Master's thesis

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Development and testing of antibiotic resistance and promoter cassettes for a versatile toolbox of conditional suicide plasmids in *Marinobacter algicola*

Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Helga Ertesvåg
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Preface

This master's thesis was completed in the spring of 2024 at the Department of Biotechnology at the Norwegian University of Science and Technology in Trondheim, under the supervision of Professor Helga Ertesvåg.

I would like to thank my supervisor, Professor Helga Ertesvåg, for her guidance in writing and in laboratory work, her helpful feedback, and her availability and quick response time. Thanks to my lab group, Andreas, Vilde, and Ane, for making the long lab days enjoyable and for being great to work with.

Thanks to my uni mates for making these five years at NTNU the best ones so far. To O-banden, for the trips, adventures, and everyday humor and lunches. To NTNUI Bandy Damer, for five amazing seasons. Thanks to my roomies, SCHOT, for making our quirky apartment into a great home, for all the laughter and shenanigans shared. And of course, my family is cool — I'm grateful for their unconditional support.

Cheers, Tuva Askmann Nærby

Abstract

This thesis is part of the development of a versatile toolbox of conditional suicide plasmids, each consisting of a cassette with different characteristics that can be easily changed to adapt to different experiments. These plasmids are designed to incorporate cassettes of genes that can be assembled to meet specific experimental needs, consisting of a promoter system, controlled replication system, reporter gene, antibiotic resistance gene, and gene for negative selection. They aim to enable precise control of gene expression and can be selectively activated or removed after their purpose is fulfilled.

This thesis focuses on the part of constructing antibiotic resistance gene cassettes that can be easily exchanged through restriction digestion and ligation. Using the plasmid pTN3 with tetracycline resistance, developed in my previous work, new plasmids pTN4, pTN5, and pTN6 were constructed, incorporating resistance genes for apramycin, kanamycin, and spectinomycin, respectively. This was done to achieve versatility, allowing their use in various gram-negative bacterial strains and serving as selectable markers. The effectiveness of gene substitution was also tested, demonstrating high efficiency by replacing kanamycin resistance with apramycin resistance in a series of plasmids.

Various promoter systems were evaluated for their ability to regulate gene expression in *Marinobacter algicola*. This assessment aimed to determine promoter strength and system behavior in both induced and non-induced states in this bacteria, and compared to similar tests conducted in other bacteria. The AraC-pBAD system proved to be the most effective, showing the highest expression of RFP, whereas other systems tested, Pm wt, Pm ML1.17, PmG5, AntR-Pant, LacI-Ptrc, and RhaSR-pRha, were less effective, resulting in lower expression levels. This underscores the potential for developing a flexible toolbox where promoter cassettes can be easily exchanged to adapt to the different requirements of bacterial strains, with the aim of optimising gene expression. Variations in growth rates and fluorescence intensities between induced and non-induced strains suggested that protein production imposed a metabolic burden on the *M. algicola*, affecting the growth rates.

Throughout this thesis, it was demonstrated that the versatile toolbox can be an effective way of tailoring conditional suicide plasmids to various conditions and needs. The potential of this easily adaptable cassette system for plasmid-based genetic modifications was highlighted.

Sammendrag

Denne oppgaven er en del av en utvikling av en fleksibel verktøykasse for betingede selvmordsplasmider, hvor hvert plasmid består av kassetter av forskjellige egenskaper som enkelt kan byttes ut for å tilpasse ulike behov. Plasmiddesignet består av flere genkassetter som kan settes sammen for å tilpasses spesifikke eksperimentelle betingelser. Disse utskiftbare kassettene er ulike promotorsystem, kontrollerte replikasjonssystem, reporter-gen, antibiotikaresistensgen og gen for negativ seleksjon. De har som mål å sikre presis kontroll av genuttrykk og kan selektivt aktiveres eller fjernes etter at formålet er oppfylt.

I denne oppgaven fokuserers det på å konstruere antibiotikaresistens-kassetter som enkelt kan byttes ut gjennom restriksjonskutt og ligering. Med utgangspunkt i plasmidet pTN3 med tetracyklinresistens, utviklet i mitt tidligere arbeid, ble nye plasmider pTN4, pTN5 og pTN6 konstruert, som innehar henholdsvis resistensgenene apramycin, kanamycin og spektinomycin. Dette ble gjort for å ha et bredere utvalg av resistens gen i verktøykassen, slik at de kan brukes i ulike gram-negative bakterier og fungere som selekterbare markører. Effektiviteten av gen-bytte ble også testet, og viste seg effektivit ved å enkelt kunne erstatte kanamycinresistens med apramycinresistens i en serie plasmider.

Ulike promotorsystemer sin evne til å regulere genuttrykk i *Marinobacter algicola* ble testet og analysert. Denne analysen hadde som mål å evaluere promotorstyrke og oppførsel av indusert og ikke-indusert tilstand i bakterien. Analysen ble også sammenlignet med lignende tester av samme systemer gjort i andre bakterier. AraC-pBAD-systemet viste seg å være det mest effektive, med høyest uttrykk av RFP, andre testede systemer, Pm wt, Pm ML1.17, PmG5, AntR-Pant, LacI-Ptrc og RhaSR-pRha, var derimot mindre effektive og viste lavere uttrykksnivå. Dette understreker potensialet for å utvikle en fleksibel verktøykasse hvor promotor-kassetter enkelt kan byttes ut for å tilpasses de ulike bakteriestammenes behov, med mål om å optimalisere genuttrykket. Variasjoner i vekstratene og uttrykt fluorescens intensitet mellom induserte og ikke-induserte stammer antydet at proteinproduksjon er en metabolsk belastning for *M. algicola*, som da vil påvirker vekstratene.

Gjennom denne oppgaven ble det vist at den fleksible verktøykassen kan være en effektiv måte å tilpasse betingede selvmordsplasmider til ulike behov og forhold. Det ble fremhevet hvilke potensial et slikt enkelt tilpasningsdyktig kassettsystem kan ha innen plasmidbaserte genetiske modifikasjoner.

Abbriviations

Am	Apramycin
Am^R	Apramycin resistant
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
bp	Base pair
cAMP	Cyclic adenosine monophosphate
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
CRP	cAMP receptor protein
DAP	Diaminopimelic acid
ddNTP	Di-deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
dsDNA	Double-stranded DNA
DSB	Double-strand breaks
FI	Fluorescence intensity
GMO	Genetically modified organism
gRNA	guide RNA
HR	Homologous recombination
HDR	Homology-directed repair
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobases
Km	Kanamycin
Km^R	Kanamycin resistance
LA	Luria agar
LB	Luria broth
mRNA	Messenger RNA
NHEJ	Non-homologous end joining
OD	Optical density
OD_{600}	Optical density read at 600 nm
ON	Overnight
Ori	Origin of replication
OriT	Origin of transfer
OriV	Origin of vegetative replication
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
QS	Quorum sensing
RFP	Red fluorescent protein
RO	Reverse osmosis
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulfate
SOC	Super optimal broth
Sp	Spectinomycin
Sp^R	Spectinomycin resistance
ssDNA	Single-stranded DNA
TAE	Tris-acetate-EDTA
TALEN	Transcription Activator-Like Effector Nucleases
Tc	Tetracycline
Tc^R	Tetracycline resistance
Tm	Melting temperature
TOPO	PCR Blunt-II Topoisomerase
ZFN	Zinc Finger Nucleases
λH	λ-DNA cut with HindIII
λP	λ-DNA cut with PstI

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1 Introduction

1.1 Background and Aims

Ultimately, this thesis aims to contribute to the development of a versatile toolbox-kit of cassettes to assemble conditional suicide plasmids for an adaptable and efficient system of plasmid based genetic modification. These plasmids are to be derived from RK2 plasmids and designed to easily test the different sets of standardised cassettes for gen expression, which are composed of antibiotic resistance genes, promoter systems, *trfA*, and selective markers, as illustrated in Figure 1. By providing this modular framework, researchers can efficiently swap these elements to tailor the final plasmid to specific experimental needs.

The ability to change cassettes within the vector simplifies the process of testing different genetic configurations, thereby hopefully accelerating research and discovery. As part of this, Wagle is testing the *trfA* systems for the replication system cassettes for the toolbox[1]. Controlling the *trfA* gene is essential in regulating the plasmid replication, ensuring stability and compatibility across different bacterial hosts. Additionally, Vold[2], Haaland[3], and Wesche[4] have previously worked on different promoter systems to assess their strength and adaptability as cassettes in the toolbox.

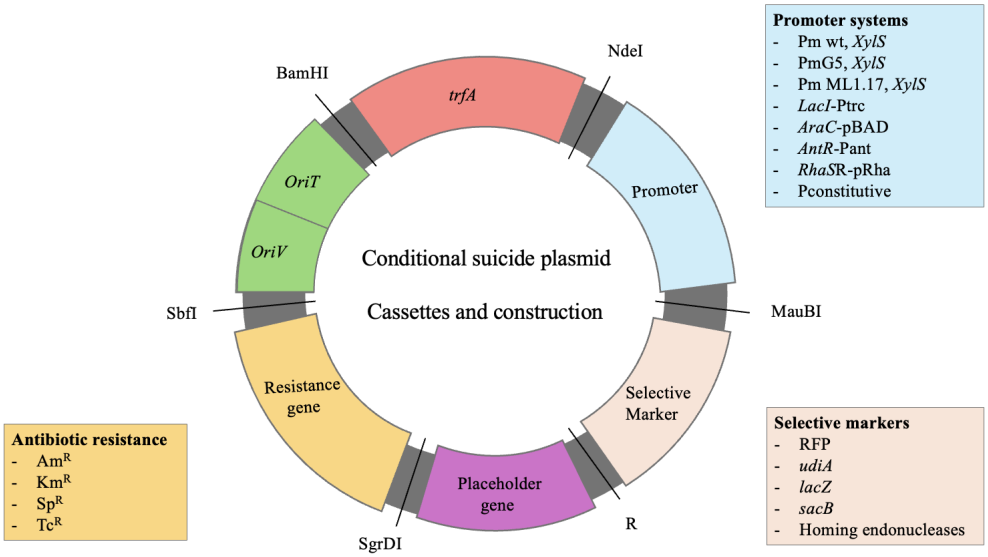


Figure 1: Illustration of the different interchangeable cassettes of the conditional suicide plasmids, which are the end-goal of the overall project. The "R" denotes a restriction enzyme not decided, where the substitution of genes can occur through digestion and ligation. The placeholder gene can be replaced by any gene of interest.

As a segment of the greater initiative, this thesis aims to construct different resistance markers as cassettes for the toolbox, allowing for the selection and maintenance of plasmid holding bacteria and the compatibility with various gram negative bacteria. To construct these plasmids, a previously designed tetracycline resistant (Tc^R) plasmid, pTN3[5], is used as backbone for the insertion of new antibiotic resistance genes. These new plasmids, pTN4, pTN5 and pTN6, encodes apramycin (Am), kanamycin (Km), and spectinomycin (Sp) resistance, respectively. The utility of such flexibility is valuable for optimising gene expression and enhancing the efficiency of genetic experiments. Following the assembly of these plasmids, previously constructed promoter systems was to be tested in *Marinobacter algicola* for the purpose of investigating their strength in this species.

This thesis chose to focus on *M. algicola*, a gram-negative, γ-proteobacterium , as limited genetic tools have been developed for this organism. The aim of this is to measure which systems in the toolbox that provide the most reliable and controlled gene expression in this specific bacteria.

The introduction will cover the necessary background for understanding the overall project aims and the methods used in this thesis. It will provide context on the development of the genetic toolbox, the rationale behind the use of conditional suicide plasmids and the importance of the different cassettes. As this thesis is a continuation of my previous project "To be and then to be gone: Developing generalized protocols for conditional suicide plasmids focusing on antibiotic resistance"[5], both the introduction and method sections are based on those from my previous work.

1.2 Genome editing in bacteria

Genome editing are techniques that precisely modifies deoxyribonucleic acid (DNA) sequences to alter gene expressions. The application of genome editing are used across various fields, including biotechnology, agriculture, clinical medicine, and research[6][7]. By adding, deleting, or altering DNA within an organism's genome, scientists can manipulate gene expression to unravel genetic functions and their implications in complex biological processes, including disease pathogenesis[8]. This technological advancement not only exceeds in identifying therapeutic targets but also has industrial applications, such as the engineering of microorganisms for the production of pharmaceuticals, biofuels, and chemicals[6]

Chemical biology provides a spectrum of approaches for genome editing through programmable nucleases such as Zinc Finger Nuclease (ZNFs), Transcription Activator-Like Effector Nucleases (TALENs), and CRISPR-Cas9 systems. These nucleases induce double-strand breaks (DSBs) at specific genomic sites, leading to gene disruption or gene replacement through cellular repair mechanisms like non-homologous end-joining (NHEJ) or homology-directed repair (HDR)[9]. Homologous recombination (HR), a natural repair mechanism, facilitates the exchange of genetic material between similar DNA sequences, allowing the precise integration of genetic changes by replacing a target gene with an engineered variant. This method requires the introduction of a DNA template carrying the desired modification and is often used in combination with genome editing technologies such as CRISPR-Cas systems[10].

1.2.1 Recombinant DNA technology

Recombinant DNA technology is a foundational method for editing and manipulating DNA, enabling the creation of new genetic sequences by combining DNA from different sources.

Topoisomerase cloning is a specific recombinant DNA technology that simplifies the construction of DNA constructs by using the enzyme topoisomerase I. This enzyme cuts and ligates DNA, allowing for rapid insertion of DNA fragments into plasmid vectors without the use of restriction enzymes or ligases. This method offers high efficiency, seamless cloning[11], and as a method used in this thesis will be further detailed in Section 2.9.

In addition to topoisomerase cloning, multiple other methods are used within recombinant DNA technology. These include sequence and ligation independent cloning, Gibson assembly, seamless ligation cloning extract, and gateway cloning, among others. These methods creates single-strand overhangs, recombining homology arms, or amplifies inserts as mega-primers to facilitate genetic modifications[12]. Recombinases such as RecE and RecT are involved in homologous recombination processes in vivo, facilitating the insertion of specific DNA sequences into target genomes. Often, specialized *Escherichia coli* host cells induced to express these recombinases are used, allowing for precise and efficient genetic modifications[12].

Despite its potential, recombinant DNA technology also presents challenges, including technical complexities in cloning and design, as well as with transformation efficiency and stability. Additionally, there is a need for stringent regulatory frameworks to manage its application safely and responsibly[13].

1.2.2 Homologous recombination in bacteria

Homologous recombination in bacteria is primarily a DNA repair mechanism, involving the exchange of genetic information between two DNA molecules or segments with similar sequences. This process is crucial for maintaing genetic integrity and facilitating genome evolution in bacteria[14]. By harnessing this innate mechanism, researchers can introduce specific alterations into the DNA of an organism to make targeted genetic modifications[15].

To use HR as a genome editing technique, a DSBs and a donor DNA molecule are introduced into the cell. This donor template, is designed to be nearly identical to the target DNA sequence and includes the desired modifications. The modifications could be a simple single base pair change or the insertion of a new gene or sequence[16][17]. As illustrated in Figure 2, the donor DNA with sequences A and B aligns with the chromosomal sequences A' and B', facilitating the recombination process. Once the DSB is created, the cell's natural DNA repair mechanisms, the homologous recombination pathway, are recruited to repair the break. The RecA protein binds to single-stranded DNA (ssDNA) and facilitates strand breakage, where the ssDNA pairs with a complementary sequence in an undamaged DNA molecule. This leads to the formation of a Holliday junction, a four-way junction where two double helices connect. It acts as a intermediat in the prossess of DNA recombination and is then resolved to complete the repair[14]. The cell uses the donor DNA as a template for repair, incorporating the new sequence into the genome in place of the original sequence at the break site.

In the second step, to ensure the removal of the vector and retain only the desired genetic of interest, a second recombination event occurs. The vector is designed to lack the ability to replicate autonomously within the host cell, which ensures it will be lost after cell division. The integration of a linked reporter gene within the donor sequences facilitates the identification of successfully edited cells. These cells can then be tracked and selected based on the expression of the reporter gene, ensuring that only cells with the desired genetic modification are used in further experiments[16]. A toxic selection system is employed. Such systems could be *sacB*-based where the activation by sucrose, expresses the *sacB* gene to produces a toxic polymer, killing cells retaining the plasmid. Thus, only cells that have undergone both recombination steps will survive, achieving negative selection[16]. Understanding HR mechanisms in bacteria allows researchers to harness this process for genome editing, enabling targeted modifications.

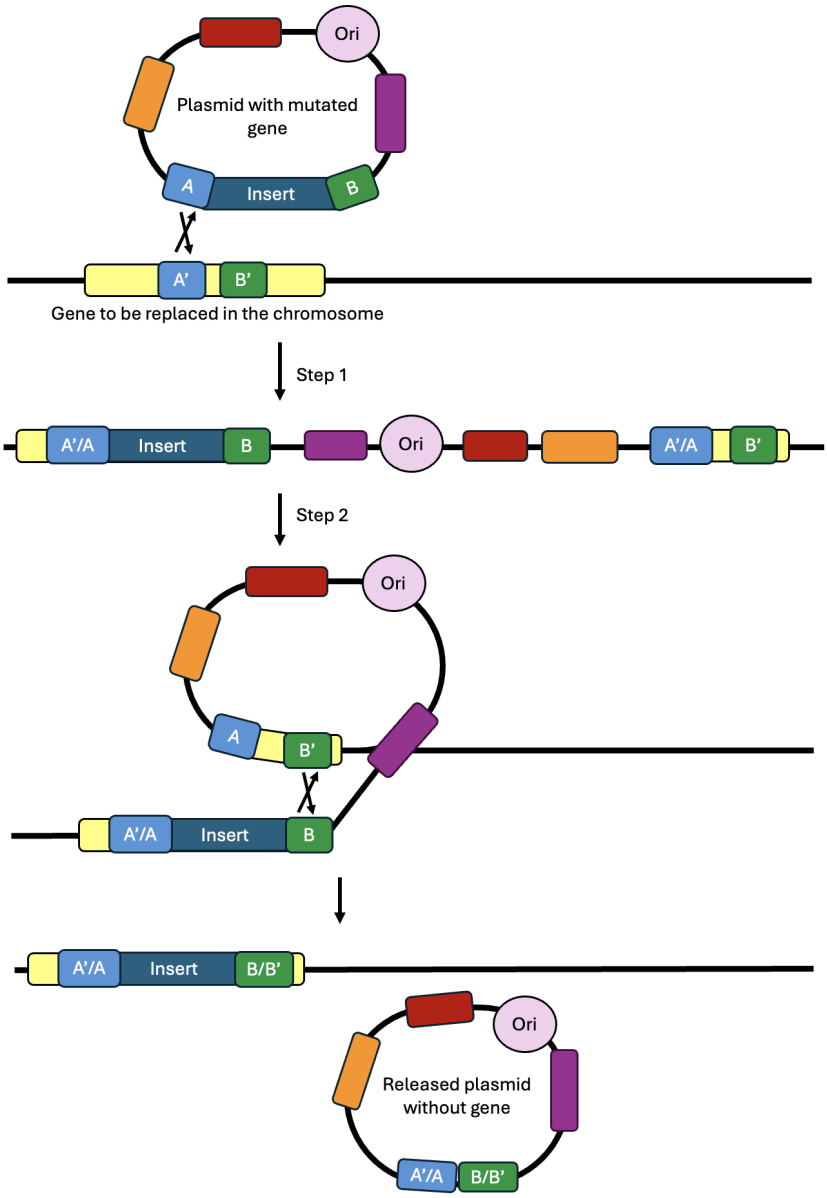


Figure 2: Illustration of the two steps in HR, where firstly the gene of interest in a vector has similar sequences, A and B, to the chromosomal sequences, A' and B', and is inserted by recombination. In the second step, the vector is removed through another recombination event. The released plasmid which lacks the ability to replicate, is lost by the cell. The illustration is inspired by a figure from Holm[18]

In summary, HR facilitates the exchange of DNA segments between two molecules across regions of identical sequences, thereby enabling precise genetic interventions such as the replacement, insertion, deletion, or modification of specific genes or genetic elements[16].

1.2.3 Clustered Regularly Interspaced Short Palindromic Repeats

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology is derived from prokaryotic immune defense system, utilising guide RNA (gRNA) to direct the Cas9 protein to specific DNA sequences, creating DSBs at these locations[7]. In natural settings, this mechanism allows for the destruction of invasive DNA fragments. In research and clinical applications, these DSBs are used for genome editing where they can be repaired by NHEJ, which can result in inser-

tions or deletions, or by HDR, a method that enables precise genetic modifications by incorporating a donor DNA sequence to replace or alter the original nucleotide sequence[19]. The advantages of CRISPR/Cas9 include its high success rate, efficiency, and that the guide RNA is relatively easy to design and can be quickly synthesized. However, challenges include achieving high HDR rates and off-target effects, where Cas9 may bind to and cut unintended parts of the genome, potentially leading to unwanted mutations or chromosomal rearrangements[19].

