGACCAGCCCGACGT
4 MucRsekv2   --------------------------------------------------------------------------------
5 MucRsekv1   GGTGATTTCCAGGGTCAGGCTGTTGGCCGGCAACTGGTGACGTTCCAGGGCCGCGGCCACGCTTTTGACCAGCCCGACGT

           881                                                                              960
1 pSV7        GGCAGAACTGCAACGCGGACAGGTTCACGGCGATGCGCCAGTGTTCGTAACCTTCTGCATACCACAGGCTCATTTGACGA
2 M13forward  --------------------------------------------------------------------------------
3 M13reverse  GGCAGAACTGCAACGCGGACAGGTTCACGGCGATGGGCCAGTGTTCGTAACCTTCTGCATACCACAGGCTCATTTGACGA
4 MucRsekv2   --------------------------------------------------------------------------------
5 MucRsekv1   GGCAGAACTGCAACGCGGACAGGTTCACGGCGATGCGCCAGTGTTCGTAACCTTCTGCATACCACAGGCTCATTTGACGA

           961                                                                             1040
1 pSV7        CAGGCTTCGTTAAGCACCCATTCGCCGATGGG---AATGATCAGCCCGGTCT-TTTCCGCCATGTCGATAAACATCGCCG
2 M13forward  --------------------------------------------------------------------------------
3 M13reverse  CAGGCTTCGTTAAAGCACCCATTCGCCGAATGGGAAATGATCAGCCCGGTCCTTTTCCGCCATGTCGAATAAAACATTCG
4 MucRsekv2   --------------------------------------------------------------------------------
5 MucRsekv1   CAGGCTTCGTTAAGCACCCATTCGCCGATGGG---AATGATCAGCCCGGTCT-TTTCCGCCATGTCGATAAACATCGCCG

          1041                                                                             1120
1 pSV7        GCAG----CAGC-AGGCCCTGTTGCGG-ATGCTCCCAGCGCA--GCAGCGCTTCGGCACCCACCGGGATACCGCTGAGC-
2 M13forward  --------------------------------------------------------------------------------
3 M13reverse  CCGGGCAGCAGCAAGGCCCTGTTGCGGAATGCTCCCAGCGCAAGCAGCCGCTTCGGCACCCACCGGGGAATACCGCTGGA
4 MucRsekv2   --------------------------------------------------------------------------------
5 MucRsekv1   GCAG----CAGC-AGGCCCTGTTGCGG-ATGCTCCCAGCGCA--GCAGCGCTTCGGCACCCACCGGGATACCGCTGAGC-

          1121                                                                             1200
1 pSV7        -----GCATCGAACTTGGGCTGGTAGTACAGGCGGAACTGCTGCTGCT---CCACCGCGTTGCGCA---AGTCCTGCAA-
2 M13forward  -----------------AGAGGGTCGTACGGGCGAA--------------------------------------------
3 M13reverse  ACCGCATCCAAAATTGGGGCTGGTAGTAACAGGGGGAAAACGGCTGCTGGCTCCACCCGCTTGGCGCAAAGTCCTGGCAA
4 MucRsekv2   --------------------------------------------------------------------------------
5 MucRsekv1   -----GCATCGAACTTGGGCTGGTAGTACAGGCGGAACTGCTGCTGCT---CCACCGCGTTGCGCA---AGTCCTGCAA-

          1201                                                                             1280
1 pSV7        --CAACTGCAATTGTTTGCG---CGCATTGGTGTTCATCGACGCATCAAAGAAGCTGTAGCCGTTCTTGCCCATGCCCTT
2 M13forward  ---------------------------TT---------------------------------------------------
3 M13reverse  CAAACTTGCAAATGTTTTGCGCCGCATTGGTGGTTCAT--CCAACCCCATCAAAAAAAAACCTGGTAGCCCGTTTCTTG
4 MucRsekv2   --------------------------------------------------------------------------------
5 MucRsekv1   --CAACTGCAATTGTTTGCG---CGCATTGGTGTTCATCGACGCATCAAAGAAGCTGTAGCCGTTCTTGCCCATGCCCTT