CRISPR interference (CRISPRi) is another technique, derived from CRISPR, that uses the CRISPR system to inhibit the expression of specific genes. It consists of a deactivated Cas9 protein, which cannot digest DNA but is still directed to specific DNA sequences by a gRNA. When deactivated Cas9 is targeted to a gene promoter region by a gRNA, it interferes with the transcription of that gene, leading to inhibited/suppresed gene expression[20]. This method allows for reversible and specific regulation of gene expression without altering the original DNA sequence, and has been used in various organisms, including bacteria, fungi, and other pathogens[20], and is illustrated in Figure 3.

In addition, CRISPR activation (CRISPRa) represents another application of the CRISPR-Cas system, focusing on gene activation rather than repression or editing. CRISPRa utilizes a nuclease-deactivated Cas protein that is fused to transcriptional activators, which can be directed to specific gene regions by a guide RNA. This approach facilitates the targeted activation of genes, enabling gene identification and expression optimization. CRISPRa also has the ability of targeting multiple genes simultaneously, and is suitable for temporary gene activation[21]

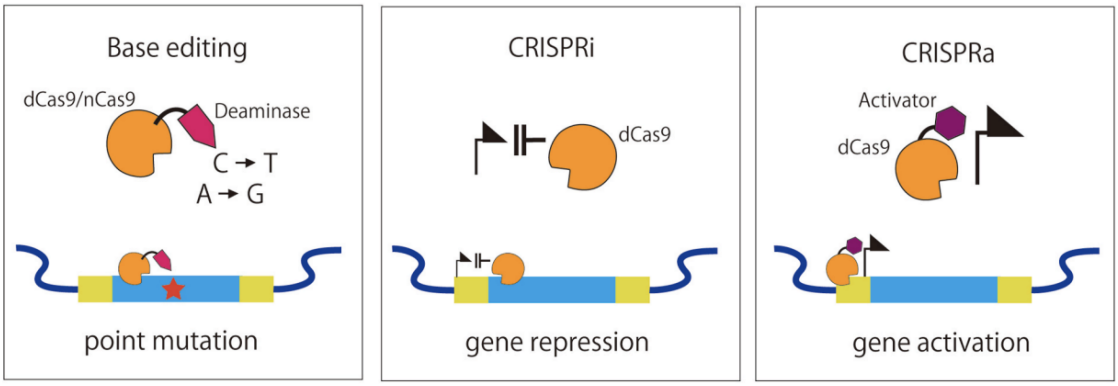


Figure 3: Illustration of CRISPR-derived editing tools, using DNA deamination to introduce precise point mutations. CRISPRi suppressing gene expression by inhibiting transcription, while CRISPRa enhances gene expression by recruiting transcriptional machanism to specific sites. Figure copied from Nishida and Kondo[21].

1.2.4 Zinc Finger Nucleases

Zinc Finger Nucleases combine zinc finger DNA-binding domains with a FokI nuclease cleavage domain, tailored to recognize specific DNA triplets, thereby achieving high specificity in targeting DNA sequences for gene corrections and knockouts. With this technology, HR can be enhanced, facilitating gene targeting and editing with efficiencies up to 29%, which is vital for applications ranging from basic research to clinical gene therapy[22][23]. ZFNs offer adaptability across various cell types and organisms due to their design flexibility, which allows for precise retargeting and optimization[24].However, the precision engineering is a considerable challenge, where the design must accurately avoid off-target effects[25]. Moreover, developing efficient delivery methods is crucial to unlock their full potential[23].

1.2.5 Transcription activator-like effector nucleases

Transcription Activator-Like Effector Nucleases, which fuse a TAL effector DNA-binding domain with the FokI nuclease domain, achieve targeted DNA cleavage through the specifity of TAl effectors from *Xanthomonas* bacteria[9]. TAL effectors, contain a central repeat region where each repeat correlates with a specific DNA base, enabling targeted DNA sequence recognition, triggering cellular repair processes such as NHEJ or HDR for gene editing[26]. TALENs offer high specificity and versatility with customised DNA-binding domains that enhance targeting accuracy and minimise off-target effects, and are effective across a broad range of organisms. However, designing TALENs is complex and labour-intensive because each repeat unit must match a specific DNA base, making them time-consuming to construct. Although they generally reduce off-target cleavage, they are not risk-free and efficiently delivering TALENs into target cells poses challenges[26].

1.3 Plasmid vectors

Plasmid vectors are extrachromosomal DNA molecules capable of autonomous replication within their host due to their inclusion of origins of replication (Ori). They may serve as foundational components for a wide range of genetic engineering applications. Plasmids naturally facilitate the transfer of genetic material between cells via mechanisms such as conjugation or transformation, thereby enhancing horizontal gene transfer across various bacterial species[27][28].

An important aspect of plasmid vectors is their ability to carry additional genetic elements that confer different functionalities to the host bacteria. These functionalities include mechanisms for copy number control, multimer resolution, active partitioning systems, and post-segregational killing. The ability to regulate their own copy number is particularly valuable for controlled gene expression and stability within the host[27].

For effective gene delivery and expression, plasmid DNA (pDNA) must be produced in a stable and pure form. The design and production of pDNA vectors is therefore focused on optimizing these properties to increase, enhancing the efficiency of gene transfer techniques[29]. Essential components of plasmid vectors include selection markers, commonly antibiotic resistance genes, which facilitates the maintenance and identification of transformed cells. Additionally, these vectors contain specific promoters and regulatory elements that control the expression of target genes[30].

Plasmids often exhibit instability in host cells, leading to the loss or deletion of cloned genes, resulting in the disappearance of phenotypic expressions. This instability can be influenced by several factors. One significant factor is the host cell's growth rate, as rapidly dividing cells might not replicate the pDNA as efficiently, resulting in plasmid loss over time. Genetic characteristics of the host can influence the stability by for example mutations in replication or repair genes, or factors like environmental stress with change in temperature, pH or nutrient availability can stress the host cell affecting their ability to maintain plasmids[28].

Additionally, plasmids may encounter barriers like nuclease activity that can degrade foreign DNA, such as plasmids, reducing their stability. Plasmids also face challenges related to replication control, transcription, translation, which can reduce expression of the genes carried by the plasmid. Other cellular processes, such as distrubution of plasmid DNA during cell divison, can also impact their stability and effectiveness as vectors for gene expression[28].

1.3.1 RK2 based vectors

The RK2 plasmid, with a size of approximately 60 kilobases (kb), demonstrates the ability to replicate within a wide range of bacterial species, facilitating the use of a consistent vector system across different bacterial hosts[31]. RK2 acts as a vehicle for the transfer of genetic material, such as antibiotic resistance genes, between bacterial cells, accomplished through conjugation[32].

The essential replicative component of the RK2 plasmid includes a gene encoding the trans-acting replication protein, TrfA. This protein binds to the iteron-containing, cis-acting Origin of Vegetat-

ive Replication (OriV), which together are sufficient to support replication across various bacterial species[32][33][34].

During conjugation, RK2 facilitates the transfer of its genetic material from a donor cell to a recipient cell. This transfer is supported by a suite of plasmid-encoded genes that produce the proteins necessary for establishing and conducting the conjugation process. Initially, a mating pair is formed between the donor and recipient cells, followed by the transfer of the plasmid DNA via a specialized structure known as the pilus[35].

To ensure its stability within the host cells and effective segregation during cell division, the RK2 plasmid incorporates a partition gene, integral to its partitioning system. This system, organized around a central control region, contains elements encoding proteins and a centromere-like DNA site. One of the proteins binds the centromere-like site, thereby linking the plasmid to a partitioning ATPase that energizes the plasmid's movement during cell division. This partitioning system ensures that each daughter cell receives a copy of the plasmid, thus maintaining plasmid stability across generations[36].

Upon entry into the recipient cell, RK2 can independently replicate using its own replication machinery, separate from the host's chromosomal DNA. This autonomous replication capability ensures that RK2 can be maintained and passed on to subsequent generations of cells[33][34].

The RK2-based vectors are relatively small in size and are designed to include the minimal replicon of RK2, consisting of *oriV* and *trfA*. They feature multiple cloning sites and have the ability to modify copy numbers by mutations in the *trfA* gene, which is essential for the replication of RK2. These modifications make RK2 vectors useful for studying the expression of different genes in a variety of gram-negative bacteria, and some gram-positive[37].

1.3.2 Conditional suicide plasmids

There exists key differences between suicide plasmids and conditional suicide plasmids in terms of their function and purposes. Suicide plasmids do not replicate in specific host bacteria, functioning as one-time-use tools in genetic engineering[38]. In contrast, conditional suicide plasmids are designed for controlled gene expression, regulation, or conditional gene knockout under specific conditions[39]. Their replication or dormancy is dictated by the presence of inducers or repressors, granting precise control over genetic experiments.

An innovation in plasmid vector technology involves the manipulation of plasmid replication dynamics through inducible regulatory systems. This approach utilises inducible promoters, such as the Pm promoter, linked with specific inducer molecules to modulate plasmid replication in response to environmental cues. The integration of an inducible promoter to control the *trfA* gene, which is critical for plasmid replication, exemplifies this method[31]. Activation of the *trfA* gene by the Pm promoter in the presence of an inducer results in the synthesis of the TrfA protein, thereby initiating replication and increasing plasmid copy number. Conversely, in the absence of the inducer, the promoter remains inactive, which suppresses TrfA expression and consequently reduces plasmid replication[31].

Temperature-sensitive elements can also be used in conditional suicide plasmids to control plasmid stability and gene expression. By incorporating temperature-sensitive replication proteins or regulatory elements, these plasmids remain stable at lower, permissive temperatures and becomes unstable at higher, non-permissive temperatures. This characteristic can lead to the their elimination from the cell population, and allows for temporal control over the absence or presence of the plasmid within the host. Temperature shifts can therefore be used strategically to induce plasmid loss or to activate lethal genes, enhancing the versatility and control in genetic engineering[40].

Conditional suicide plasmids are constructed by controlling the expression of a replication protein to levels insufficient for each daughter cell to always inherit a copy of the plasmid. This is achieved by using a dual control system with specific promoters to regulate the expression of the replication protein. The plasmid is designed to be stable when induced and unstable when uninduced, leading to its elimination from the cell population. This approach allows for the selection of recombinants

while simultaneously ensuring the loss of the plasmid. The construction of these plasmids involve steps of selection of suitable promoters, testing of promoter strengths, and the incorporation of a conditional replication system[41].

In the realm of genetic engineering, an alternative method designs the conditional suicide plasmids to self-destruct within the host cell after performing a specific function, such as facilitating site-specific mutagenesis, enabling gene knockout experiments, or deliver Cas9. These plasmids are equipped with a counter-selection marker, which could be a toxic gene or one that requires specific growth conditions. This feature allows for the identification of cells that have successfully shed the plasmid, and their conditional nature ensures precise control over genetic alterations[41].

Conditional suicide plasmids enable the separation of plasmid transfer from the recombination process, increasing the number of transconjugants compared to those produced with non-replicating plasmids. The replication of these plasmids within a host is controlled by the introduction of specific external inducers, aligning the conjugation process with conditions conducive to plasmid replication[41]. This temporal separation between gene transfer and recombination significantly improves the efficiency of site-specific mutagenesis.

Conditional suicide plasmids are usful as they offer a higher number of transconjugants compared to non-replicating plasmids, facilitating homologous recombination. These plasmids, can serve as vectors for delivering transposons or genome-editing systems such as the CRISPR-Cas system, making them valuable for synthetic evolution where specific genetic characteristics are introduced, modified, or removed[41]. Since conditional suicide plasmids are lost at a high frequency when not selected for, their gene expression is time limited. This characteristic allows for controlled removal of the plasmid after it has served its purpose, facilitating experiments that require temporary genetic modifications[41].

1.4 Selection markers in genetic engineering

Selection markers are indispensable tools in genetic engineering, facilitating the identification of cells that have successfully incorporated foreign DNA. These markers typically include genes that confer resistance to antibiotics, produce visible color changes in cells, or satisfy specific nutritional requirements. The incorporation of a selectable marker gene allows for the differentiation of transformed cells by their ability to prosper in environments containing selective agents, which inhibit or eliminate non-transformed cells[42][5].

1.4.1 Antibiotic resistance as a selective marker

In genetic experiments, genes that confer resistance to antibiotics are commonly used as selective markers. These genes, when co-introduced with desired genetic material into host cells, enable the cells to grow in media containing specific antibiotics. As a result, only cells that have obtained the antibiotic resistance gene will proliferate, whereas those that have failed to incorporate this gene will be eliminated by the antibiotic. This selective pressure facilitates the isolation and analysis of genetically modified cells[43][44].

1.4.2 Toxic gene for negative selection

sacB
The *sacB* gene encodes levansucrase, which converts sucrose into levan, a polymer toxic to most bacteria. This characteristic makes sucrose a negative selection marker, as it causes cell death or growth inhibition in bacteria harboring the *sacB* gene. Employed in suicide vectors for genetic editing, *sacB* provides dual-negative selection. Following a single-crossover event, bacteria with the integrated vector are positively selected, in a subsequent crossover, *sacB* facilitates negative selection by promoting growth on sucrose-containing media, ensuring that only desired clones are retained[41][45][5].

CRISPR-based endonucleases
Endonucleases, such as CRISPR-Cas9, may be used as selective markers in bacteria by inducing targeted double strand breaks at specific locations. When Cas9 is directed to cleave an essential gene or a gene of antibiotic resistance, the resulting DSBs can lead to cell death if not properly repaired. Cells that maintain an intact essential gene will survive, whereas those with successful CRISPR-induced disruptions will not, ensuring that only bacteria lacking the targeted gene are eliminated[46].

The CRISPR-Cas9 system might be designed to target a plasmid encoding both a toxin gene and an antibiotic resistance gene. When Cas9 cleaves the plasmid at the resistance gene, cells that lose the plasmid due to failed repair are eliminated by the toxin, allowing only successfully edited cells to proliferate. This selective pressure enhances the efficiency of genetic modifications by ensuring that only the desired genetic changes survive in the bacteria[46].

Homing endonucleases
Homing endonucleases are site-specific enzymes that facilitate the movement of specific DNA sequences, such as introns, between similar genomic locations. These enzymes function by recognizing and binding to specific target sites within a host gene and creating a double-strand break. This initiates a homing process, where the intermediate sequence is transferred and subsequently duplicated within the recipient allele of the host genome. This mechanism ensures the amplification of the intermediate sequence and maintains high fidelity by minimising off-target cleavage within the host genome[47]. The homing endonucleases are encoded by open reading frames within these mobile genetic elements and are able at cleaving closely related variants of their target sites, providing a flexible yet precise recognition mechanism[47].

These enzymes can be effectively used as negative selection markers by utilizing their ability to catalyse DSBs, which activates the cell's own repair machinery and increases the frequency of homologous recombination at the targeted site. When homing endonucleases like I-SceI are employed alongside a survival mechanism, they can be instrumental in selecting cells that exhibit the desired genetic alterations. For instance, in a system where the expression of a toxic protein is coupled with the expression of a homing endonuclease, only cells that successfully express the endonuclease and thus cleave the plasmid encoding the toxin will survive. This selective pressure ensures that only cells with active homing endonuclease capabilities survive[48].

Homing endonucleases like I-SceI enhance genetic modifications by creating targeted DSBs, which facilitate homologous recombination. The study by Cianfanelli et al. demonstrated the use of I-SceI in a dual-negative selection system to improve the efficiency of suicide vector resolution in bacterial genomes. By combining I-SceI with the *sacB* gene, which confers sucrose sensitivity, they achieved effective counter-selection of unwanted recombinants, increasing the yield of desired genetic modifications. This method suggested effective generation of deletions, insertions, and point mutations in various bacteria, including multidrug-resistant strains, showing the versatility of homing endonucleases[45]. However, their requirement for specific recognition sequences can limit their application in some genomes, requiring the construction of modified variants with broader or altered characteristics to expand their use in genome engineering[45].

1.4.3 Selection using colour, *lacZ*, *udiA*, and fluorescent protein

Selection using colour, colourimetric markers, in plasmids are to visually identify the genetically modified cells. These markers facilitate the differentiation of colonies based on a color change. The gene responsible for the colour change produces the colour when expressed, while insertions or deletions in the sequence result in the absence of colour[49].

The *lacZ* gene, encoding β-galactosidase, plays a role in lactose metabolism by breaking it down into glucose and galactose. In genetic engineering, *lacZ* is also utilized as a selective marker since it can hydrolyze the substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). X-gal is colorless, but its cleavage by β-galactosidase produces a blue product, enabling visual identification of cells containing an active *lacZ* gene on indicator plates[50][51].

Similarly, the *udiA* gene encodes β-glucuronidase, which is capable of cleaving the colorless sub-

strate 4-methylumbelliferyl-β-D-glucuronide into the blue-fluorescent product 4-methylumbelliferone, which is visible under ultraviolet light[52].

Additionally, fluorescent proteins, like mCherry, serves as a robust tool for cellular studies. This red fluorescent protein (RFP), used as a marker for cellular localization, emits red fluorescence when excited by a specific wavelength of light, 558 nm and 583 nm. The emitted fluorescence can be visualized and quantified through microscopy or a plate reader, allowing researchers to track the localization of specific proteins within cells and to monitor changes in fluorescence intensity. As a colorimetric marker, mCherry offers a sensitive, specific, and non-invasive technique to observe and quantify biological processes, enhancing the understanding of cellular dynamics[53].

The use of these markers offers several advantages, including the quick visual screening process of identifying colonies with the desired genetic modifications, high throughput for analyzing large numbers of colonies simultaneously, and being non-invasive. However, the technique can produce false positives or negatives due to incomplete digestion or small in-frame inserts, leading to incorrect identification.

1.5 Promoter systems and regulation used in this work

The promoter system is fundamental to understanding how genes are regulated and expressed within an organism. Promoters are specific DNA sequences located upstream of a gene, providing essential binding sites for the RNA polymerase. In prokaryotes, RNA polymerase binds to the promoter, repressors bind to the operator, and activators bind to the activator binding site. This assembly initiates transcription, setting the stage for gene expression[54].

Adjusting a promoter's characteristics can enhance control over gene expression, making it inducible or repressible based on external conditions. Alterations can also adjust the promoter's strength to either increase or decrease expression levels, which is essential for applications needing high protein production or reduced metabolic burden[55]. Moreover, enhancing the compatibility of gene expression systems with various host organisms broadens the applicability of genetic tools across different biological systems. This can ensure more reliable gene expression, which is essential for consistent phenotypic outcomes in genetically modified organisms.

Alternatively, testing different promoter systems provides insights into their structural profiles and characteristics. This approach helps in investigating the role of promoter DNA properties in transcription, thereby revealing the gene expression characteristics associated with specific promoters. It allows for comparisons of structural profiles across different prokaryotic species, enabling a better understanding of how these promoters function in various genetic contexts[56].

The advantages of testing different promoter systems including the insights into the behaviour of downstream genes, is to improve the accuracy of promoter prediction tools and provide new perspectives for future research on uncharacterised functional elements prokaryotes[56]. However, there are disadvantages such as the potential bias of promoter prediction tools, based on sigma factor recognition or gene expression classes. In addition, it can be challenging to cover all the parts of the genome when testing promoters, which might reduce the effectiveness of the test. Furthermore, different experimental methods used to find transcription start sites can give varying results, affecting the reliability and accuracy of the results[56].

An example of gene regulation in bacteria is quorum sensing, which is a regulatory mechanism that allows bacteria to coordinate gene expression in response to cell-population density[57]. This ensures that certain genes are expressed only when it is beneficial for the bacterial community.Examining the quorum sensing reveals the dynamic nature of bacterial gene regulation and underlines the importance of promoter systems in facilitating complex interactions within bacterial populations. By studying these systems, we gain insights into bacterial communication and behavior[57].

Choosing the best expression system depends on specific needs, such as the importance of transcription level, protein functionality, host flexibility, and control precision[58].

1.5.1 Pconstitutive promoter system

Constitutive promoters are always active and continuously drive the expression of their downstream genes without the need of specific inducers or repressors[54]. They rely on the availability of RNA polymerase and its interaction with promoter-specific parameters, such as maximum promoter activity (V_{max}) and the RNA polymerase concentration required to reach half of the maximum activity (K_m). It is a system where gene expression depends significantly on the cellular concentration of RNA polymerase, which varies according to the cell's growth rate and environmental conditions[59]. This results in generally steady and predictable levels of gene expression, essential for maintaining basic cellular functions and applications requiring constant protein production.

In this thesis, a specific constitutive promoter, Pconstitutive, was used. This promoter is a strong synthetic promoter derived from the gram-positive bacterium *Streptomyces lividans* TK24, designed to drive high levels of gene expression constitutively. Despite the promoter originally was selected for gram-positive bacteria, previous studies have confirmed its expression in *Pseudomonas fluorescens*[3] and *Azotobacter vinelandii*[60].

1.5.2 Regulatory promoter systems

While constitutive promoters maintains constant gene expression, biological systems often require more refined regulation mechanisms to adapt to the changing environmental conditions and cellular needs. Regulatory promoter systems are flexibile by enabling gene expression to be turned on and off in response to specific signals and conditions[54].