          1281                                                                             1360
1 pSV7        GGCGTGGTACATCGCGGCGTCGGCGTTCATCAACAGCTCCTGGGGGCTGTTGCCATTGCCGGGGAACATGGCGATGCCGA
2 M13forward  --------------------------------------------------------------------------------
3 M13reverse  GCCATGG-------------------------------------------CCCCTTGGGCGGGGTAAATCCCGGGGCGTC
4 MucRsekv2   --------------------------------------------------------------------------------
5 MucRsekv1   GGCGTGGTACATCGCGGCGTCGGCGTTCATCAACAGCTCCTGGGGGCTGTTGCCATTGCCGGGGAACATGGCGATGCCGA

          1361                                                                             1440
1 pSV7        CACTGGCGGAGATCTTCAACTCATGCTCGGCCACCCGGAAGGCGCGGTTGATCAGCACCACCTGGCGTTCGGCGAGGCCG
2 M13forward  -------GGGCCCTCTAGATGCATGCTCGAGCGGC-----------------------------------CG-----CCA
3 M13reverse  CGG--GCGTTCCATCAAAAAGCTTCCTGG---------GGGCCTGTTGCCCTT---TGCCGGGGGAAAATTGGGCCA
4 MucRsekv2   --------------------------------------------------------------------------------
5 MucRsekv1   CACTGGCGGAGATCTTCAACTCATGCTCGGCCACCCGGAAGGCGCGGTTGATCAGCACCACCTGGCGTTCGGCGAGGCCG
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1441        :       .       .       .       .       5       .       . 1520
1 pSV7          AGGGCAT-CGTCCGGCTGGGTCAGTTGCACCAGCAACACAAACTCATCGCCGCCGATGCGCGCCAGGGTGTCCTGGCTGC
2 M13forward    GTGTG---ATGGATATCTGCAGAATTCGGCCTTAGTCATATGCTCATCGGTAGTTATTCACCCTCACTGGTCGTGATTTC
3 M13reverse    ATGCCAAAACCTGGGCGGAAAAATTTTTAAA-----------------TCCATGGGTTCGGGCCCCC----CCGGAAAGG
4 MucRsekv2     ------------------------------------------------TGAAAGGCCAGCGGGCAGGGTGTCCTGGCTGC
5 MucRsekv1     AGGGCAT-CGTCCGGCTGGGTCAGTTGCACCAGCAACACAAACTCATCGCCGCCGATGCGCGCCAGGGTGTCCTGGCTGC

1521        .       .       :       .       .       .       .       6 1600
1 pSV7          GCAGGTCTTCGCGCAGGCGCACCCCTACTTCGCTCAGCAACTGGTCACCCATATGA---------TGGCCGAAGGCATCG
2 M13forward    GCTGTTCGTCGCGATCCTGGCTTCCTACACCGCGCTCGACCTTTCCGGCCGGATCGCCACCGCCAAGGGACGCGGTGTCT
3 M13reverse    GGGGG-GTTTGATTAAC-ACCCCCCT------------------------------------------------------
4 MucRsekv2     GCAGGTCTTCGCGCAGGCGCACCCCTACTTCGCTCAGCAACTGGTCACCCATATGA---------TGGCCGAAGGCATCG
5 MucRsekv1     GCAGGTCTTCGCGCAGGCGCACCCCTACTTCGCTCAGCAACTGGTCACCCATATGA---------TGGCCGAAGGCATCG

1601        .       .       .       .       :       .       .       . 1680
1 pSV7          TTGACCGGCTTAAAGCCATCCAGGTCAATAAACATCAGTGCGAAGCAGCCACCCTGCTCCTTCACCGCCTGCATGGCTTG
2 M13forward    ATTTATGGATAACCGGCG----GCGCCATGGCGATGGGCGTGGGCGTGTGGTCGATGCACTTCATCGGCATGCTGGCGTT
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     TTGACCGGCTTAAAGCCATCCAGGTCAATAAACATCAGTGCGAAGCAGCCACCCTGCTCCTTCACCGCCTGCATGGCTTG
5 MucRsekv1     TTGACCGGCTTAAAGCCATCCAGGTCAATAAACATCAGTGCGAAGCAGCCACCCTGCTCCTTCACCGCCTGCATGGCTTG