Regulatory promoters are classified based on their mechanisms of action and which signals they respond to. For example, inducible promoters are activated by the presence of specific molecules, which leads to gene expression only when needed. In contrast, repressible promoters are turned off in response to specific signals, avoiding unnecessary gene expression. Additionally, there are synthetic promoters which can be designed to respond to a different inputs, providing desired and specialised control over gene expression[54]. In this thesis, different promoter systems were tested, Pm, Pm ML1.17, PmG5, Ptrc, pBAD, AntR, and RhaSR, each of which is described in the following sections.

1.5.3 XylS/Pm regulator/promoter system

The XylS/Pm promoter system is an inducible system, derived from *Pseudomonas putida* TOL plasmid pWWO, consisting of the Pm promoter and the *xylS* regulator gene. The system regulates the expression of genes involved in degradation of aromatic hydrocarbons[55]. This promoter system regulates genes essential for metabolizing toluene and xylenes into simpler compounds, and is inducible by various benzoic acid deviates. The *xylS* gene is transcribed from two promoters, Ps1 and Ps2, where Ps1 is σ54-dependent and inducible, while Ps2 is σ70-dependent and provides constitutive, low-level expression of XylS[55]. Upon activation of the benzoic acid derivates, which can enter cells by passive diffusion and operate in a dose-dependent manner providing induction at graded levels, the XylS binds to the Pm promoter and initiates transcription[61][55].

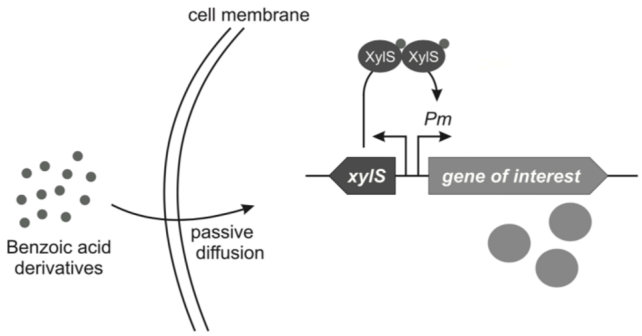


Figure 4: Illustration of the XylS/Pm promoter system coiped from Braustad[62]. The inducer molecules passively diffuse into the cells, bind to, and activate XylS. This activation subsequently enhances transcription from the Pm promoter.

The Pm ML1.17 variant is a derivative of the XylS/Pm system designed to increase recombinant protein production, showing improvements over the wild-type[58]. A distinctive characteristic of Pm ML1.17 is its flexibility, as it is does not require specific properties in the host cell for efficient protein production. This feature makes Pm ML1.17 a versatile tool for a range of bacterial hosts, as it achieves a better balance between transcriptional and translational mechanisms, resulting in increased protein yield per transcript. The improved performance is primarily due to mutations in the core region, which is essential for the enhanced protein production capabilities[58]. In a comparative study conducted in *E. coli*, the Pm ML1.17 variant along with LacI/PT7lac, generally produced the highest levels of functional protein[58]. While these studies were conducted in *E. coli*, the design suggests it can be efficiently used in various gram-negative bacteria, as Vold confirmed for *P. fluorescens* in her thesis[2].

The PmG5 promoter is a mutated derivative of the wild type XylS/Pm system in *Pseudomonas fluorescens*. This promoter system was developed for incresed ability to control gene expression with lower background activity in the absence of an inducer, compared to its wild type counterpart[63]. The PmG5 promoter operates by controlling the transcription of downstream genes, regulated by the *algC* and *algL* genes, which are involved in the alginate biosynthesis. The promoter is inducible by m-toluate, allowing regulation of gene expression[63].

1.5.4 LacI-Ptrc promoter system

The Ptrc promoter is a hybrid promoter derived from the *lac* and *trp* promoters, designed for regulated gene expression in *E. coli*, and is illustrated in Figure 5. It was derived from the -35 region of the *trp* promoter and the -10 region of the *lacUV5* promoter/operator. This inducible system is activated by the inducer isopropyl-β-D-thiogalactopyranoside (IPTG). When IPTG is present, it binds to the lac repressor protein, LacI, causing a structural alteration that prevents the repressor from binding to the trc promoter. This release allows RNA polymerase to access the promoter, initiating the transcription of the downstream target gene. As a result, the gene is translated, facilitating the production of its corresponding protein[64].

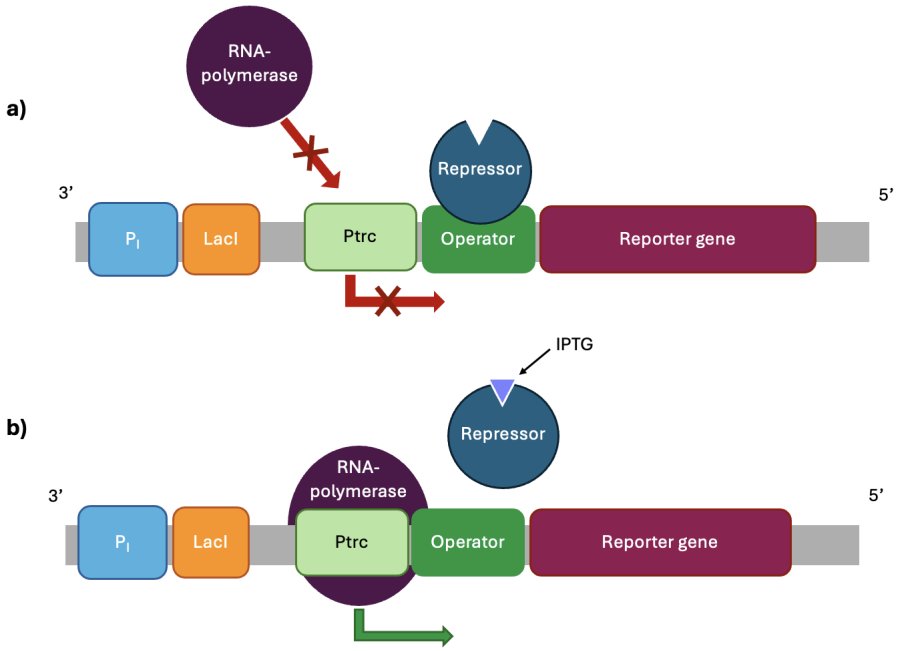


Figure 5: Illustration of the LacI-Ptcr promoter system where **a)** shows the system in absence of IPTG, the LacI repressor binds to the operator, blocking RNA polymerase from transcribing the reporter gene, resulting in no expression. **b)** IPTG is present, it binds to the LacI repressor releasing it from the operator. This allows RNA polymerase to access the promoter and transcribe the reporter gene, leading to expression of the reporter gene

1.5.5 AraC-pBAD promoter system

The pBAD promoter system regulates gene expression in *E. coli* by responding to the presence of arabinose. Central to this system is the AraC protein, a dimeric regulator whose activity is modulated by arabinose[65]. The negative and positive regulation of the AraC-pBAD system is illustrated in Figure 6. In the absence of arabinose, AraC is inactive with a low DNA binding affinity, resulting in low gene expression levels. Conversely, the presence of arabinose induces a conformational change in AraC, enhancing its DNA binding affinity and activating it. The active AraC then binds to the pBAD promoter, significantly increasing the transcription of arabinose metabolism genes. This control mechanism is useful for studies that require regulated gene expression, such as those investigating metabolic pathways and recombinant protein production, allowing researchers to accurately manipulate the timing and intensity of gene expression in bacterial cultures[65].

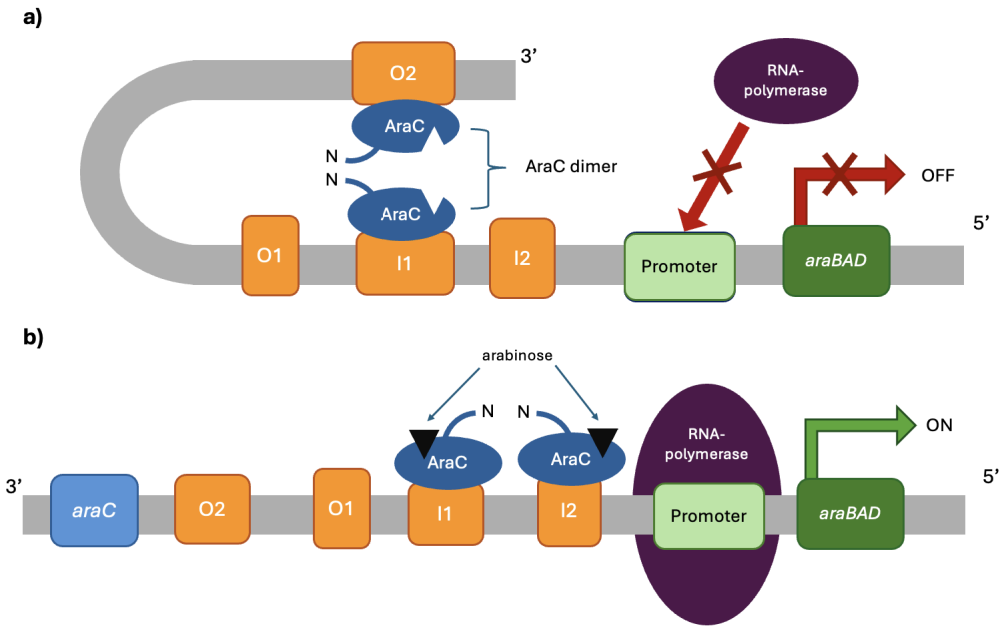


Figure 6: Illustration of **a)** negative regulation of the arabinose operon with the AraC protein where transcription is supressed. **b)** positive regulation of arabinose where the RNA polymerase binds to the promoter and AraBAD gets transcribed.

1.5.6 AntR-Pant promoter system

The AntR-Pant promoter system used in this study originates from *P. fluorescens*, and plays an essential role in regulating the anthranilate dioxygenase operon, essential for metabolizing anthranilate and benzoate. AntR, a transcriptional activator, binds to the Pant promoter, enhancing the transcription of the *antABC* genes necessary for the break down of anthranilate[66]. Experimental evidence suggests that the effectiveness of AntR is a limiting factor for the operon's expression, as its overexpression significantly increases the Pant promoter's activity[66]. Anthranilate is an aromatic amine, and in addition to serving as an inducer, it is naturally used to metabolise aromatic hydrocarbons. The processes converts anthranilate to catechol by anthranilate 1,2-dioxygenase, and can further enter metabolic pathways in the bacteria[66].

In *Pseudomonas aeruginosa*, the metabolism of anthranilate is regulated through a complex of quorum qensing (QS) regulators, including *LasR*, *RhlR*, and *QscR*. These regulators influence the expression of genes, such as *antABC* involved in the degradation of anthranilate, and modulate the levels of anthranilate and its derivatives. The QS system ensures that anthranilate is metabolised efficiently during different growth phases. This regulation is essential for both degradation of anthranilate and bacterial communication[67]. Given the importance of anthranilate metabolism and quorum sensing in *P. aeruginosa*, it is interesting to investigate how the AntR-Pant system functions in different bacterial strains, in this study *M. algicola*.

1.5.7 RhaSR-pRha promoter system

The RhaSR-pRha promoter system originates from *E. coli* and regulates the catabolism of L-rhamnose through an interaction of regulatory proteins and responsive elements, essential for adapting to environmental sugar availability[68]. Central to this system is the RhaS protein, which activates the *rhaBAD* operon essential for L-rhamnose breakdown. RhaS, which is a member of the AraC family of transcription activators, binds specifically to a region within the *rhaBAD* promoter downstream of position -84 relative to the transcription start point[69]. This binding is required for initiating transcription in the presence of L-rhamnose, directly leading to the production of enzymes involved in the catabolism of this sugar[69].

Catabolite repression is the regulatory mechanism that prioritises glucose as the primary source of carbon, by inhibiting transcription pathways of less favourable sugars[70]. The pathway is mediated by the cyclic adenosine monophosphate (cAMP) and the cAMP receptor protein (CRP). When glucose levels are low, cAMP binds to CRP, which binds to a specific site on the *rhaBAD* promoter, initiating the transcription of genes necessary for L-rhamnose metabolism[70][68]. This process is further regulated by the presence of L-rhamnose, which causes the activation of the RhaR protein. RhaR then induces the transcription of the *rhaSR* genes, leading to the accumulation of RhaS[68]. Once enough RhaS accumulates, it further enhances the transcription of the *rhaBAD* operon by binding near its promoter, potentially overlapping with another inverted repeat[68].

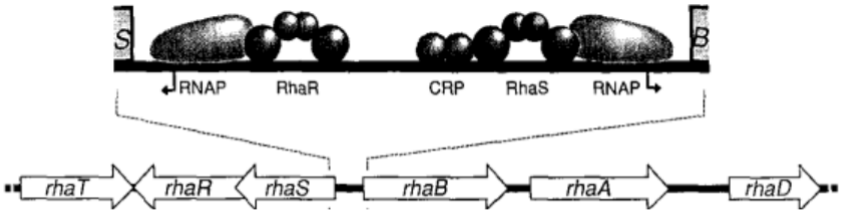


Figure 7: This figure shows the rhaS-rhaB regulatory area along with the suggested activator proteins and RNA polymerase, and the *rhaT* gene encoding the L-rhamnose transport protein. This illustration is copied from Egan et al.[68].

This complex regulatory mechanism allows the bacteria to respond dynamically to changes in L-rhamnose levels in the environment. RhaR and RhaS play distinct roles, where RhaR initiates the cascade by binding upstream of *rhaSR*. RhaS completes the cascade by activating *rhaBAD* transcription. Together, these activities optimises gene expression and also ensures that the pathway is only active when L-rhamnose is available. This system demonstrates the bacteria's ability to regulate gene expression in response to nutritional signals[68].

1.6 Use of red fluorescent protein as reporter gene

A reporter gene is a gene that expresses an easily detectable product, that can be used to measure the activity of a specific promoter. Such genes were first developed in early the 1980s to measure promoter-driven transcriptional activity, allowing quantification and visualisation of gene expression by connecting the reporter gene to specific promoters and thereby detecting the activity[71].

In this thesis, the red fluorescent protein gene was used to measure promoter strength in plasmids introduced into *M. algicola*. The *RFP* gene, initially identified as DsRed from the coral *Discosoma*, has been a significant advancement in molecular biology and biotechnology due to its distinct spectral properties[72].

The fluorescence of RFP is typically measured using techniques like fluorescence microscopy or flow cytometry, where the intensity of the emitted light is quantitatively analyzed. This measurement provides information regarding the expression levels of the *RFP* gene, the location and dynamics of the tagged proteins, and the interactions within cells.This protein is characterized by excitation and emission peaks at 558 nm and 583 nm, respectively. In practical applications, the fluorescence measurement of RFP can be used to monitor gene expression, protein localization, and cellular processes in real-time, providing a dynamic view of biological events as they unfold[72].

Enhancements and engineering of the original DsRed protein for improved performance in biological experiments, have led to the development of variants such as mOrange, mStrawberry, and mCherry. Of these, mCherry has emerged as the most widely used. Notably, mCherry generally requires extended time to achieve full chromophore maturation, as it undergoes two oxidation steps. A study on the *E. coli* Colicin E2 system shows mCherry maturation varies between strains, from 59.4 $\pm$ 7.5 min in C strain(E2C-BZB1011) to 77.7 $\pm$ 8.5 min in R strain (E2R-BZB1011). The maturation time correlates positively with growth rate, faster growth rates resulting in longer maturation times[73]. The chromophore maturation is essential for its fluorescence capabilities[73].

It is widely used for studying gene expression and protein localization, especially in challenging environments, and is valued for its pH stability and high photostability[74][75].

Developed from progress in fluorescent protein technology, mCherry was designed to address specific scientific needs, such as improved stability, faster maturation, and reduced aggregation compared to its predecessor, DsRed[74]. Another notable RFP is TurboRFP, derived from the sea anemone Entacmaea quadricolor. This variant is a dimeric protein known for its rapid maturation at 37°C and brightness, with excitation and emission peaks at 553 nm and 574 nm[76]. These developments underscore progress in the evolution of fluorescent proteins, broadening their applicability across a range of research disciplines.

1.7 *Marinobacter algicola*

Marinobacter algicola is a gram-negative, aerobic, moderately halophilic bacterium known for its ability to degrade hydrocarbons. This rod-shaped, halotolerant bacterium typically occurs as single cells, in pairs, or as short chains. It exhibits motility via a single non-sheathed polar flagellum and has a draft genome sequence with a DNA guanine/cytosine content of 59.2%[77]. The optimal growth conditions for *M. algicola* include temperatures between 25-30°C and a NaCl concentration ranging from 3-6%[78].

Identification of *M. algicola* in laboratory settings has been achieved through both phylogenetic and phenotypic analyses. These include examining colonies grown on marine agar, determining gram reaction and cell morphology, and conducting various biochemical tests to assess enzyme activity and substrate utilization[78][77].

A part of the motivation of using *M. algicola* in this study is due to its underexplored status compared to other bacterial strains, particularly regarding alginate production. Importantly, *M. algicola* possesses an operon homologous to the alginate biosynthesis operon found in other bacteria[78][79]. Although the production of alginate in *M. algicola* remains uncertain, further investigation could unravel its capabilities in alginate production and lead the way to new biotechnological applications. Hence, further research on *M. algicola* shows potential for broadening our knowledge of its biological functions and utilizing it in industrial applications. Conjugating conditional suicide plasmids into *M. algicola* is of interest due to its potential as a source of alginate, a valuable polysaccharide with industrial applications. This approach enables precise genetic modifications to enhance alginate production, offering an alternative to sources like *Pseudomonas sp.* and *Azotobacter vinelandii*[79].

In a previous thesis written by Instefjord[80], an attempt was made to introduce suicide plasmids for HR into *M.algicola*, but it was unsuccessful. A potential reason could be too low recombination frequency. Therefore, *M.algicola* remains a suitable candidate for further investigation using conditional suicide plasmids.

2 Methods and material

The descriptions of the media, solutions, buffers, and other materials utilized are detailed in Appendix A. Additionally, an overview of the plasmids and bacterial strains employed is provided in Table 1 below. Many of the procedures in this thesis are refinements of those also used in my previous work[5].

2.1 Bacterial strains and plasmids used

The previous work focused on the development of conditional suicide plasmids, with a key aspect being the construction of a series of plasmids that confer antibiotic resistance. The pTN3 vector was derived from pSV2 with an inserted Tc^R gene, and is used as a backbone vector for further development of these plasmids in this thesis. Maps showing the compositions of each of the applied plasmids are illustrated in Appendix F.

During the course of this thesis, a variety of bacterial strains were utilized, the details of which are provided in Table 1. These strains, including multiple derivatives of *E. coli* and *M. algicola*, were selected for their genetic characteristics.

Table 1: Bacterial strains used during this thesis

Strain	**Characteristics**	**Tm [°C]**	**Reference**
E.coli S17.1	RP4 2-Tc::Mu-Km::Tn7 pro res mod*	37	[81]
E.coli DH5α	*lac*ZΔM15, *rec*A1, *end*A1	37	[82]
E.coli JKE201	MFDpriΔmcrAΔ(mrr-hsdRMS-mcrBC)aac(3)IV::lacI^q,Dap$^-$	37	[83]
M. algicola DG893	wild type	30	[78]

E. coli S17.1 is a suited donor strain for plasmid transfer due to its modifications with a chromosomally integrated RP4 plasmid, which facilitates efficient gene transfer. Additionally it lacks the *E. coli* K12-specific DNA restriction system that enhances the uptake of cloned foreign DNA[81]. For plasmid transfer, the origin of transfer (*oriT*) sequence is essential, as it initiates the transfer of plasmids between bacteria. In this system, plasmids with *tra* genes provide the necessary mechanism for transferring the plasmids containing an *oriT* sequence to recipient bacteria. Further, λ phage conjugation uses bacteriophage λ in the transfer of genetic material. The integration of a λ phage into the bacterial chromosome allows for maintenance and transfer, and facilitates for gene insertion and transposon delivery[81].

In addition to these bacterial strains, a series of plasmids were employed and developed to facilitate the genetic manipulations required for this thesis. The specifics of these plasmids are summarized in Table 2 below, including both provided plasmids and constructs created in this study.

Table 2: Plasmids used during this thesis

Plasmid	**Characteristics**	**Antibiotic resistance**	**Reference**
pCR-blunt II-TOPO	pUC *Ori*, *LacZα*, *ccdB*	Km^R	ThermoFisher[84]
pSV2	*XylS*-Pm wt	Km^R	Vold S[2]
pSV3	RK2 based, *LacI*-Ptrc	Km^R	Vold S[2]
pSV4	RK2 based,*XylS*-Pm ML1.17	Km^R	Vold S[2]
pSV5	RK2 based,*AraC*-pBAD	Km^R	Vold S[2]
pSV6	RK2 based,*XylS*-PmG5	Km^R	Vold S[2]
pHH108	RK2 based, pConstitutive	Km^R	Haaland H[3]
pAFW4	RK2 based,*AntR*-Pant	Km^R	Wesche A[4]
pAFW5	RK2 based, *RhaS*R-pRha	Km^R	Wesche A[4]
pTN3	Derivative of pSV2	Tc ^{R}	Nærby T[5]
$pTN4_T$	Derivative of pCR-blunt II-TOPO, *ccdB*	Am^R	Nærby T[5]
$pTN5_T$	Derivative of pCR-blunt II-TOPO, *ccdB*	Km^R	This study
$pTN6_T$	Derivative of pCR-blunt II-TOPO, *ccdB*	Sp^R	Nærby T[5]
pTN4	Derivative of pTN3	Am^R	This study
pTN5	Derivative of pTN3	Km^R	This study
pTN6	Derivative of pTN3	Sp^R	This study
pTN7	Derivative of pSV2. *XylS*-Pm wt,	Am^R	This study
pTN8	Derivate of pSV3, *LacI*-Ptrc,	Am^R	This study
pTN9	Derivate of pSV4, *XylS*-Pm ML1.17	Am^R	This study
pTN10	Derivate of pSV5, *AraC*-pBAD	Am^R	This study
pTN11	Derivate of pSV6, *XylS*-PmG5	Am^R	This study
pTN12	Derivate of pHH106, pConstitutive	Am^R	This study
pTN13	Derivate of pAFW4, *AntR*-Pant	Am^R	This study
pTN14	Derivate of pAFW5, *RhaS*R pRha	Am^R	This study

2.1.1 Storage of strains

For short-term storage of plasmids, *E. coli* strains harboring the desired plasmid constructs was maintained at 4°C on Luria agar (LA) plates supplemented with the appropriate antibiotic. This method typically preserves the bacterial cultures for a couple of weeks.

The *E. coli* strains containing the verified plasmids were long-term stored at -80°C in a 20% glycerol solution. To prepare for freezing, 1 mL of cell culture, inoculated ON in Luria broth (LB) medium, was combined with 300 μL of 60% glycerol in a cryotube. This glycerol mixture is crucial as it lowers the freezing point and inhibits the formation of damaging ice crystals, thereby preserving the cells' structure, viability, and functionality[85]. To revive frozen bacterial cultures, the bacteria were first spread onto agar plates. They were then incubated until colonies became visible. Following this, the colonies were transferred to liquid media to continue their growth.

2.2 DNA transfer

In this study, standard bacterial cultivation procedures were employed. Bacterial strains were grown either in liquid LB or on LA plates, depending on the specific requirements of the experiment. Additionally, the appropriate antibiotics and supplements, as described in the Table 1 and 2, were added to the media to ensure selective growth or supplementation of essential nutrients. Furthermore, each strain's optimal growth temperature, was considered to ensure favorable conditions for bacterial growth and viability.

2.2.1 Preparation of competent *E.coli*

Competent cells are essential in genetic engineering because they have been treated to easily take up foreign DNA from their environment. This ability is crucial for the transformation procedures

involving *E. coli* and plasmid DNA integration, allowing the introduction and expression of new genetic material in cells. This process enables the manipulation and study of genes, making competent cells essential for molecular genetics, molecular cloning, and biotechnology research[86]. Due to their significant negative charge, DNA molecules cannot attach to the negatively charged surfaces of most bacterial cells, nor can they penetrate the wall-membrane complex to enter the cytoplasm. For successful transformation and the incorporation of heterologous DNA into bacteria, it is essential to first make the cells competent[86]. The treatment with calcium chloride ($CaCl_2$) and rubidium chloride (RbCl) plays a role in transformation efficiency, where $CaCl_2$ helps attach DNA to the cell surface, while RbCl enhances the efficiency[87]. This is followed by a rapid thermal shock that alters the cell membrane's permeability, facilitating the passage of DNA into the cytosol.. Consequently, cells are induced into a state of competence, enabling them to acquire the capability to absorb foreign DNA[88][89].

Procedure[5]
The *E. coli* starter culture was inoculated overnight (ON) in 10 ml Psi medium and incubated at 37°C and 225 rpm. 2 ml of the ON culture was transferred to 200 ml Psi medium and incubated at 37°C until the optical density (OD) reached 0.40-0.45. OD was read at 600 nm (OD_{600}), and the culture was cooled on ice for 15 minutes.

The culture was then centrifuged at 4000 rpm (alternatively 2500 xg) for 5 minutes at 4°C, followed by removal of supernatant. Further, the cell pellet were resuspended in 80 ml cooled transformation buffer 1 (TFB) solution, and incubated for 5 minutes on ice. Centrifugation was repeated, supernatant discarded, and the cells resuspended in 6 ml of cooled TFB2 solution. 100 μl of cells were then distributed into pre-chilled eppendorf tubes, and briefly snap-frozen with liquid nitrogen for 10 seconds. The competent cells were then stored at -80°C.

2.3 Transformation of DNA by heat shock

Heat shock transformation is a technique utilized to introduce foreign DNA into bacterial cells. This process involves exposing bacterial cells to a short-term temperature elevation, followed by a rapid cooldown. Such thermal fluctuation increases cell membrane permeability, thereby enhancing the uptake of exogenous DNA molecules like plasmids into the bacterial cells. The transient increase in temperature is thought to induce a temporary disruption of the cell membrane, making it sufficiently porous for the foreign DNA to pass through. This process enables the bacteria to acquire new genetic traits through the incorporation of the introduced material into their own genomes or the maintenance of the DNA as an independent plasmid[90].

Procedure[5]
The competent cells were thawed on ice to prepare for the transformation process. 10 μl DNA was gently added to 100 μl of competent cells and mixed gently without pipetting. The mixture was then incubated on ice for an additional 30 minutes up to an hour, followed by a heat shock in a water bath at 37°C for a period of 2 minutes. Immediately afterward, the tubes were returned to ice for an additional 2 minute cooling period. Then 900 μL of pre-warmed super optimal broth (SOC) medium at 37°C was added to each tube, before they were sealed tightly and agitated horizontally at 225 rpm at 37°C for 1-2 hour. Post-incubation, 100 μL of the mixture was spread onto one agar plate supplemented with the specific antibiotic suited for the target plasmid, while the remaining volume was spread onto another.

Additionally, positive and negative control plates were prepared for each new batch of competent cells. The negative control, competent cells without any added plasmid DNA, tests for the natural resistance of the cell's to the antibiotic, ensuring that any colonies that grow on the antibiotic plates are due to successful transformation rather than contamination or inherent resistance of the cells. For the positive control the competent cells were transformed with a plasmid known to successfully confer antibiotic resistance. This control verifies the effectiveness of the transformation protocol, including the competency of the cells and the functionality of the antibiotic in the agar plates. The plates were then incubated overnight at 37°C for *E. coli*, after which colonies were available to be selected for further analysis.

2.3.1 Conjugation to *M.algicola*

Bacterial conjugation is a process of genetic material transfer between bacterial cells through direct contact[91]. The process is divided into several stages. During the specific pair formation stage, the donor and recipient cells come into contact and form stable unions, facilitated by pili or cellular attachments[91]. This is followed by effective pair formation, where a cellular connection is established between the donor and recipient cells. The next stage is chromosome mobilization, where the circular donor chromosome or conjugal fertility factor is prepared for transfer. One pDNA molecule is replicated during the conjugation, and one strand is transferred to the recipient whilst the other remains in the donor[92].This occurs when a cleavage is made at the oriT on the donor pDNA and the 5' end of the cleaved strand is transported through the cell membrane into the recipient[92]. Simultaneously, the uncleaved strand is replicated in the donor cell. The transferred strand is then synthesised into a complete plasmid in the recipient cell, becoming circular again and allowing the formation of genetic recombinants[93].

Finally, parts of the transferred donor chromosome integrates into the recipient cell's genome, resulting in genetic recombinants[91]. This enhances the efficiency of the transfer process and enables manipulation of the genome.

Procedure
Conjugation was performed to transfer plasmids from the donor strain to the recipient bacteria. In this study, the donor strain was *E. coli* JKE201, a bacterium that requires DAP for growth, while the recipient strain was *M. algicola*.

To transfer the donor plasmids into the recipient, initial cultures were established. *M. algicola* was introduced into 25 ml LB medium and incubated at 30°C. Simultaneously, *E. coli* JKE201, carrying the desired plasmid, was cultured in 10 ml LB medium, supplemented with 1 mM DAP and the respective antibiotics and incubated at 37°C ON. The following day, 500 μl of the recipient precultures were transferred to 25 ml growth medium and incubated at 30°C for 2 hours. After two hours, 200 μl of the JKE201 cultures were transferred to 10 ml LB medium containing DAP, and incubated at 37°C.

Upon both cultures reaching the exponential phase around $OD_{600} = 0.40$, 3 ml of the recipient strain was combined with 3 ml of the donor strain in sterile tubes, and centrifuged for 5 minutes at 5000-7000 rpm. The supernatant was discarded, leaving approximately 0.1 ml of liquid to resuspend the cell pellet. These suspensions were then applied as drops onto LA plates, and incubated at 30°C ON. Following this incubation, a dilution series was prepared by scraping cell growth from the plates and transferring it into 1 ml liquid medium. Subsequently, 100 μl of the dilutions (undiluted, 10^{-2}, 10^{-4}, and 10^{-6}) were plated on LA plates with the appropriate antibiotic but without DAP, and incubated at 30°C for 2 days.

2.4 Isolation of plasmid DNA from *E. coli*

In the context of cloning, it is essential to purify pDNA from the chromosomal DNA within the cell. The foundation for modern plasmid purification was set by Birnboim and Doly in 1979 when they introduced the alkaline lysis method, which is still widely used today due to its effectiveness in distinguishing between plasmid and genomic DNA based on their denaturation properties[94][95]. In the current study, the plasmid DNA was isolated from *E. coli* using the ZR Plasmid Miniprep-Classic kit by Zymo Research, which adopts a refined version of the original alkaline lysis technique[84].

Alkaline lysis involves the resuspension of cells followed by the application of a lysis buffer, which typically contains sodium dodecyl sulfate (SDS) and NaOH. The SDS acts as a surfactant, compromising the cell wall integrity and denaturing proteins to facilitate subsequent removal. The elevated pH from NaOH additionally contributes to the damage of the cell wall, denaturing proteins, fragmenting genomic DNA, and dissolving the hydrogen bonds in double-stranded DNA (dsDNA)[96]. Gentle handling during this phase is crucial to prevent the genomic DNA from fracturing into small fragments that could potentially reanneal and contaminate the plasmid DNA.

The process is completed by neutralizing the mixture with an acidic potassium acetate solution, which enables the renaturation of the plasmid DNA due to its supercoiled nature, allowing it to remain intact. The more substantial genomic DNA strands are unable to renature correctly under these neutral conditions, resulting in their aggregation and subsequent precipitation out of the solution. In addition, the high salt concentration in the buffer causes the precipitation of high molecular weight RNAs and protein-SDS complexes[97]. Through this step, contaminants are effectively segregated, yielding purified plasmid DNA suitable for downstream applications.

The column is designed to purify DNA by using gravity flow to enhance the separation process. The column matrix consists of a silica-based membrane, that selectively binds pDNA based on its size, type, and the volume of bacterial culture used. The binding capacity of the column depends on plasmid copy number, type, and size[98]. The purification process involves loading the supernatant onto the equilibrated column, allowing the DNA to bind to the silica membrane. Subsequent wash steps remove impurities while the plasmid DNA remains bound. Finally, elution buffer is added to release the purified plasmid DNA from the column matrix[98].

Procedure[5]
Plasmids were retrieved from individual colonies cultivated on agar plates through inoculation into 3 mL of antibiotic-supplemented growth medium. The inoculated cultures underwent overnight orbital shaking at 37°C at 225 rpm. Following incubation, the cultures were centrifuged, the supernatant discarded, and the resultant cell pellets subjected to plasmid extraction using the EZ Plasmid Miniprep-Classic kit from Zymo Research[84] [98].

The purification commenced with the resuspension of cell pellets in 200 μL of P1 Buffer. Lysis was achieved by adding 200 μL of P2 Buffer and inverting the tubes 2-4 times, resulting in a clear, purple, and viscous lysate. Subsequent neutralization was indicated by the addition of 400 μL P3 Buffer and a color transition to yellow. The neutralized lysate was then incubated at room temperature (RT) for 2 minutes[84] [98].

The neutralized lysate was transferred to a Zymo-Spin™ IIN column for purification. After a centrifugation step, the flow-through was eliminated. The column received 200 μL of Endo-Wash Buffer, centrifuged, followed by the addition of 400 μL of Plasmid Wash Buffer and another centrifugation[98].

Plasmid DNA was eluted by adding 50 μL of DNA Elution Buffer to the column, incubating for 1 minute at RT, and centrifuging for 1 minute to collect the DNA.

2.5 Determining the DNA concentration

The NanoDrop One Microvolume UV-Vis Spectrophotometer is a tool for quantifying DNA, RNA, and protein concentrations, utilizing just 1–2 μL of sample to provide results without requiring dilutions. The instrument uses UV-Vis technology, where UV light is directed through a small volume of sample placed on a measurement pedestal. By analyzing the absorbance of UV light at specific wavelengths, the concentration of DNA in the sample is accurately calculated using the Beer-Lambert Equation 1. The concentration of DNA, denoted as C and measured in molarity (M), can be calculated using the UV absorbance, represented as A in absorbance units (AU), the wavelength-dependent molar absorptivity coefficient, denoted as ϵ ($M^{-}1$ $cm^{-}1$), and the light path length, L, measured in centimeters (cm). For double-stranded DNA, the molar absorptivity coefficient ϵ is 50,000 M^{-1} cm^{-1}[99].

$$C = \frac{A}{\epsilon L} \tag{1}$$

This NanoDrop One is equipped with Thermo Scientific™Acclaro™Sample Intelligence technology, enhancing the user's ability to assess sample quality comprehensively before proceeding with downstream applications. Acclaro technology incorporates features embedded sensor and digital image analysis to maintain measurement integrity. It provides instant feedback on sample quality, which

is critical for making informed decisions regarding sample suitability. Additionally, the presence of RNA in the sample will also contribute in the evaluation[99].

Procedure
The concentration of dsDNA was quantified using the NanoDrop One Spectrophotometer. Initially, the instrument's pedestal was cleansed with 2 μL of distilled water. Subsequently, 1 μL of Zymo DNA Elution Buffer was employed to establish the blank reference. Following this, 1 μL of the dsDNA sample was applied to the pedestal, and spectral analysis was performed. Upon completion of the measurements, the instrument was cleansed with distilled water to remove any residual sample.

2.6 Enzymatic digestion of DNA by restriction endonucleases

Restriction endonucleases have emerged as fundamental tools in genetic engineering due to their ability to precisely cleave DNA at specific sequences. These enzymes utilize a recognition mechanism to identify specific 4-8 base pair palindromic sequences, which may be continuous or interrupted by nucleotides[100]. Type II restriction endonucleases typically function as homodimers and require magnesium ions as cofactors for activity. They bind non-specifically to the DNA backbone initially, with facilitated diffusion aiding the search for their specific target sites. Once bound, the enzyme-DNA interaction triggers significant conformational changes, enabling the catalytic sites to cleave the DNA, producing fragments with 3'-hydroxyl and 5'-phosphate ends, as illustrated in Figure 8. These fragments can have blunt or sticky ends, influencing subsequent cloning applications. This specificity not only aids in cloning but also in the verification of plasmid sequences, ensuring the fidelity of genetic manipulations[96][100].

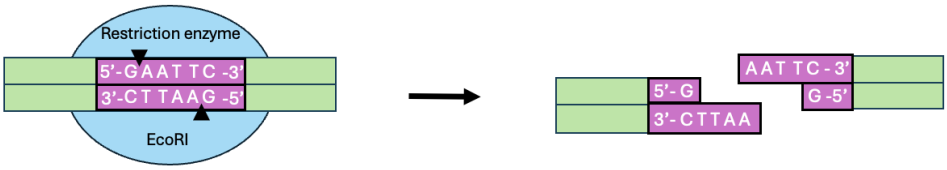


Figure 8: Illustration of EcoRI, a type II restriction enzyme, binding to and cleaving its specific recognition sequence (GAATTC) on a dsDNA. Resulting in the formation of two DNA fragments with complementary overhanging ends.

Procedure
For conducting restriction enzyme digestions, the selection of appropriate enzymes was facilitated by Benchling[101]. The standard reaction setup involved mixing 150-200 ng of DNA with 2 μL of an appropriate buffer, either 10X NEB Buffers 3.1 or CutSmart. An additional 0.5 μL of enzyme and sufficient reverse osmosis (RO) water were added to achieve a total volume of 19.5 μL. The mixture was then incubated at 37°C for at least 1 hour. Post-incubation, these mixtures could either be stored at -20°C or immediately analyzed by gel electrophoresis for the assessment of cleavage efficiency.

2.7 Separation of DNA fragments by gel electrophoresis

Gel electrophoresis is a method used to separate DNA fragments by the size of the molecules, and is frequently used to analyse DNA following a polymerase chain reaction (PCR) or restriction enzyme reaction. This method leverages the molecular filtering properties of agarose gels, which, based on their pore sizes, allows effective separation of DNA fragments varying in size from 100 base pairs (bp) to 25 kb[102]. The separation process is facilitated by Tris-acetate-EDTA (TAE) buffer, of 40 mM Tris-acetate and 1 mM EDTA, which provides essential ions and maintains a stable pH throughout electrophoresis. EDTA binds to and removes cations that otherwise could activate

nucleases and interfere with the separation process. This creates an optimal environment for DNA fragment separation within the electric field constructed between a positive and a negative electrode. Due to the negatively charged phosphate backbone of DNA molecules, they migrate towards the positive electrode[102][103]. The separation of DNA fragments is influenced by multiple factors, critical among them being the molecular weight and conformation of the DNA, as well as the composition and operational parameters of the electrophoresis setup. For size-based separation, samples are introduced into a gel matrix composed of cross-linked polymers. There exists an inverse relationship between the distance a DNA molecule travels through the gel and its molecular weight. Smaller molecules navigate through the gel more swiftly, while larger fragments display less mobility and progress more slowly, hindered by the gel's pores[96]. Additionally, the DNA conformation affects its migration rate, for instance negatively supercoiled *E. coli* plasmid DNA navigates through the gel more readily compared to its nicked or linearized counterparts, with the nicked exhibiting a decreased migration rate and the linearized showing an intermediate rate[96]

Operational factors such as the applied voltage, the buffer composition, and the use of DNA stains further influence the migration rate. In this thesis, GelGreen was selected over traditional stains like ethidium bromide (EtBr) for its safety and compatibility with blue light visualization, reducing the risk of UV-induced DNA damage and avoiding the toxicity associated with EtBr[102][103][5].

Procedure[5]
The agarose gel, composed of TAE, 0.8% agarose and GelGreen, was poured into a gel tray containing the required number of wells. The mixture was then left to solidify at RT for a minimum of 15 minutes. The comb was removed, and the gel put in an electrophoresis chamber covered with TAE buffer.

Samples consisting of 1 μl 10X purple Loading Dye, as DNA usually is colourless, 5 μl of autoclaved deionized water and 5 μl of isolated DNA were prepared. The standard samples were prepared similarly, but with only 3 μl of the standard DNA solution. The samples were then loaded into each well. The standards used in this study was either λ-DNA cut with PstI (λP) or HindIII (λH) restriction enzymes, which are shown in Appendix B. The standards were used as reference fragments of known sizes to confirm the unknown DNA-fragments. All samples and standards were then carefully loaded into the wells of the gel.

Electrophoresis was conducted using the BioRad PowerPac Basic Power Supply was programmed to a voltage of 100 V, current of 400 MA, and a run-time of 40-60 minutes. Following the completion, the gel was gently transferred for imaging under blue light[104].

The Molecular Imager ChemiDoc^{TM}XRS+ (Bio-Rad) was then used to visualize the fragments. The machine utilised blue light to excite the GelGreen dye. Upon exposure to blue light, the GelGreen-DNA complex fluoresced, enabling identification and visualisation of DNA fragments separated by the electrophoresis. The ChemiDoc^{TM}XRS+ system captured images of the fluorescent DNA bands, which were analysed by using the Image Lab 6.0.1 software. When needed, bands of interest were extracted from the agarose gel, and purified[104].

2.8 Ligation of DNA fragments

DNA ligases forms the phosphodiester bond between DNA fragments, and ligation enzymes utilize replication, repair, and recombination processes. To form bonds between the "juxtaposed" free 5' phosphate end and the 3' hydroxyl ends of DNA fragments, energy is supplied by NAD^+ in bacteria and by adenosine triphosphate (ATP) in animals and bacteriophages[96]. In this thesis, the ATP-dependent T4 DNA ligase was used for both sticky and blunt ends, even though it ligates sticky ends more efficiently. Figure 9 illustrates the ligation process in the three steps of enzyme adenyl, transfer of adenosine monophosphate (AMP) and the formation of the phosphodiester bond. Hydrolysis of pyrophosphate supplies the energy needed to drive the reaction, where ATP forms a bond with a lysine residue in the enzyme's active site, constructing an enzyme-AMP intermediate which activates the 5'-phosphate end. Further, the 3'-hydroxyl end acts as a nucleophile and releases AMP as the phosphodiester bond is formed between the two ends[105][106].