1681        .       7       .       .       .       .       :       . 1760
1 pSV7          CTGAATACGGTCAGCGAGCAAAGTGCGGTTGGGCAGACCGGTGAGCATGTCGTGCAGGGCCAGGTGGGTCAGCTCTTGGT
2 M13forward    GCGTTTGCCGTTTGCC------CTGGGGTTCGACGTAGGCAT------------------------------CACTGCGT
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     CTGAATACGGTCAGCGAGCAAAGTGCGGTTGGGCAGACCGGTGAGCATGTCGTGCAGGGCCAGGTGGGTCAGCTCTTGGT
5 MucRsekv1     CTGAATACGGTCAGCGAGCAAAGTGCGGTTGGGCAGACCGGTGAGCATGTCGTGCAGGGCCAGGTGGGTCAGCTCTTGGT

1761        .       .       .       8       .       .       .       . 1840
1 pSV7          TGGCTTGGGTCAGGGAGTCGGCAAGCACCGCAGTGCGGGCTTCCAGACGCGCATCGAGCAACGACGTCAGCAAAGCGATG
2 M13forward    TGTCGTTGTT----------------------------------------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     TGGCTTGGGTCAGGGAGTCGGCAAGCACCGCAGTGCGGGCTTCCAGACGCGCATCGAGCAACGACGTCAGCAAAGCGATG
5 MucRsekv1     TGGCTTGGGTCAGGGAGTCGGCAAGCACCGCAGTGCGGGCTTCCAGACGCGCATCGAGCAACGACGTCAGCAAAGCGATG

1841        :       .       .       .       .       9       .       . 1920
1 pSV7          GCCAGCACCGCCAGGGTCGTCACCAGCACCAGATTATCCAGGCCCTTGCCGCTCAGGCCGTTCAGCGCAGCACCGCAGAA
2 M13forward    -----------------------GATTGCGGTGTTGTCCAGCGGCTTTGCCTTGTGGCTGGTCAGC--------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     GCCAGCACCGCCAGGGTCGTCACCAGCACCAGATTATCCAGGCCCTTGCCGCTCAGGCCGTTCAGCGCAGCACCGCAGAA
5 MucRsekv1     GCCAGCACCGCCAGGGTCGTCACCAGCACCAGATTATCCAGGCCCTTGCCGCTCAGGCCGTTCAGCGCAGCACCGCAGAA

1921        .       .       :       .       .       .       .       0 2000
1 pSV7          ACTCTCATCGGCAAACCGTGCCGCCGCCATGCCGGTGTAATGCATGCCGACGATGGCAAAACCCATCACCACCGCCGCCC
2 M13forward    --------------------------------------------------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     ACTCTCATCGGCAAACCGTGCCGCCGCCATGCCGGTGTAATGCATGCCGACGATGGCAAAACCCATCACCACCGCCGCCC
5 MucRsekv1     ACTCTCATCGGCAAACCGTGCCGCGGCCATGCCGGTGTAATGCATGCCGACAAGGC-AAAACCCATCACCACCGCCGCCC

2001        .       .       .       .       :       .       .       . 2080
1 pSV7          CACCGCGCGCGAGGCGTACGTACGGGGTATTGCGCCGCAGGTTATAGGCGATCCACAGCGCAGCGCCGGACGCCGCGACC
2 M13forward    --------------------------------------------------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     CACCGCGCGCGAGGCGTACGTACGGGGTATTGCGCCGCAGGTTATAGGCGATCCACAGCGCAGCGCCGGACGCCGCGACC
5 MucRsekv1     CACCGGCGGCGAGGCGTACGTACGGGGTATTGCGCCGCAGGTTAAAGGCGATCCCAAGGCCACGCCGGAACCCGGAACCG

2081        .       1       .       .       .       .       :       . 2160
1 pSV7          GCGATCAGCAGCGAAGCGCCGAATAAGGCGGGGTCGTAGTCGATACCCGGCG--TCATCCGCATCGCGGCCATGCCGGTG
2 M13forward    --------------------------------------------------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     GCGATCAGCAGCGAAGCGCCGAATAAGGCGGGGTCGTAGTCGATACCCGGCG--TCATCCGCATCGCGGCCATGCCGGTG
5 MucRsekv1     GAATACAAACG--AAACGCCAAAAAGGGGGGT---CTAAACAAAACCGGGCTCCCCCTCCGCCCAGCGGGGAAGGAGGGG