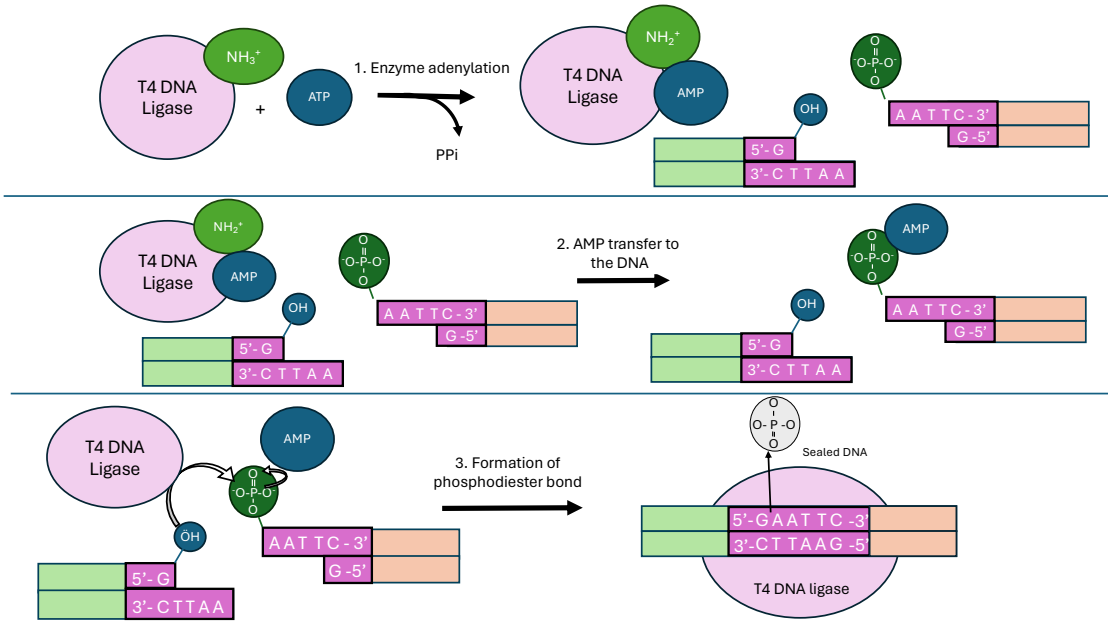


Figure 9: Illustration of sequential steps of DNA ligation by T4 DNA Ligase. Step 1 showing ATP being consumed to adenylate the enzyme, preparing it for reaction. Step 2, the adenyl group is transferred to the 5' phosphate end of the DNA, activating it for ligation. Step 3, the activated 5' end undergoes a nucleophilic attack on the 3' hydroxyl end, leading to the formation of a phosphodiester bond that seals the DNA strands.

Procedure[5]
The vector to insert was maintained at 3x molar ratio, totaling 17 μL of DNA, comprising 14 μL of insert and 3 μL of vector. Additionally, 2 μL of NEB 10X T4 DNA Ligase Buffer and 1 μL of NEB T4 DNA Ligase were added to the mixture, which was then incubated for 4-16 hours at 16°C.

2.9 Cloning of PCR product by TOPO isomerase I

For this thesis the Zero Blunt® TOPO® PCR Cloning Kit is employed, which is a kit designed for simplicity and efficiency in cloning DNA sequences without the need for ligases[84]. Topoisomerase I-mediated cloning, which facilitates the direct insertion of blunt-end PCR products into a plasmid vector, will hereafter be referred to as TOPO cloning.

The core of TOPO cloning technology is its use of DNA topoisomerase I, an enzyme derived from the Vaccinia virus. DNA topoisomerases are enzymes that manage DNA supercoiling by making transient breaks in the DNA strands. These breaks allow for the adjustment of DNA tension through strand rotation or passage, followed by religation of the DNA[107][108]. In the context of TOPO cloning, topoisomerase I specifically recognizes and cleaves the DNA sequence 5'-CCTT-3'[109]. This cleavage separates the DNA strand and also preserves energy by forming a covalent bond between the enzyme's tyrosyl residue and the 3'-phosphate of the cleaved DNA strand. This stored energy is a subsequent steps in the cloning process[107][110].

The plasmid used in TOPO cloning are linearized PCR-Blunt II-TOPO, where topoisomerase I is pre-attached to the 3' ends of the vector. This process is illustrated in Figure 10. The setup allows for the insertion of a blunt-end PCR product in either orientation relative to the vector backbone[84]. Once the insert is positioned, the energy conserved in the topoisomerase-

DNA complex facilitates the insertion by enabling the enzyme to release, thereby sealing the insert within the plasmid[110][109][84].

The vector is designed to include the lethal *E. coli* gene *ccdB*, which is attached to the C-terminus of the LacZα fragment. This setup ensures that only *E. coli* cells that have successfully incorporated the plasmid with the inserted DNA fragment will survive, eliminating the need for additional screening techniques like blue/white screening and thus simplifying the selection process[110][109]. Furthermore, the vector contains EcoRI cut sites flanking the insertion site for easy excision of the inserted DNA, and it carries both kanamycin and zeocin resistance genes to facilitate selection in *E. coli*. M13 forward and reverse primer sites, detailed in Table 10, are included to assist in sequencing the inserted DNA fragment[110][109][84].

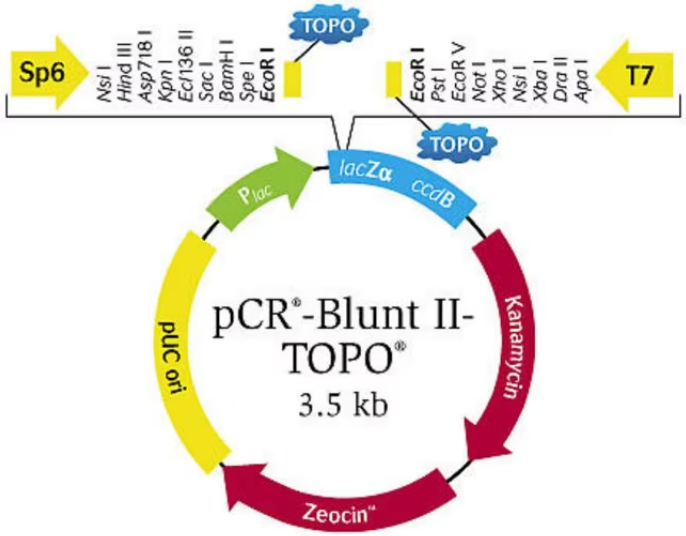


Figure 10: An illustration of the pCR-Blunt II-TOPO vector used in TOPO cloning reactions featuring the *ccdB* gene linked to the C-terminus of the LacZα fragment. Adjacent to the insertion site are EcoRI restriction sites, facilitating easy extraction of inserted DNA. The vector also includes kanamycin and zeocin resistance genes for antibiotic selection[111].

Procedure[5]
The TOPO Cloning reaction was prepared by combining 2 μL PCR product, 1 μL PCR-Blunt II-TOPO vector, and 0.5 μL salt solution. The mixture was incubated at room temperature for 20-30 minutes. After incubation, the reaction mixture was either immediately cooled on ice for transformation or stored at -20°C[84].

2.10 DNA amplification by Polymerase Chain Reaction

Polymerase Chain Reaction is an enzymatic method developed in the mid-1980s by Kary Mullis, which is used extensively for amplifying specific DNA sequences[112]. This technique involves cycles of heating and cooling to melt DNA and then enzymatically synthesize millions of copies from specific sequences. PCR employs a heat-stable DNA polymerase that can withstand the high temperatures needed for DNA strand separation. This polymerase catalyzes the formation of new DNA strands starting from a DNA primer, which attaches to a specific target sequence on the ssDNA[113][96].

The process leverages the natural mechanisms of DNA replication but is distinguished by its repetitive, cyclical nature, which exponentially amplifies the target DNA, producing large quantities sufficient for subsequent applications.This process ensures the exponential amplification of DNA, potentially resulting in over a billion precise copies of the target DNA segment, where each cycle doubles the quantity of DNA as schematically represented in Figure 11.

The introduction of Taq polymerase from the thermophilic bacterium Thermus aquaticus was a pivotal development in PCR technology, enhancing its efficiency by allowing continuous DNA synthesis across cycles without enzyme degradation[96]. However, for this project the Q5 high-fidelity DNA polymerase was used, because it exhibits a lower error rate, ensuring precise DNA amplification. Unlike Taq polymerase, Q5 includes a proofreading ability that corrects errors during DNA synthesis and produces blunt-ended DNA, making it suitable for cloning procedures that require blunt-ended PCR products[96].

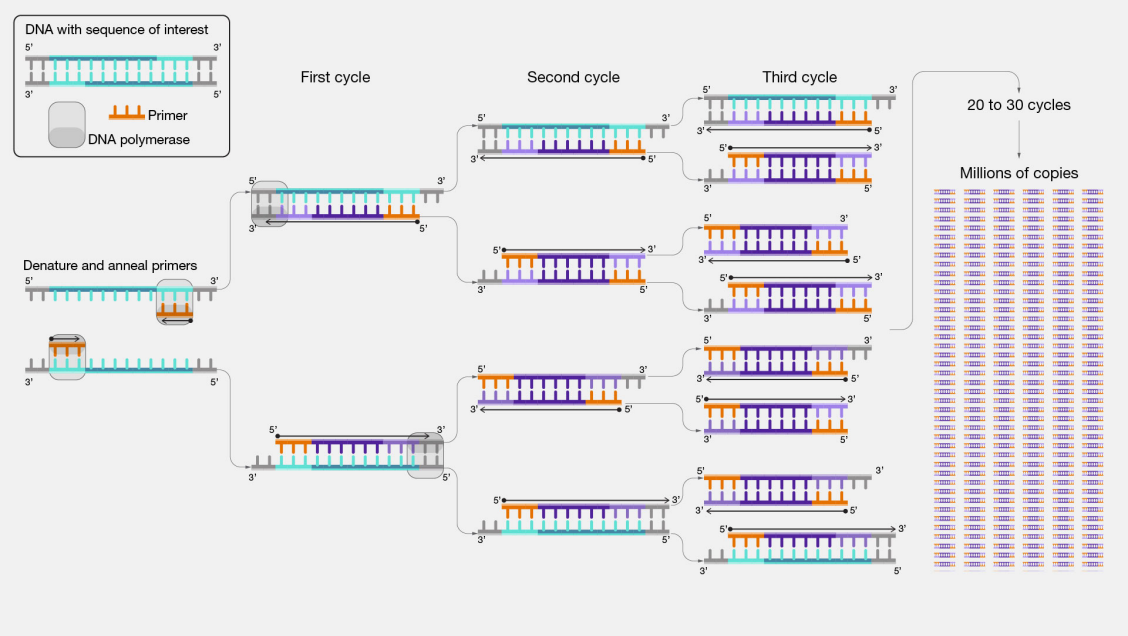


Figure 11: A schematic representation of the polymerase chain reaction process illustrating the sequential steps involved in amplifying a specific DNA sequence of interest over multiple cycles, copied from NIH[114]. Starting with the initial DNA strand, primers anneal to target regions, and DNA polymerase extends the new strands. Each cycle, consisting of denaturation, annealing, and extension phases, doubles the number of DNA copies. This exponential amplification results in millions of copies of the target DNA sequence, enabling further analysis and applications.

Procedure[5]
0.2 μL of the PCR reaction mixture, detailed in Appendix´A, was transferred to a 0.2 mL PCR tube and kept on ice. The PCR procedure was carried out over 35 cycles, beginning with an initial denaturation at 98°C for 3 minutes to separate the DNA strands. This was followed by an annealing step at a temperature 3°C below the lowest melting temperature (Tm) of the primers used, allowing the primers to bind to their respective complementary DNA strands. The elongation phase then took place at 72°C, where Q5 DNA polymerase synthesized new DNA strands from the available templates. After completing the cycles, a final elongation step was conducted at 72°C for 2 minutes, and the samples were subsequently held at 4°C to prevent any degradation of the newly formed DNA.

2.11 Sequencing of DNA

DNA sequencing is a technique utilized to identify the exact sequence of nucleotides in a DNA molecule. Following procedures such as PCR and TOPO-cloning, sequencing is used for confirming the absence of unintended mutations in the cloned DNA sequences. The method predominantly used for such applications is the Sanger sequencing technique, named after its developer, Frederick Sanger, who introduced this method in 1977[115].

Sanger sequencing operates on the principle of selective incorporation of chain-terminating nucleotides during DNA synthesis. A typical reaction setup includes a DNA template, a primer, DNA

polymerase, and a mix of regular deoxynucleotide triphosphates (dNTPs) and specially modified di-deoxynucleotide triphosphates (ddNTPs). These ddNTPs lack the 3' hydroxyl group required for forming a phosphodiester bond, thereby terminating the DNA strand extension once incorporated. Originally, the process required separate reactions for each type of radioactively labelled ddNTP, but modern techniques employ a single reaction mixture where each ddNTP is tagged with a distinct fluorescent dye[115][116].

The process starts with the preparation of the sequencing sample, which involves mixing purified plasmid DNA with specific primers and a stabilizing agent, typically dimethyl sulfoxide (DMSO), and diluting with RO water to achieve the desired volume. These prepared samples are then submitted for automated sequencing, employing the Sanger method facilitated by Eurofins Genomics GATC through their LightRun service.

In the automated Sanger sequencing process, DNA polymerase extends from the primer, incorporating nucleotides that are complementary to the template strand. This includes both regular dNTPs and the fluorescently labeled ddNTPs. The ratio of dNTPs to ddNTPs is carefully controlled, typically around 100:1, to allow partial incorporation of ddNTPs and thus varying lengths of DNA fragments[115]. Once the extension is complete, the reaction mixture contains a spectrum of DNA fragments terminated at each position where a ddNTP was incorporated. These fragments are then separated by capillary gel electrophoresis based on size. As they pass through a laser detector, the fluorescent tags emit signals that are captured and translated into a chromatogram. This chromatogram visually represents the sequence of bases by displaying peaks of different colors corresponding to the four nucleotides[116]. The precise sequence of the DNA is determined by analyzing the order of these peaks.

The preference for Sanger sequencing in certain applications, such as verifying single gene sequences or examining cloned DNA inserts, underscores its reliability and precision. Despite the advent of next-generation sequencing technologies, Sanger's method provides a high level of accuracy and remains a high standard for tasks requiring detailed and specific genetic analysis[117]. This approach ensures that researchers can confidently assess and utilize the genetic information derived from their experimental workflows, maintaining the integrity of their scientific investigations.

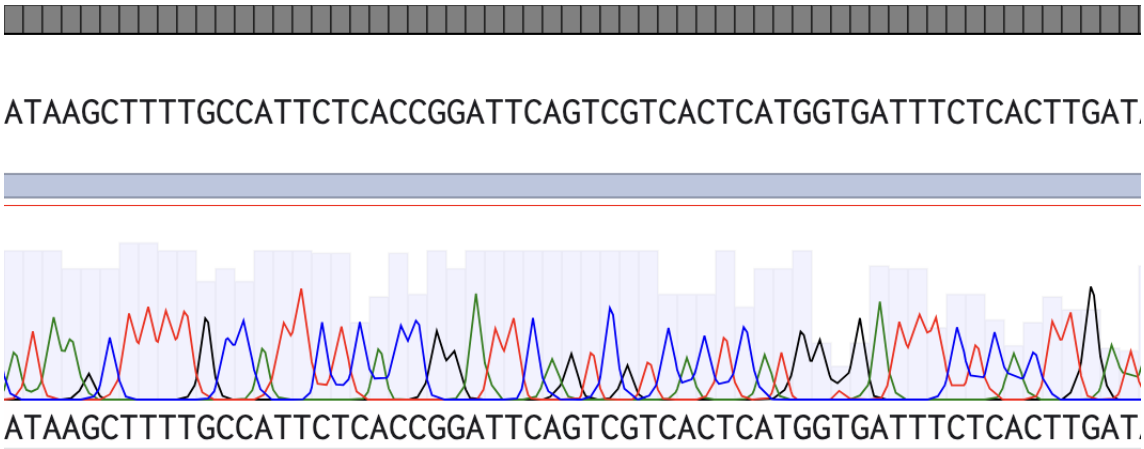


Figure 12: A display of a segment of the DNA sequence alignment from the Sanger sequencing file obtained from Eurofins, aligned against the pSV2 plasmid DNA sequence in Benchling[101]. The alignment illustrates the precise matching and mismatches of nucleotide sequences, confirming the sequence accuracy or revealing mutations. In the alignment, each row represents a contiguous segment of DNA, with the top row indicating the reference pSV2 DNA sequence and the bottom row showing the sequenced sample. Discrepancies between the sequences would have been highlighted, facilitating easy identification of any genetic variations or errors introduced during the sequencing process.

Procedure[5]
Initially, 1.75 μL of the appropriate primer was combined with 600-750 ng of plasmid DNA, along

with 0.75 μL of DMSO. This mixture was then diluted with RO water to achieve a final volume of 15 μL, ensuring the correct concentrations for optimal sequencing reactions. Following the preparation, the samples were sent to Eurofins Genomics GATC in eppendorf-tubes for automated Sanger sequencing with their LightRun service. The results were subsequently interpreted employing Benchling to verify sequence accuracy and identify any potential mutations or discrepancies in the cloned DNA segments.

2.12 Cultivation in plates for measurement of growth and fluorescent protein expression

The investigation of cell growth and fluorescence necessitates control over environmental conditions. A custom-designed labware device was utilized, tailored for compatibility with 96-well plates and facilitating controlled lighting conditions for growth experiments. The device was designed and manufactured by Bruder et al. using OpenSCAD, the device included LED matrix adapters and a universal plate-holder for incubators, ensuring accurate regulation of light intensity and duration[118].

OD_{600} measurements are used to assess the growth rate of microorganisms by quantifying the turbidity, or cloudiness, of a culture, which correlates with cell density. The wavelength of 600 nm is selected because it's within the visible spectrum and effectively avoids the absorption peaks of most biological molecules, thus providing measurements primarily influenced by the scattering of light by cells[119]. In this thesis a 96-well plate with black walls and a clear bottom is used. Light at a wavelength of 600 nm is directed through the clear bottom of the wells, and the amount of light that passes through is measured. The black walls of the wells prevent light from entering or exiting the sides, ensuring that measurements are not influenced by adjacent wells or external light sources. The reduction in light intensity, due to scattering and absorption by the cells, is used to calculate the OD_{600} value, which is proportional to the cell density in the well[119].

Fluorescence measurements assess the expression of fluorescent proteins, such as mCherry. In this case, the cells are exposed to light at a specific excitation wavelength that mCherry can absorb, leading to the emission of light at a longer wavelength, characteristic of the fluorescent protein. For mCherry, the excitation light is typically in the red part of the spectrum, at 587 nm, and the emitted light is measured at a slightly longer wavelength, at 610 nm[119]. The setup uses a programmable LED matrix to provide controlled light exposure to the samples from below, through the clear bottom. This arrangement enables precise control over the light quality, such as color, quantity, including intensity, timing, and pulse frequency, for both the excitation and measurement phases.

Procedure
Measurements were conducted in a CLARIOstar Plus plate reader by BMG LABTECH, with the CLARIOstar MARS program.

Plasmids that had previously been constructed and conjugated into *M. algicola* was used. Each strain was cultivated in 25 mL of LB medium supplemented with the appropriate antibiotic. They were then incubated over a period of 12-14 hours at 30°C with agitation at 225 rpm. After the incubation, the OD_{600} of the pre-cultures was measured. Then the cell cultures were then diluted into 25 mL of fresh LB containing the appropriate antibiotic to achieve an OD_{600} value of 0.1. From these diluted cultures, 150 μL was transferred to wells in a 96-well plate for measuring, as illustrated in Figure 13.

Plate 1

Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	pTN7 1 m-tol	pTN7 2 m-tol	pTN7 3 m-tol	Blank	pTN7 1	pTN7 2	pTN7 3	Blank	pTN8 1 IPTG	pTN8 2 IPTG	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	pTN8 3 IPTG	Blank	Blank
Blank	pTN9 1 m-tol	pTN9 2 m-tol	pTN9 3 m-tol	Blank	pTN9 1	pTN9 2	pTN9 3	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	pTN8 1	pTN8 2	Blank
Blank	pTN11 1 m-tol	pTN11 2 m-tol	pTN11 3 m-tol	Blank	pTN11 1	pTN11 2	pTN11 3	Blank	pTN8 3	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Figure 13: Layout of a 96-well plate showing *M. algicola* cultures arranged in triplicate for each condition, with and without inducers.

To analyse the strength of the inducers, the relevant samples were measured both with and without inducers. Anthranilate and m-toluic acid were dissolved in 96% ethanol, isopropyl-β-D-thiogalactopyranoside (IPTG), arabinose and rhamnose were dissolved in water. These were added to the appropriate samples in the well plate to achieve desired concentrations, as detailed in Table 3. Each sample was cultivated in three replicate wells, with "blank" wells containing only LB medium placed between the different triplicates to prevent cross-contamination. OD and fluorescence were measured each hour, or every other hour, for the first 12 hours, followed by additional measurements at hour 24 to 36, and again between hours 48 and 60.

Table 3: Overview of plasmids, conjugated in *M.algicola*, with their respective inducer concentrations. As pTN12 is a constitutive plasmid, it is not induced.

Plasmid	**Inducer**	**Concentration of inducer**
pTN7	m-toluate	0.5 mM
pTN8	IPTG	0.5 mM
pTN9	m-toluate	0.5 mM
pTN10	arabinose	13 mM[2]
pTN11	m-toluate	0.5 mM
pTN12	-	-
pTN13	anthranilate	10 mM[4]
pTN14	rhamnose	10 mM[4]

2.13 Bioinformatics

To connect, interpret and analyze the genetic information and sequences in this thesis, bioinformatic tools was employed as a multidisciplinary approach. In this thesis the bioinformatic sofware Benchling[101] was used for analyzing genetic information, understanding biological processes, and predictions of biological systems and enhancing interpretative capabilities.

2.13.1 Benchling

Benchling is a comprehensive bioinformatics platform, and was emplyoed in this thesis to streamline the design, management, and analysis of experiments. It facilitated tasks such as DNA sequence analysis, identification of restriction enzyme cut-sites, sequence alignment, plasmid mapping, and the execution of virtual digests and assemblies[101][5].

3 Results

3.1 Construction of RK2-based broad host range plasmids with different antibiotic resistance cassettes

One aim for this thesis was to develop a versatile toolbox of conditional suicide plasmids, each consisting of a cassette with different characteristics that are easily changeable to adapt to various experiments.Constructing cassettes of different antibiotic resistance genes is essential to achieve versatility, which allows their use in various gram negative bacterial strains and as selectable markers. Having a set of RK2-based plasmids with different antibiotic resistance genes allows for easy testing of which resistance genes are effective in the desired bacteria.

In my earlier work, a vector plasmid, pTN3, was derived from pSV2 and inserted with a tetracycline resistance (Tc^R) gene from pLit28Tc to facilitate as a vector plasmid, where the resistance genes can easily be replaced by using SgrDI or SalI and SbfI or PstI[5]. Further, resistance cassettes, of apramycin (Am), and spectinomycin (Sp), were constructed by using PCR-Blunt II TOPO Clones of a template DNA[5], denoted here as $pTN4_T$, and $pTN6_T$, respectively.

In this thesis, the cassette of $pTN5_T$ with kanamycin resistance (Km^R) was constructed from a pSV2 template. The template DNA was digested with StuI and DraIII to extract the 1.2 kb, as shown in Figure 14a. The Km^R was then purified and amplified by PCR with primers KmSgrDI and KmSbfI. Subsequently, amplified fragment of 1.0 kb was subject to agarose gel electrophoresis, purified, as shown in Figure 14b. The fragment was then cloned into a TOPO vector, resulting in the construct $pTN5_T$. $pTN5_T$ was then sequenced to verify the integrity of the gene of interest and confirm that no unintended mutations had occurred, as shown in Appendix G.

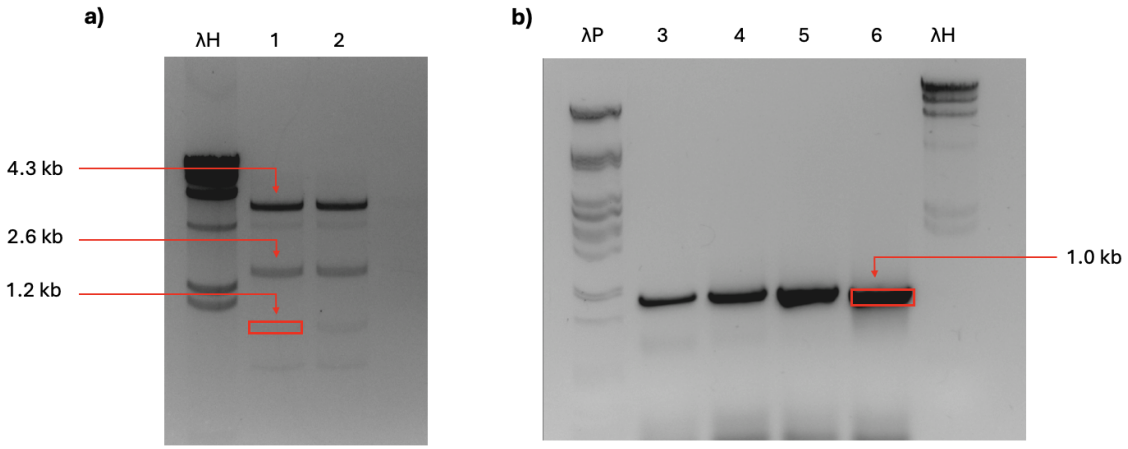


Figure 14: Agarose gel electrophoresis of Km^R derived from pSV2, where the boxed fragments in and were excised and purified. **a)** pSV2 digested by DraIII and StuI, and **b)** Km^R fragment amplified by PCR followed by agarose gel electrophoresis. λH and λP denotes standard DNA-ladders of HindIII and PstI, respectively.

These three TOPO cloned plasmids, $pTN4_T$, $pTN5_T$, and $pTN6_T$ were further enzymatically digested, to isolate the resistance gene, and prepared for ligation with pTN3. The plasmid maps of vector pTN3, and the constructed plasmids, are listed in Appendix F. A flowchart of the construction of pTN4, pTN5 and pTN6 is illustrated in Figure 15.

To make these cassettes for the toolbox, the plasmids pTN4, pTN5, and pTN6 were constructed with resistance to Am, Km and Sp, respectively, as illustrated in Figure 15. PCR Blunt II TOPO cloning technology were employed to construct the inserts for these three plasmids, $pTN4_T$, $pTN5_T$, and $pTN46_T$, and were subsequently ligated with the pTN3 backbone.

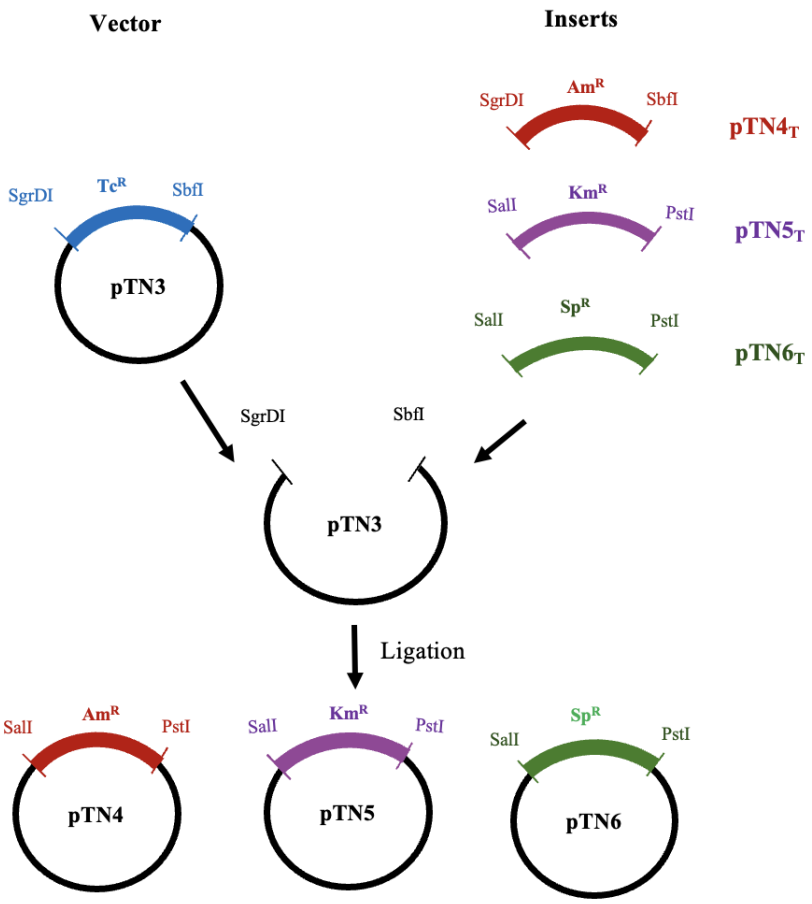


Figure 15: Flowchart of the construction of plasmids pTN4, pTN5, and pTN6 from the common backbone plasmid pTN3. Each plasmid is distinguished by its own antibiotic resistance gene. The figure is an adjusted version of the flowchart used in my previous work[5].

The Km^R and Sp^R genes, $pTN5_T$ and $pTN6_T$ respectively, were extracted from their vectors by digestion with restriction enzymes PstI and SalI, while Am^R, $pTN4_T$, was digested with SgrDI and SbfI, as shown in Figure 16a, 16b, and 16c. The pTN3 backbone was subjected to enzymatic cleavage using SgrDI and SbfI to remove the Tc^R gene, and purifying it for ligation with the other resistance genes as shown in Figure 16d. These fragments were then extracted and purified from the agarose gel and prepared for ligation.

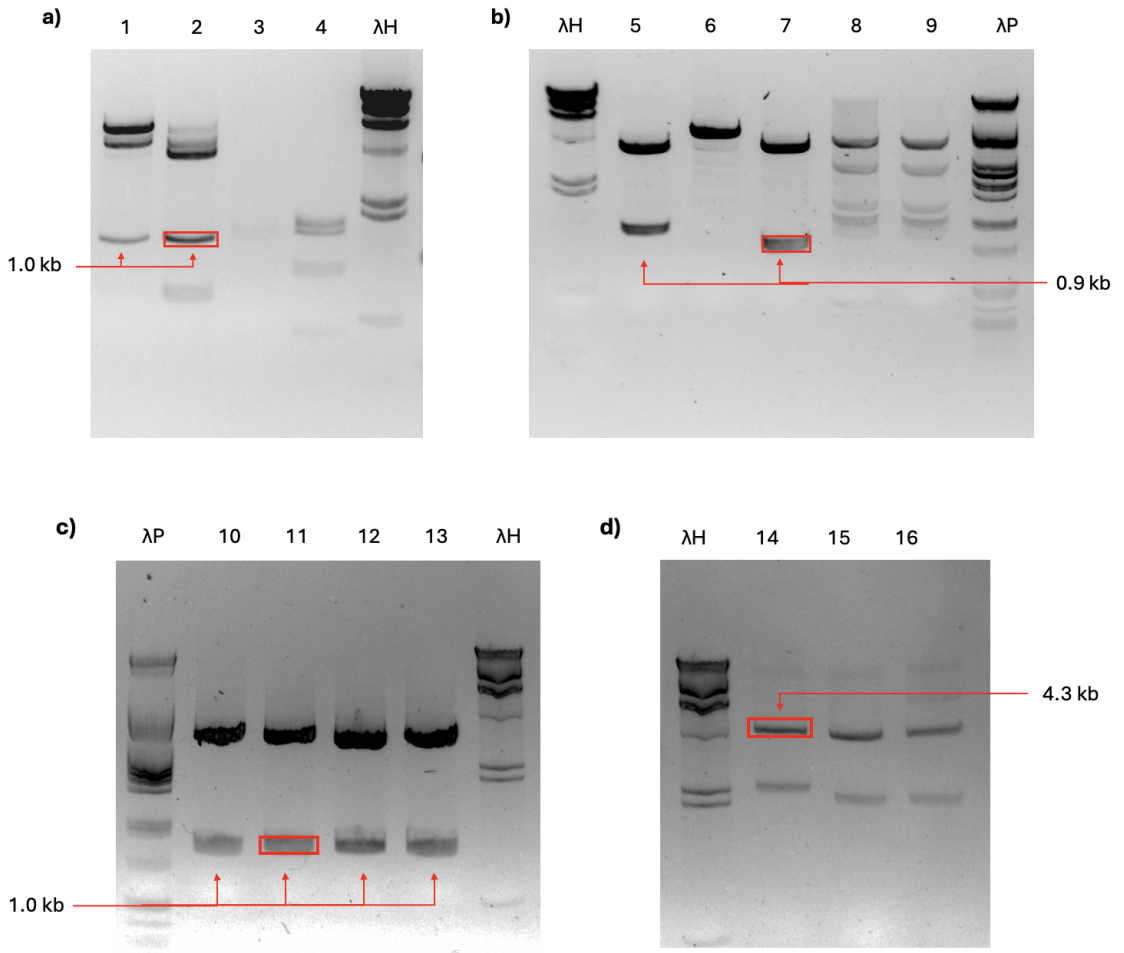


Figure 16: Agarose gel electrophoresis for extracting fragments of pTN3 backbone and insertions $pTN4_T$, $pTN5_T$, and $pTN6_T$ for the construction of pTN4, pTN5 and pTN6. **a)** Well 1 and 2 contain $pTN6_T$ digested by enzymes PstI and SalI. **b)** Well 5-6 contain $pTN4_T$, digested by SgrDI and SbfI. **c)** $pTN5_T$ digested with PstI and SalI. **d)** pTN3 digested by SgrDI and SbfI. The boxed fragments were extracted and purified for further use. λH and λP denotes standard DNA-ladders of HindIII and PstI.

Subsequently, the ligation products were transformed into chemically competent *E. coli* cells. Specifically, strains S17.1 were used for pTN4 and pTN5, carrying Am^R and Km^R, respectively. However, the Sp^R plasmid pTN6 was transformed into *E. coli* DH5α, as strain S17.1 is resistant to spectinomycin.

Transformed *E. coli* colonies were selected on LA plates containing the corresponding antibiotic for each plasmid construct. Only cells that successfully took up the plasmid constructs would grow, indicating the presence of the antibiotic resistance genes. Plasmid DNA was extracted from antibiotic-resistant colonies, digested with restriction enzymes, and the resulting patterns analyzed by gel electrophoresis to validate the successful construction of each plasmid. The specifics of the restriction enzymes, expected fragment sizes for successful construction, as well as unwanted fragment sizes indicating the presence of parental plasmids, are detailed in Table 4.

Table 4: Overview of the constructed plasmids, detailing the specific enzymes used for digestion and subsequent verification via gel electrophoresis.

Construct	**Fragments from TOPO clone**	**Verification enzymes**	**Expected fragments [kb]**	**Parental fragments*** **[kb]**	**Verification gel**
pTN4	Am^R, 0.8 kb Figure 16b	EcoRI, SfoI	3.5, 1.7	4.0, 1.4, 0.86 2.8, 0.76, 0.57	Figure 17
pTN5	Km^R, 0.9 kb Figure 16c	Xmal, SalI	4.7, 0.57	4.9, 1.1, 0.36 2.4, 1.5, 0.57	Figure 18a
pTN6	Sp^R , 1.0 kb Figure 16a	NcoI, HindIII, XhoI	2.7, 2.6	3.7, 2.7 1.6, 1.4, 1.1, 0.44	Figure 18b

*Parental fragments refers to the sized fragments that would show if the parental plasmids had been digested with the respective enzymes. The upper line represents pTN3 backbone, and the lower line represents the TOPO-clones.

The digested plasmids were analyzed using gel electrophoresis, and the results are shown in Figure 17, 18a and 18b. For each ligation at least one plasmid provided a fragment sizes that matched the expected sizes and differ from both parental plasmids. This confirms the correct ligation of pTN3 with each insert $pTN4_T$, $pTN5_T$, and $pTN6_T$. In the gel photos, the bands indicating successful ligation are marked with arrows. The colonies corresponding to these wells, which show correctly ligated plasmids, were selected for further use and stored for subsequent use.

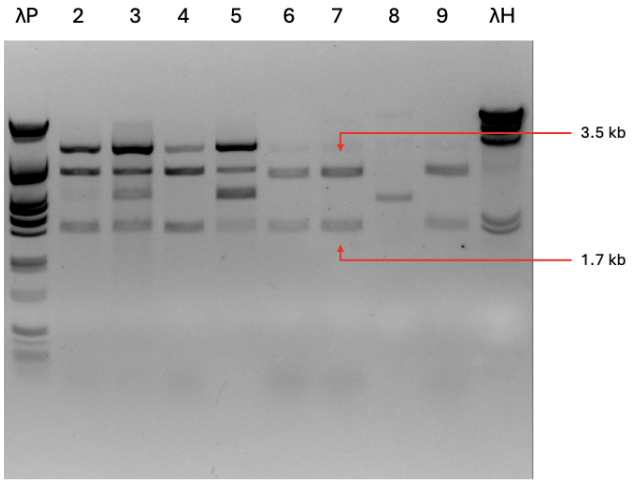


Figure 17: Validation of pTN4, with candidates digested using EcoRV and SfoI restriction enzymes. Wells 4 and 5 contain standard DNA ladders. The two bands at 3.5 kb and 1.7 kb in well 7 confirm successful ligation of pTN4.

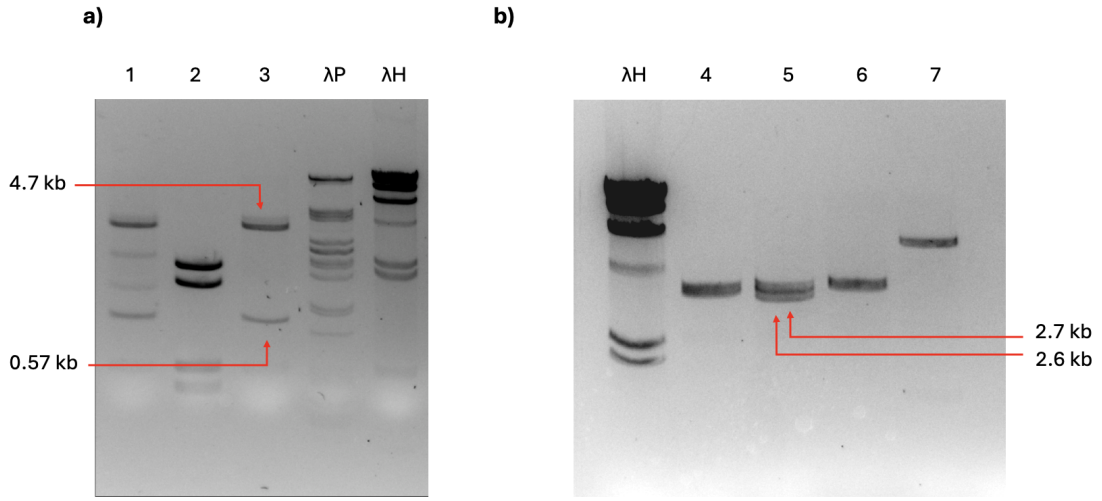


Figure 18: Agarose gel for verification of pTN5 and pTN6. **a)** pTN5 digestion with XmaI and SalI restriction enzymes showing two distinct fragments of expected sizes confirming the successful ligation in well 3. **b)** Digestion of pTN6 with NcoI, HindIII, and XhoI restriction enzymes. The bands in well 5 might indicate the correct sizes of 2.7 kb and 2.6 kb, however the image is not conclusive, hence sequencing was performed.

For Figure 18 b, the band sizes did not quite match the expected ones. However, in samples with a higher amount of DNA in the standard lane, DNA fragments often migrate relatively faster. This could impact the interpretation of the results, as it may lead to difficulties in accurately comparing the sizes of the DNA fragments. Thereby, sequencing of the inserted genes were performed to confirm the correct orientation and integrity of each cloned antibiotic resistance gene. These sequences of pTN4, pTN5 and pTN6 provided in Appendix G, confirm successful cloning and ligation without mutations that interfere with the gene of interest.The observed mutations are outside the gene of interest sequences and thus do not impact the functionality of the antibiotic resistance cassettes, which are the essential part for this project.

3.2 Assessment of antibiotic resistance genes in *M. algicola*

pTN3, pTN4, pTN5 and pTN6 were successfully transformed into *E. coli* JKE201, which was chosen due to its requiring of diaminopimelic acid (DAP) supplementation for growth. Subsequently, conjugation between *E. coli* JKE201 containing the plasmids and *M. algicola* was performed. To select for the presence of the plasmids in *M. algicola*, a dilution series of the conjugated cells was plated onto LA supplemented with the appropriate antibiotic corresponding to each plasmid. Additionally, DAP was omitted from the media to ensure that only *M. algicola* cells containing the conjugated plasmids survived. Notably, plasmids pTN3, pTN4, and pTN6 were successfully transferred to *M. algicola*, demonstrating efficient conjugation and selection processes. Colonies that appeared on the lowest selective diluted plates were further analyzed, respectively $10{-}5$ dilution for pTN3 (Tc^R), $10{-}3$ for pTN4 (Am^R) and pTN5 (Km^R), and $10{-}1$ for pTN6 (Sp^R).

The conjugation of pTN5 into *M. algicola* was unsuccessful, possibly due to the kanamycin resistance gene, as it is otherwise identical to pTN3, pTN4 and pTN6.

Another aspect of creating the toolbox of plasmids with interchangeable cassettes, was testing different promoter systems in *M. algicola*. The plasmids with various promoter systems to be tested, pSV2, pSV3, pSV4, pSV5, pSV6, pHH108, pAFW4 and pAFW5, were constructed by previous master students[2] [3] [4] and are detailed in Table 5.

Table 5: Overview of additional plasmids with characteristics to be transformed into *E.coli* JKE201, followed by conjugation into *M.algicola*.