2161        .       .       .       2       .       .       .       . 2240
1 pSV7          TAATGCATGCTGGCAATGCCGGTGCCCATGATCAGCGCGCCCAATGCCAGCTGCCAGGCCGGTAAGCGCGGCTGGCTGAC
2 M13forward    ---------------------------------------------------------------CAGCCGCGCTTACCGGC
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     TAATGCATGCTGGCAATGCCGGTGCCCATGATCAGCGCGCCCAATGCCAGCTGCCAGGCCGGTAAGCGCGGCTGGCTGAC
5 MucRsekv1     GAAAGCGGGGCCAAAAA-------------ACCCCCAAGCCCTTT-CCGGG-AAAGGGGGGGTAAAC-------------
```

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                2241       :       .       .       .       .       3       .  2320
1 pSV7          CAGCCACAAGGCAAAGCCGCTGGACAACACCGCAATCAACAACGACAACGCAGTGATGCCTACGTCGAACCCCAGGGCAA
2 M13forward    CTGGCAGC------------TGGCATTGGGC-------------------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     CAGCCACAAGGCAAAGCCGCTGGACAACACCGCAATCAACAACGACAACGCAGTGATGCCTACGTCGAACCCCAGGGCAA
5 MucRsekv1     -----ACAAAGAAACC----------------------------------------------------------------

                2321       .       .       :       .       .       .       .       4  2400
1 pSV7          ACGGCAAACGCAACGCCAGCATGCCGATGAAGTGCATCGACCACACGCCCACGCCCATCGCCATGGCGCCGCCGGTTATC
2 M13forward    --------------------------------------------------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     ACGGCAAACGCAACGCCAGCATGCCGATGAAGTGCATCGACCACACGCCCACGCCCATCGCCATGGCGCCGCCGGTTATC
5 MucRsekv1     --------------------------------------------------------------------------------

                2401       .       .       .       .       :       .       .       .  2480
1 pSV7          CATAAATAGACACCGCGTCCCTTGGCGGTGGCGATCCGGCCGGAAAGGTCGAGCGCGGTGTAGGAAGCCAGGATCGCGAC
2 M13forward    --------------------------------------------------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     CATAAATAGACACCGCGTCCCTTGGCGGTGGCGATCCGGCCGGAAAGGTCGAGCGCGGTGTAGGAAGCCAGGATCGCGAC
5 MucRsekv1     --------------------------------------------------------------------------------

                2481       .       5       .       .       .       .       :       .  2560
1 pSV7          GAACAGCGAAATCACGACCAGTGAGGGTGAATAACTACCGATGAGCATATGACTAAGGGCGAATTCTGCAGATATCCATC
2 M13forward    --------------------------------------------------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     GAACAGCGAAATCACGACCAGTGAGGGTGAATTACTACCGATGAGCATATGACTAAGGGCGAATTCTGCAGATATCCATC
5 MucRsekv1     --------------------------------------------------------------------------------

                2561       .       .       .       6       .       .       .       .  2640
1 pSV7          ACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCG
2 M13forward    --------------------------------------------------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     ACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCCTATAGTGAGTCGAATTACAATTCACTGGCCGTCG