Plasmid	Promoter system	Reporter gene	Resistance	Inducer	Source
pSV2	Pm wt, xylS	RFP	Km^R	m-toluate	Vold S[2]
pSV3	LacI-Ptrc	RFP	Km^R	IPTG	Vold S[2]
pSV4	Pm ML1.17, xylS	RFP	Km^R	m-toluate	Vold S[2]
pSV5	AraC-pBAD	RFP	Km^R	arabinose	Vold S[2]
pSV6	PmG5, xylS	RFP	Km^R	m-toluate	Vold S[2]
pHH106	pConstitutive	RFP	Km^R	-	Haaland H[3]
pAFW4	AntR-Pant	RFP	Km^R	anthranilate	Wesche A[4]
pAFW5	RhaSR-pRha	RFP	Km^R	rhamnose	Wesche A[4]

These plasmids all contain the Km^R gene. They were transformed into *E. coli* JKE201, but conjugation into *M. algicola* was again unsuccessful. Since plasmids pTN3, pTN4, and pTN6 successfully conjugated, this differential outcome indicates that the kanamycin resistance gene in these plasmids are not expressed well in *M. algicola*. As evidenced by its inability to survive on LA containing even small doses of kanamycin down to [10μl/ml], the strain showed sensitivity to this antibiotic. Testing *M. algicola*'s intrinsic resistance on LA plates with varying kanamycin concentrations revealed that it survived at concentrations up to 10μl/ml, hence lower concentrations could not be used. Based on these findings, this kanamycin resistance promoter and gene system is likely not compatible with *M. algicola* as a selection marker.

3.3 Exchange of the resistance genes in the promoter reporter plasmids

Since the Km^R cassette could not be used in *M. algicola*, the resistance gene in the promoter reporter plasmids was replaced with the Am^R from pTN4. The modification of these plasmids, later named pTN7 to pTN14, involved enzymatic digestion of the plasmids with restriction enzymes detailed in Table 6, followed by ligation with the Am^R gene. The Am^R gene was extracted from pTN4, digested by restriction enzymes NotI and SalI. These new plasmids were then successfully transformed into *E.coli* S17.1, and the transformants grew on LA plates with Am confirming the uptake of Am^R.

Table 6: Overview of modified plasmids resulting from enzymatic digestion and ligation.

New plasmids	Backbone plasmid*	Insertion plasmid	Restriction enzymes	Insertion fragment size
pTN7, Pm wt	pTN4	pSV2, Figure 19a	NotI, SalI, DraI	3.5 kb
pTN8, LacI-Ptrc	pTN4	pSV3, Figure 19b	NotI, SalI, SfiI	3.2 kb
pTN9, ML1.17	pTN4	pSV4, Figure 19b	NotI, SalI, StuI, SfiI	3.5 kb
pTN10, AraC-pBAD	pTN4	pSV5, Figure 19b	NotI, SalI	2.7 kb
pTN11, PmG5	pTN4	pSV6, Figure 19b	NotI, SalI, StuI, SfiI	3.5 kb
pTN12, -	pTN4	pHH106, Figure 19b	NotI, SalI	4.2 kb
pTN13, AntR-Pant	pTN4	pAFW4, Figure 19b	NotI, SalI, SfiI	3.8 kb
pTN14, RhaSR-pRha	pTN4	pAFW5, Figure 19b	NotI, SalI, SfiI	1.8 kb

*All pTN4 backbone plasmids underwent digestion with NotI and SalI restriction enzymes before being ligated with the respective insertion plasmids described in the table.

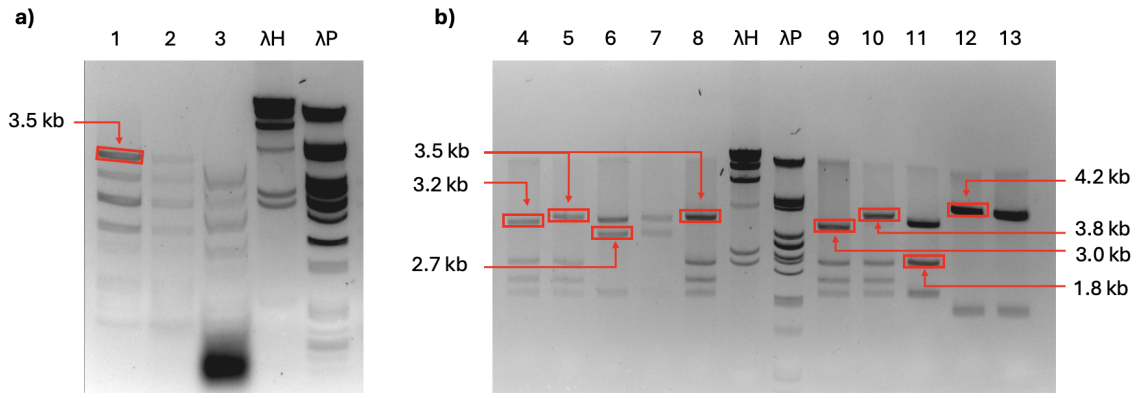


Figure 19: Gel electrophoresis of enzymatically cleaved plasmids for the construction of plasmids pTN8 to pTN14, where the boxed fragments were subsequently extracted and purified for ligation. Details of the restriction enzymes used and the resulting fragments are listed in Table 6. **a)** pSV2 digested with the restriction enzymes SalI, NotI, and DraI, for the construction of plasmid pTN7. **b)** Well 4 contains of pSV3, 5 of pSV4, wells 6 and 7 of pSV5, and well 8 contains pSV6. Well 9 contains pAFW4, well 10 contains pAFW5, well 11 contains pHH106, and wells 12 and 13 contain pTN4.

Colonies of the transformants in *E.coli* S.17.1 were then isolated and verified on gel electrophoresis to ensure correct ligation. The expected fragment sizes and restriction enzymes used for each plasmid is detailed in Table 7.

Table 7: Verification of plasmids pTN7-14, detailing the restriction enzymes used, the expected fragment sizes, and the corresponding gel electrophoresis figures, confirming correct ligation.

Plasmid	**Restriction enzyme**	**Expected fragment [kb]**	**Parental fragments*** **[kb]**	**Verification gel**
pTN7	PstI, EcoRI	3.9	3.8, 2.9, 1.2 5.2, 0.001	Figure 20a
pTN8	PstI, EcoRI	3.9, 3.5	3.8, 2.9, 1.2 5.2, 0.001	Figure 21
pTN9	PstI, EcoRI	3.9	3.8, 2.9, 1.2 5.2, 0.001	Figure 21
pTN10	PstI, EcoRI	3.9, 3.0	3.8, 2.1, 1.2 5.2, 0.001	Figure 21
pTN11	PstI, EcoRI	3.9	3.8, 2.9, 1.2 5.2, 0.001	Figure 21
pTN12	PstI, EcoRI	3.9, 1.7	3.9, 1.2, 0.8, 0.5 5.2, 0.001	Figure 22
pTN13	PstI, EcoRI	3.9, 3.4	3.9, 2.5, 1.2 5.2, 0.001	Figure 20b
pTN14	PstI, EcoRI	3.9, 3.7	3.8, 2.8, 1.2, 0.4 5.2, 0.001	Figure 22

* Parental fragments refers to the sized fragments that would indicate the presence of the parental digested with the respective enzymes. Upper line represents pTN4 (Am^R), lower line the insertion plasmids, specified in Table 6.

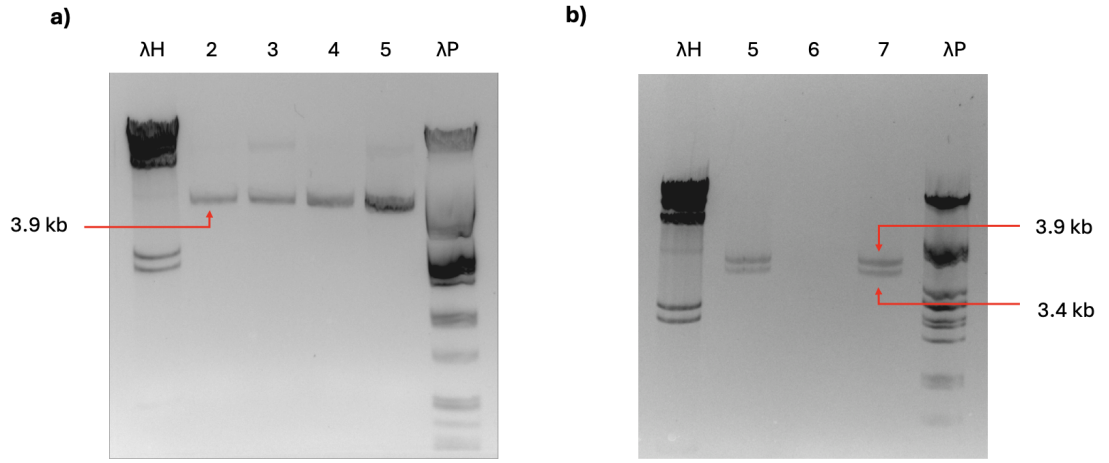


Figure 20: Agarose gel electrophoresis verifying the successful ligation and transformation of plasmids into *E. coli*. **a)** The pTN7 digested with restriction enzymes PstI and EcoRI. **b)** pTN13 digested by restriction enzymes PstI and EcoRI.

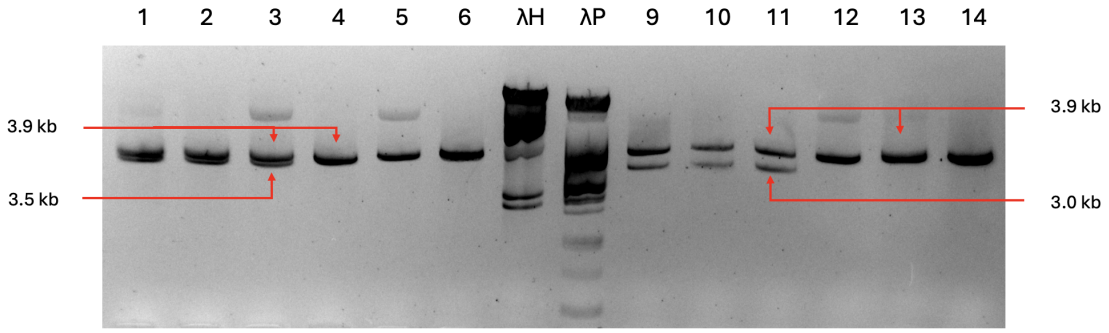


Figure 21: Gel electrophoresis verification of plasmids pTN8, pTN9, pTN10 and pTN11 through restriction digestion with EcoRI and PstI. Wells 1-3 display pTN8, 4-6 show pTN9, 9-11 contain pTN10, and wells 12-14 display pTN11. The resulting fragment sizes match the values listed in Table 7. Standard DNA ladders are denoted as λH and λP.

The standards used in the verification of plasmids in Figure 21, contained an excess amount of DNA, which caused a slight uncertainty in the credibility of the results. However, it can be observed that pTN8 is divided into two close fragments, which are more likely of the correct fragments 3.9 kb and 3.5 kb, rather than indicating religation of pSV3. The religation digested by EcoRI and PstI would give bands of 3.9 kb, 2.7 kb, and 1.2 kb, while the digestion of the pTN4 backbone would yield a single band at 5.2 kb. By comparing the sizes of other plasmid digestions and considering religation possibilities, as well as the fact that pTN8 shows ampicillin resistance and its band pattern does not match pTN4, it can be concluded with greater certainty that the plasmid is indeed pTN8, rather than relying on the standards alone.

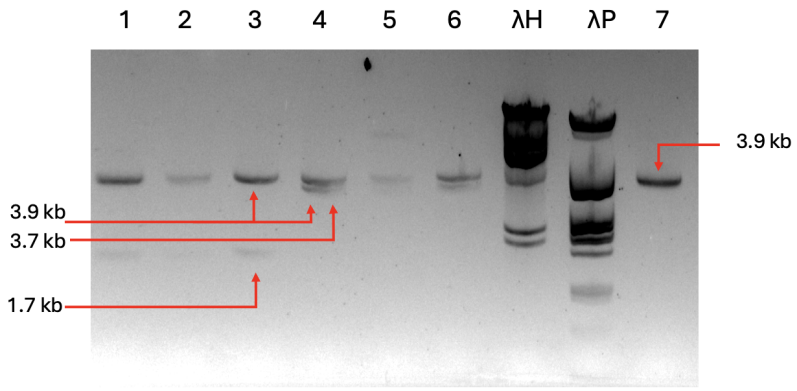


Figure 22: Confirmation of the successful construction of modified plasmids pTN12 and pTN14, both digested with restriction enzymes PstI and EcoRI. Wells 1-3 display fragments of pTN12 at 3.9 kb and 1.7 kb, while wells 4-6 show fragments of pTN14 at 3.9 kb and 3.7 kb. A 3.9 kb fragment of pTN9 in well 7 serves here as an additional reference to the DNA ladders with excess DNA.

The constructed and verified plasmids pTN7 to pTN14 were then transformed into *E.coli* JKE201. Subsequently, they were successfully conjugated into *M. algicola*. These strains were then prepared for testing to assess their promoter strength within *M. algicola*.

3.4 Growth and fluorescent protein expression assays

To evaluate the strength of the various promoter systems in *M. algicola*, 96 well cultivation plates were prepared for measurements of fluorescence and growth over time. All plasmids listed in Table 8 were conjugated into *M. algicola* and selected on LA plates with Km. Using 96-well plates allows for the simultaneous measurement of multiple samples under identical conditions, providing continuous monitoring of growth and fluorescent protein expression dynamics over time. pTN4 were also tested as a control plasmid, since it does not encode the RFP gene it would show possible background fluorescens from the bacterium.

3.4.1 Evaluating method of growth experiment

The use of a 96-well plate allows for the simultaneous testing of multiple strains, or replicates in induced and uninduced form at identical conditions. This high-throughput approach improves efficiency and ensures that all samples are subjected to uniform conditions, thereby minimising variability. The frequent measurements provide detailed time-course data, providing detailed analysis of growth dynamics and gene expression patterns. Additionally, the compact format of the 96-well plate is resource-efficient, requiring smaller volumes of reagents and culture media.

During the incubation process, the well plate was placed in a shaking incubator. To minimize cross-contamination, strains of different promoter system were strategically placed so that no two adjacent wells contained bacterial samples, set up is shown in Appendix D. The remaining wells were filled with LB and 50 μL/ml Am, and could be used to monitor any contamination transfer between wells during cultivation. The wells at the edges were not used for bacterial strains due to greater chance of evaporation.

Inter-well contamination was a potential issue due to the rotation of the plates during incubation and the handling of plates when lifting them in and out of the box. Contamination analysis was conducted simultaneously with the growth and fluorescence measurements of promoter systems in *M. algicola*. The contamination results are illustrated in Figure 23. Each well with an OD

measurement above 0.1 has the measured OD noted on the respective well, and wells containing the *M. algicola* strain with a plasmid are highlighted in pink. The figure show the measurements of growth at hour 0, and after 24 and 48 hours. The results indicate that there was some contamination between wells. However, the low values observed at hour 24 compared to the expected cultures suggest that cross-contamination between sets of samples is unlikely. This indicates that the method can be reliably used for cultivation.

Plate 1 - 0 hours

	0.12	**0.12**	**0.12**		**0.12**	**0.12**	**0.12**		**0.32**	**0.31**	
									0.32		
	0.13	**0.13**	**0.13**		**0.13**	**0.12**	**0.13**				
									0.32	**0.30**	
	0.13	**0.13**	**0.13**		**0.13**	**0.13**	**0.14**		**0.31**		
						0.112					

Plate 1 - 24 hours

0.24	0.18	0.18	0.19	0.11	0.20	0.18	0.17	0.12	0.16	0.17	0.18
0.60	**2.60**	**2.79**	**2.26**	0.27	**2.09**	**2.09**	**2.32**	0.39	**2.35**	**2.39**	0.28
0.13	0.23	0.25	0.22	0.53	0.26	0.26	0.25	0.26	**2.37**	0.55	0.21
0.19	**2.67**	**2.36**	**2.30**	0.26	**0.92**	**1.00**	**1.05**	0.47	0.24	0.21	0.19
0.18	0.26	0.26	0.38	0.27	0.28	0.28	0.27	0.23	**1.46**	**1.50**	0.24
0.21	**2.31**	**2.26**	**2.24**	0.48	**1.91**	**1.88**	**2.24**	0.24	**1.53**	0.23	0.19
0.20	0.24	0.27	0.27	0.18	0.22	0.22	0.23	0.23	0.23	0.21	0.19
0.13	0.21	0.20	0.17	0.18	0.20	0.21	0.19	0.31	0.21	0.20	0.21

Plate 1 - 48 hours

0.25	0.16	0.17	0.18	0.20	0.19	0.21	0.18	0.18	0.18	0.19	0.19
0.52	**3.17**	**3.50**	**2.87**	0.21	**2.84**	**2.85**	**2.96**	2.32	**2.77**	**2.81**	2.57
0.17	0.19	0.19	0.19	2.30	0.19	0.20	0.20	0.22	**2.87**	0.71	0.21
0.18	**3.50**	**2.89**	**2.88**	0.21	**2.89**	**2.73**	**2.71**	2.46	0.21	0.02	0.19
0.15	0.21	0.19	2.30	0.19	0.20	0.21	2.51	0.22	**2.25**	**2.74**	2.41
0.19	**2,85**	**2.87**	**2.80**	2.53	**2.77**	**2.79**	**3.09**	0.22	**2.72**	0.51	0.16
0.17	0.20	0.21	0.21	0.23	0.22	0.22	0.23	0.23	0.23	0.19	0.15
0.17	0.18	2.41	0.18	0.19	0.21	0.19	0.16	2.35	0.18	2.06	2.36

Figure 23: Contamination and growth measurements in 96-well plates. Wells initially containing *M. algicola* are highlighted in pink. Wells with OD values exceeding 0.1 are annotated on the respective well, and those with OD values exceeding 0.3 are highlighted in orange. Measurements are shown after 0, 24 and 48 hours.

Table 8: Overview of the plasmids tested with their antibiotic resistance genes, promoter systems and inducers detailed.

Plasmid	**Promoter system**	**Reporter gene**	**Resistance**	**Inducer**	**Source**
pTN4	-	-	Am^R	-	This study
pTN7	Pm wt, xylS	RFP	Am^R	m-toluate	This study
pTN8	LacI-Ptrc	RFP	Am^R	IPTG	This study
pTN9	Pm ML1.17, xylS	RFP	Am^R	m-toluate	This study
pTN10	AraC-pBAD	RFP	Am^R	arabinose	This study
pTN11	PmG5, xylS	RFP	Am^R	m-toluate	This study
pTN12	Constitutive	RFP	Am^R	-	This study
pTN13	AntR-Pant	RFP	Am^R	anthranilate	This study
pTN14	RhaSR-pRha	RFP	Am^R	rhamnose	This study

3.4.2 Comparison of overall trends in the growth and gene expression

Optical density measurements at 600 nm were recorded over a 60 hour period to evaluate the growth behavior of bacterial cultures containing the pTN7-pTN14 constructs. An equal volume of preculture diluted to an OD of 0.1 was added to three parallel wells, both with and without inducer. The plate setup is detailed in Appendix D. The results represented are average values obtained from three replicate measurements per strain, providing greater confidence in the observed data. All raw data and calculations are detailed in Appendix E and E.

The non-induced strains overall demonstrate a steady growth trend, and reached higher OD_{600} values faster than their induced counterparts, as shown in Figure 24. The induced strains showed more variations in their growth. Some of the strains reached the same density as their uninduced parallels, but needed 48 hours to do so. In contrast, induced pTN7 and pTN10 reached their stationary phase after 24 hours, similar to the uninduced strains, but at a lower density. Notably, the strains containing pTN8 and pTN14 show small differences in their induced and non-induced conditions.

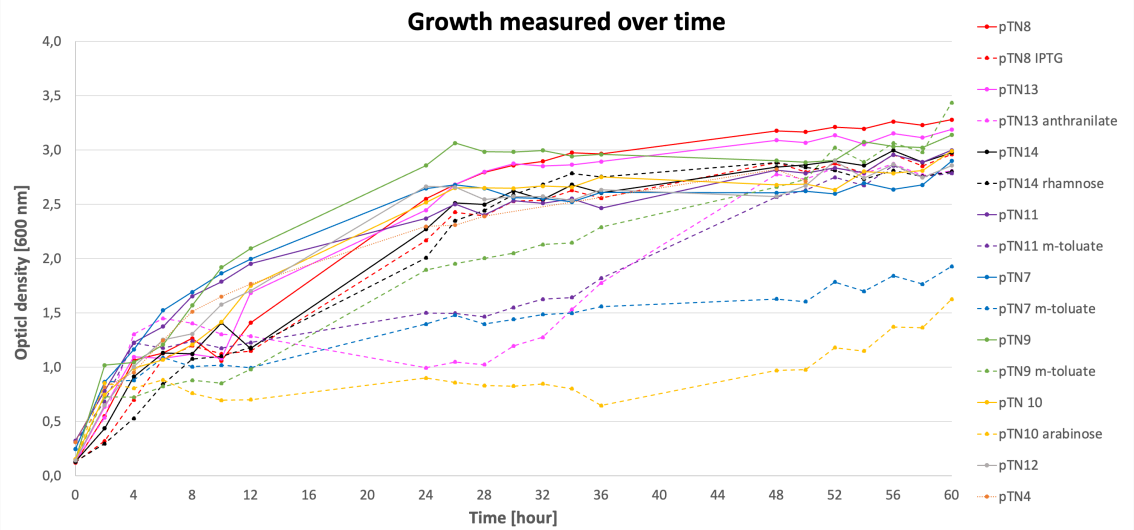


Figure 24: Growth curves of *M. algicola* strains containing plasmids pTN7 to pTN14, including both induced and non-induced conditions. Measured by OD_{600}.