5 MucRsekv1     --------------------------------------------------------------------------------

                2641       :       .       .       .       .       7       .       .  2720
1 pSV7          TTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGG
2 M13forward    --------------------------------------------------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     TTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGG
5 MucRsekv1     --------------------------------------------------------------------------------

                2721       .       .       :       .       .       .       .       8  2800
1 pSV7          CGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTATACGTACGGCAGTTTAAGGTTTACAC
2 M13forward    --------------------------------------------------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     CGTAATACGAAAAAGGCCCGAACCGATCGCCCTTTCCAACAGTTGCCCAGCCTATACGTACGGCAGTTTAGGGTTTACCC
5 MucRsekv1     --------------------------------------------------------------------------------

                2801       .       .       .       .       :       .       .       .  2880
1 pSV7          CTATAAAAGAGAGAGCCGTTATCGTCTGTTTGTGGATGTACAGAGTGATATTATTGACACGCCGGGGCGACGGATGGTGA
2 M13forward    --------------------------------------------------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     CTTTA---------------------------------------------------------------------------
5 MucRsekv1     --------------------------------------------------------------------------------

                2881       .       9       .       .       .       .       :       .  2960
1 pSV7          TCCCCCTGGCCAGTGCACGTCTGCTGTCAGATAAAGTCTCCCGTGAACTTTACCCGGTGGTGCATATCGGGGATGAAAGC
2 M13forward    --------------------------------------------------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     --------------------------------------------------------------------------------
5 MucRsekv1     --------------------------------------------------------------------------------

                2961       .       .       .       0       .       .       .       .  3040
1 pSV7          TGGCGCATGATGACCACCGATATGGCCAGTGTGCCGGTCTCCGTTATCGGGGAAGAAGTGGCTGATCTCAGCCACCGCGA
2 M13forward    GCGCTGATCATGGGCACCGGCATTGCCAGCATGCATTACACCGG------------------------------------
3 M13reverse    --------------------------------------------------------------------------------
4 MucRsekv2     --------------------------------------------------------------------------------
5 MucRsekv1     --------------------------------------------------------------------------------
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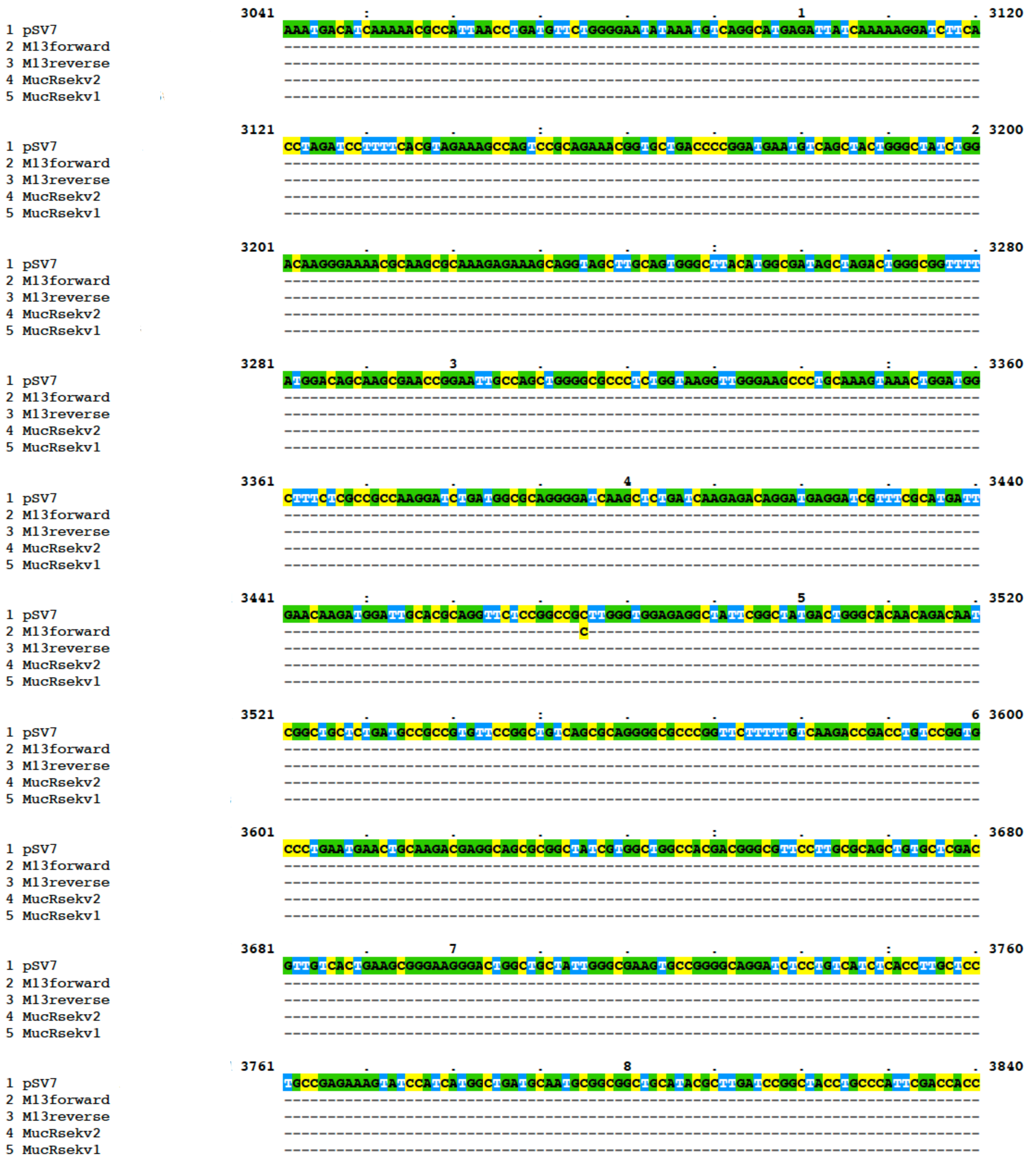

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                 4641                                                                               4720
1 pSV7           GCGGCCTGGACGAGCTGTACGCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCCATGACC
2 M13forward     GCGGTGGTGAT--------------GGGTTTTGCCATCGTCTGCATGCATTACA---------CCGGCATGGCGGCGGCA
3 M13reverse     --------------------------------------------------------------------------------
4 MucRsekv2      --------------------------------------------------------------------------------
5 MucRsekv1      --------------------------------------------------------------------------------

                 4721                                                                               4800
1 pSV7           GAGATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCACTTCGTGGCCGAGGA
2 M13forward     CGGTTTGCCGATGAGAGTTTC-TGC--GGTGCTGCACTGAACGGCCTGAGCGGCAAGGGACTGGGAAATCTGGT------
3 M13reverse     --------------------------------------------------------------------------------
4 MucRsekv2      --------------------------------------------------------------------------------
5 MucRsekv1      --------------------------------------------------------------------------------

                 4801                                                                               4880
1 pSV7           GCAGGACTGACACGTGCTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCA
2 M13forward     ---------------------------------------------GCTGGTGACGACCCCTGGCGGAGCTGGCAT-----
3 M13reverse     --------------------------------------------------------------------------------
4 MucRsekv2      --------------------------------------------------------------------------------
5 MucRsekv1      --------------------------------------------------------------------------------

                 4881                                                                               4960
1 pSV7           AAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTT
2 M13forward     --------------------------------------------------------------------------------
3 M13reverse     --------------------------------------------------------------------------------
4 MucRsekv2      --------------------------------------------------------------------------------
5 MucRsekv1      --------------------------------------------------------------------------------

                 4961                                                                               5040
1 pSV7           TTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACC
2 M13forward     --------------------------------------------------------------------------------
3 M13reverse     --------------------------------------------------------------------------------
4 MucRsekv2      --------------------------------------------------------------------------------
5 MucRsekv1      --------------------------------------------------------------------------------

                 5041                                                                               5120
1 pSV7           AACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCC
2 M13forward     --------------------------------------------------------------------------------
3 M13reverse     --------------------------------------------------------------------------------
4 MucRsekv2      --------------------------------------------------------------------------------
5 MucRsekv1      --------------------------------------------------------------------------------

                 5121                                                                               5200
1 pSV7           ACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGAT
2 M13forward     --------------------------------------------------------------------------------
3 M13reverse     --------------------------------------------------------------------------------
4 MucRsekv2      --------------------------------------------------------------------------------
5 MucRsekv1      --------------------------------------------------------------------------------

                 5201                                                                               5280
1 pSV7           AAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTG
2 M13forward     --------------------------------------------------------------------------------
3 M13reverse     --------------------------------------------------------------------------------
4 MucRsekv2      --------------------------------------------------------------------------------
5 MucRsekv1      --------------------------------------------------------------------------------

                 5281                                                                               5360
1 pSV7           CACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTC
2 M13forward     --------------------------------------------------------------------------------
3 M13reverse     --------------------------------------------------------------------------------
4 MucRsekv2      --------------------------------------------------------------------------------
5 MucRsekv1      --------------------------------------------------------------------------------

                 5361                                                                               5440
1 pSV7           CCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGA
2 M13forward     --------------------------------------------------------------------------------
3 M13reverse     --------------------------------------------------------------------------------
4 MucRsekv2      --------------------------------------------------------------------------------
5 MucRsekv1      --------------------------------------------------------------------------------
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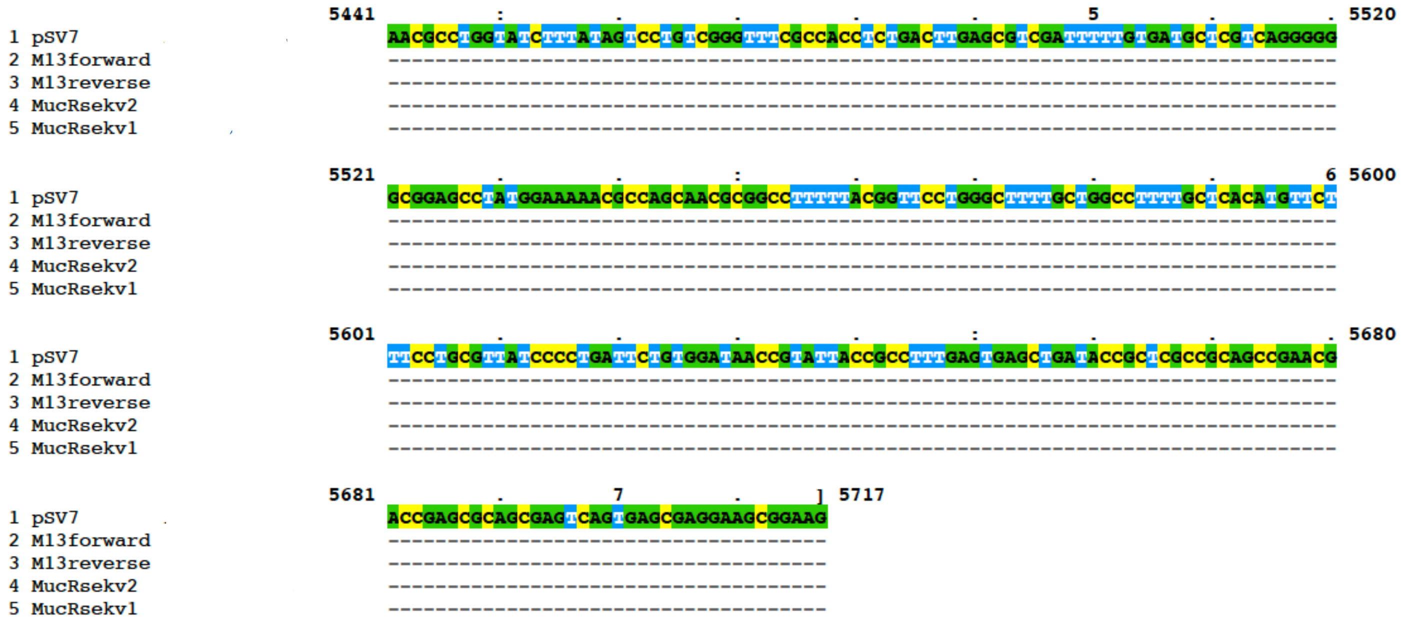

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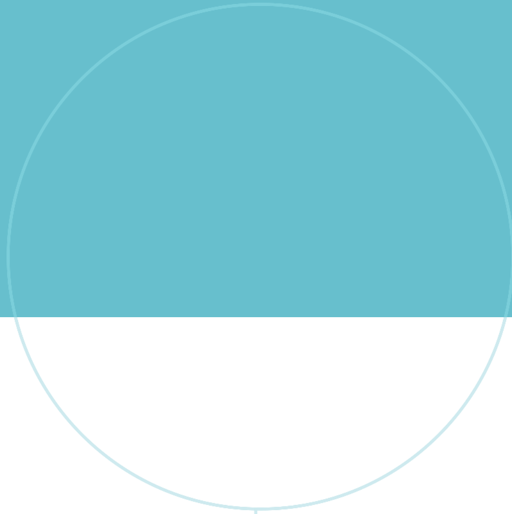
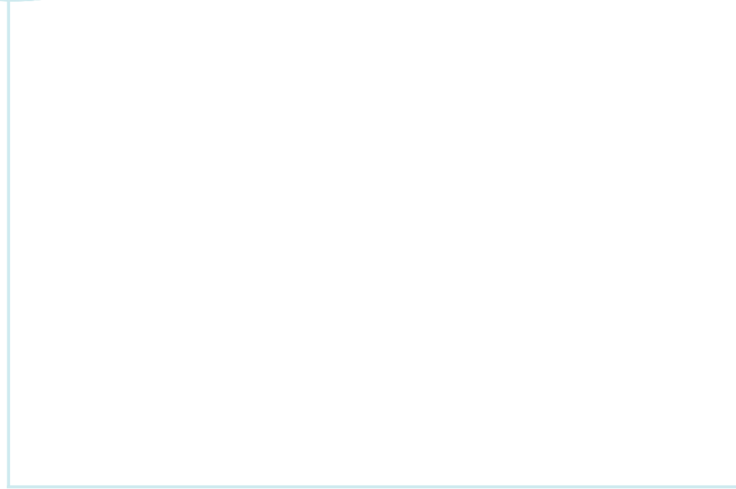

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