The fluorescence intensity of expressed RFP in *M. algicola* strains containing plasmids pTN7 to pTN14 is presented in Figure 25. The data reveal trends in fluorescence, showing an initial drop from the first to the second measurement, followed by a more stable phase and subsequent variation between each strain.

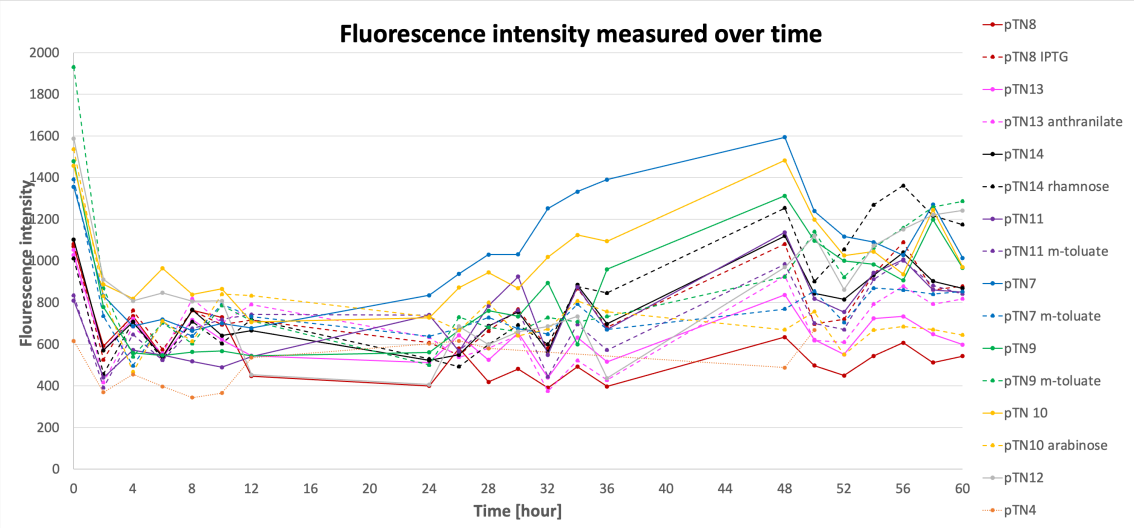


Figure 25: Fluorescence intensity measurements of RFP in *M. algicola* strains with different plasmids, including both induced and non-induced conditions.

The uninduced strains seem to express higher levels of RFP compared to the induced strains. However, since the uninduced strains have a higher cell density, this observation does not provide information about RFP production per cell. Accordingly, to obtain a more accurate assessment of promoter system performance, it is necessary to examine the FI/OD ratio, as shown in Figure 26. This normalisation accounts for differences in cell density, providing a picture of promoter efficiency relative to the number of cells. Thus, by examining the fluorescence intensity per optical density, we obtain the normalised fluorescence ratio per cell over time.

Due to the low OD values, of approximately 0.1, in the initial measurements, combined with the high initial FI readings at time 0, the resulting FI/OD values are significantly elevated at the first measurement point. Therefore, these first measurement has been excluded from these FI/OD plots to provide a clearer representation of the following measurements of the various strains under induced and uninduced conditions.

From the FI/OD it is observed high initial values which rapidly decreases within the first 6 hours. There are variations among the strains, with some displaying more significant fluctuations. Overall the trend indicates a decline in FI/OD for the first few hours, followed by a stabilisation , where the induced strains generally has a higher value than their uninduced counterparts. After approximately 24 hours, most strains reach a relatively stable phase with minor fluctuations onwards. In the following section, the results from each promoter study is shown separately.

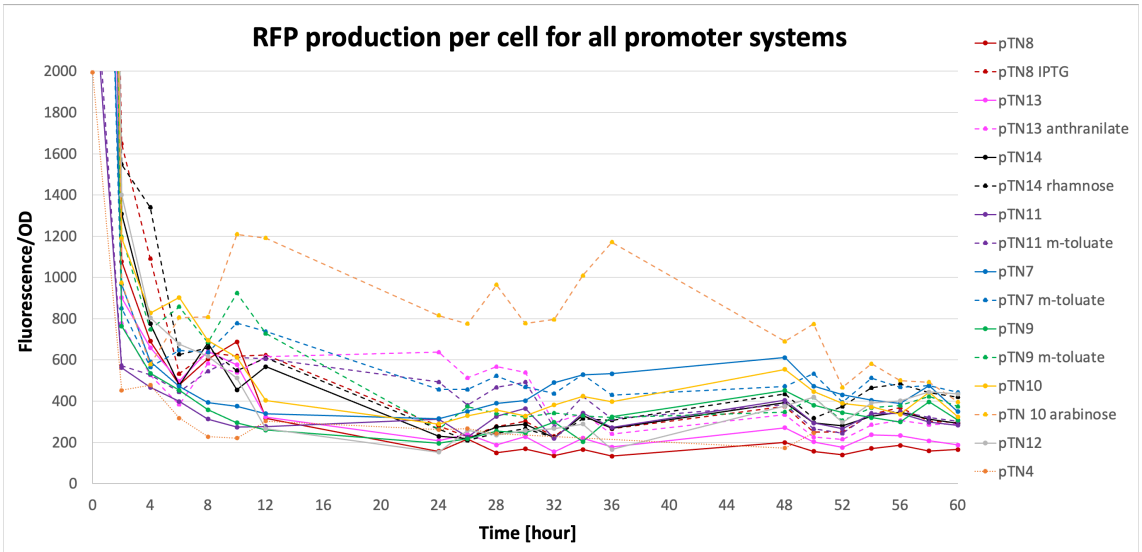


Figure 26: Normalised fluorescence intensity per optical density in *M. algicola* strains containing plasmids pTN7 to pTN14, comparing induced and non-induced conditions.

3.4.3 Activity of tested promoter systems and control strain

The graph in Figure 27 shows the relative fluorescence output of *M. algicola* strains containing the Pconstitutive strain, pTN12, and a control strain, pTN4, that does not have a reporter gene for RFP expression. The control was measured for a duration of 50 hours, whilst the pTN12 strain was measured over 60 hours.

The control strain, pTN4, maintains relatively low FI/OD values throughout the experiment, fluctuating around 200 to 250 units. The values of the control, pTN4, remained stable and express the background fluorescence expression in *M.algicola*. Hence, pTN4 is used as a baseline in comparison to the Pconstitutive, and other promoter system tested.

Initially, the Pconstitutive promoter system exhibits high relative fluorescence values, which decline for the first 12 hours. After hour 24, a stabilising trend is observed, with relative low fluorescence values fluctuating around 200. This is followed by a slow increase, reaching approximately 400 at 48 hours, after which the values stabilize, fluctuating around 400 units.

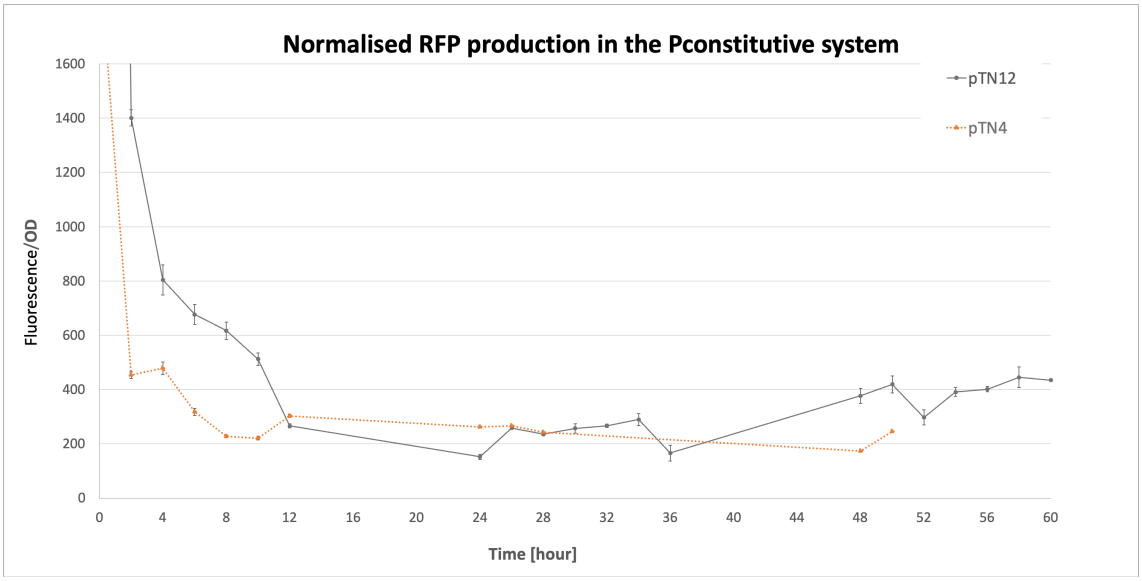


Figure 27: Normalised fluorescence intensity of RFP in *M. algicola* strains containing plasmids pTN4 and pTN12 (pconstitutive).

Regarding the Ptrc promoter, as shown in Figure 28, the induction with IPTG show an overall slightly higher relative fluorescence value compared to the uninduced strain. Both conditions follow a similar trend with decreasing FI/OD values, although, the induced strain maintains slightly higher values than the uninduced strain throughout the experiment. Notably, at hour 12, the RFP expression in the uninduced strain declines, while the IPTG-induced strain continues to exhibit higher levels of RFP expression. The next measurement is at hour 24 shows that the induced strain also declines to background levels. Additional measurements within this timeframe could reveal a period where the uninduced strain is inactive while the induced strain continues to express the gene. This would indicate potential for temporal regulation, with gene expression switched off in the uninduced strain while maintained in the induced strain during the specific time interval.

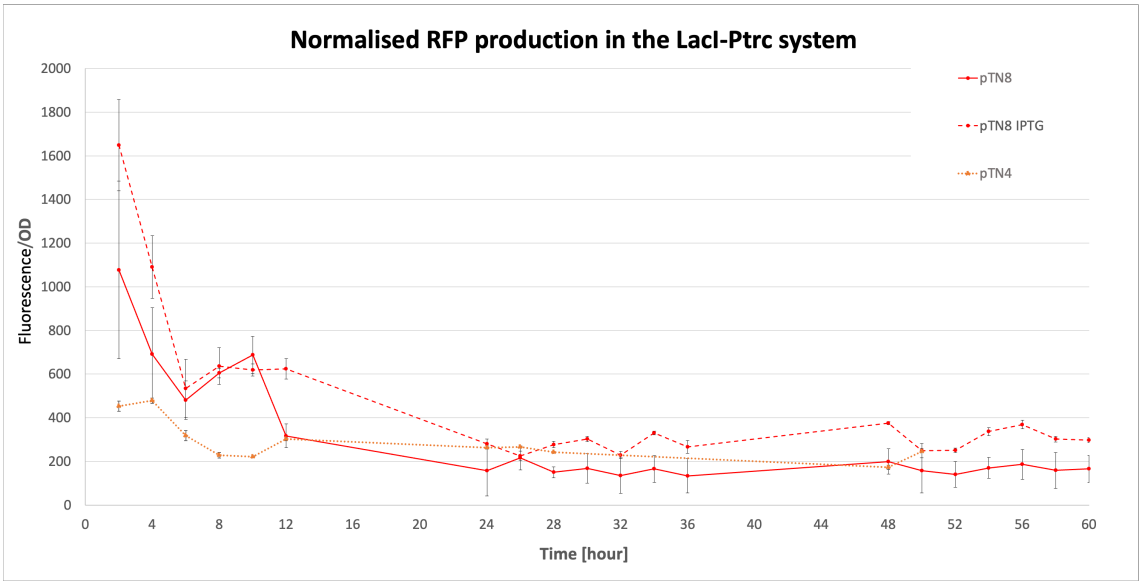


Figure 28: Relative fluorescence measurements of the LacI-Ptrc system, comparing pTN8 induced IPTG 0.5 mM, and uninduced conditions. Illustrating the promoter activity and responsiveness to induction over a 60 hour time period.

In Figure 29 the RFP expression for the AraC-pBAD promoter system is shown. Both strains

experience a decline in FI/OD values during the first 4 hours. Subsequently, the uninduced strain continues to decline, while the arabinose induced strain increases, reaching a peak of approximately 1200 units at hour 10. The induced strain then fluctuates at a higher value range than the uninduced strain, indicating higher RFP expression. Notably, irregular patterns with two distinct peaks are observed around hour 24 to 36, possibly related to low points observed in the counter measurements of the growth curve. Overall, the AraC-pBAD promoter system shows a strong response to arabinose induction in *M. algicola*, with the induced strain exhibiting higher RFP expression compared to the uninduced strain, indicating that the system works effectively in *M. algicola*.

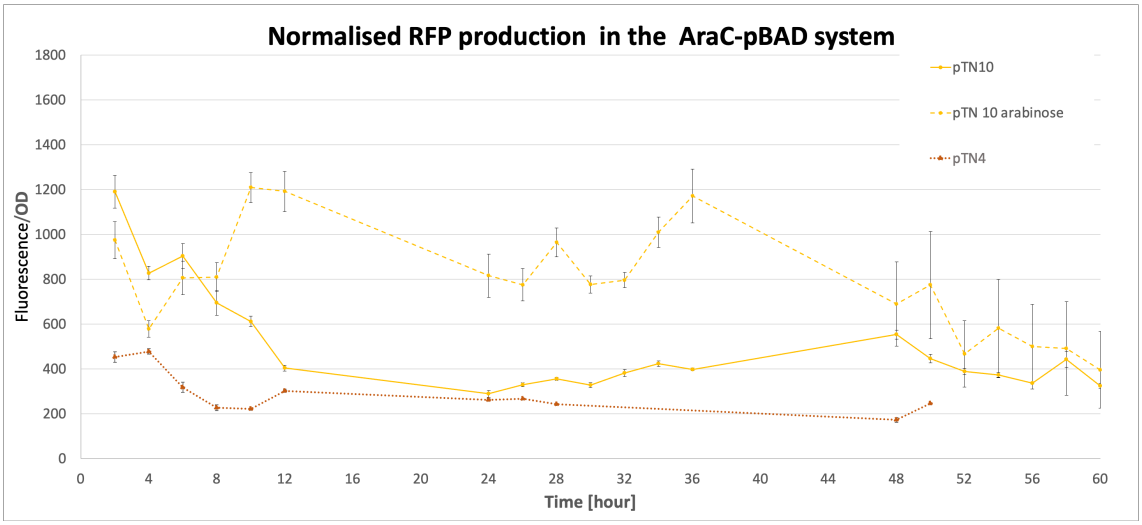


Figure 29: FI/OD measurements of the AraC-pBAD system, comparing pTN10 induced arabinose 13 mM, and uninduced conditions. Illustrating the promoter activity and responsiveness to induction over a 60 hour time period.

The RFP expression profiles for the AntR-Pant promoter system in *M. algicola* are shown in Figure 30. The graph compares strains substituted with 10 mM anthranilat inducer with uninduced conditions over time. In this strain, the uninduced pTN13 show higher values for the first 10 hours, where after 6 hours the production of RPF is greater than the degradation. After this, the induced strain surpasses the uninduced strain for the following 22 hours. Beyond hour 32, it is observed that both strains converge to base level of the pTN4 control.

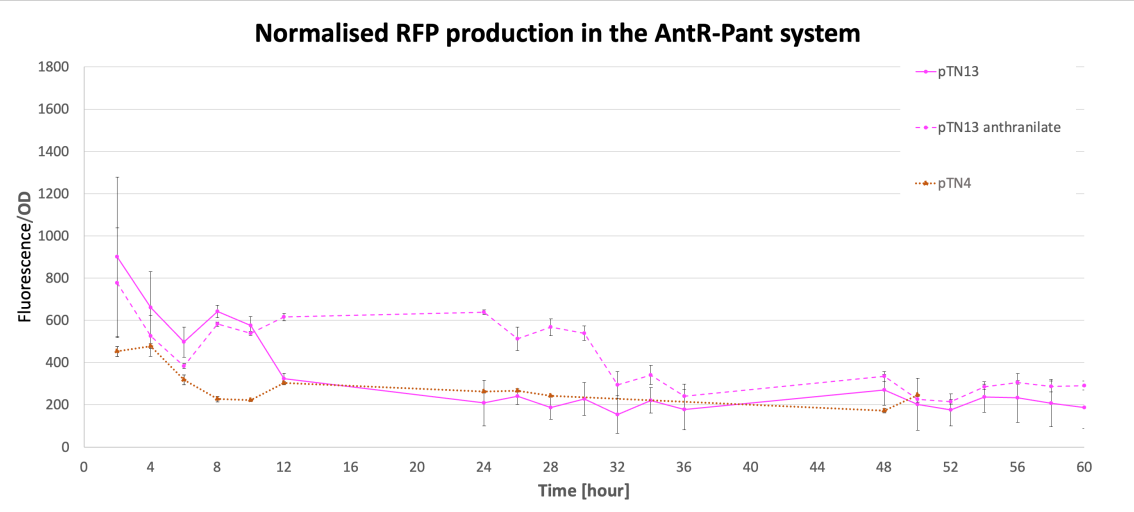


Figure 30: Normalised relative fluorescence intensity of the AntR-Pant system, comparing pTN8 induced anthranilat 10 mM, and uninduced conditions. Illustrating the promoter activity and responsiveness to induction over a 60 hour time period.

The relative fluorescence measurements of RFP expression in *M. algicola* strains containing the RhaSR-pRha promoter system, pTN14, both induced with rhamnose 10 mM and uninduced conditions, are represented in Figure 31.

Initially, over the first 6 hours, a noticeable decline in the relative fluorescence is observed for both conditions. Both strains remain relatively close to pTN4, fluctuating between 200 and 400 units, suggesting low to no RFP expression.

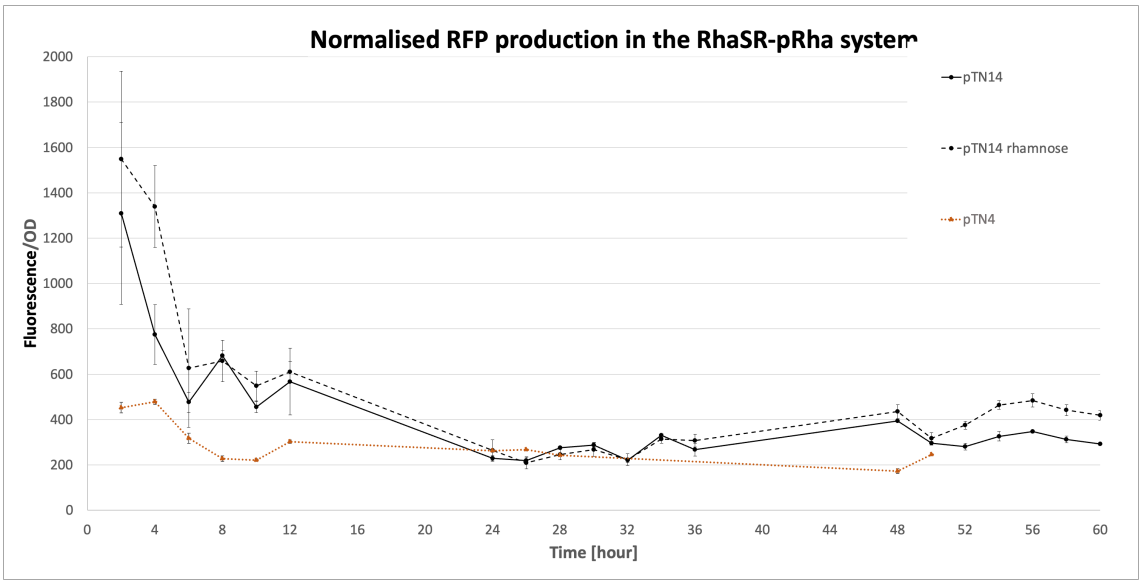


Figure 31: FI/OD measurements of the RhaSR pRha system, comparing pTN14 induced rhamnose 10 mM, and uninduced conditions. Illustrating the promoter activity and responsiveness to induction over a 60 hour time period.

The overall trend suggests that the RhaSR-pRha promoter system exhibits a weak response to the 10 mM rhamnose induction, with the pTN14 strains barely maintaining a higher FI/OD values compared to the pTN4 control strain.

Measurements of strains containing different XylS promoter system, specifically Pm wt, Pm ML1.17

and PmG5, in respectively pTN7, pTN9, and pTN11, are represented in Figure 32, 33, and 34. For plasmid pTN7, the induced condition with m-toluate shows higher initial normalised fluorescence intensity compared to the uninduced strain. There is an observed peak in RFP expression at hour 10. However, both conditions converge with fluctuations to lower levels after approximately 24 hours and the standard deviations increases.

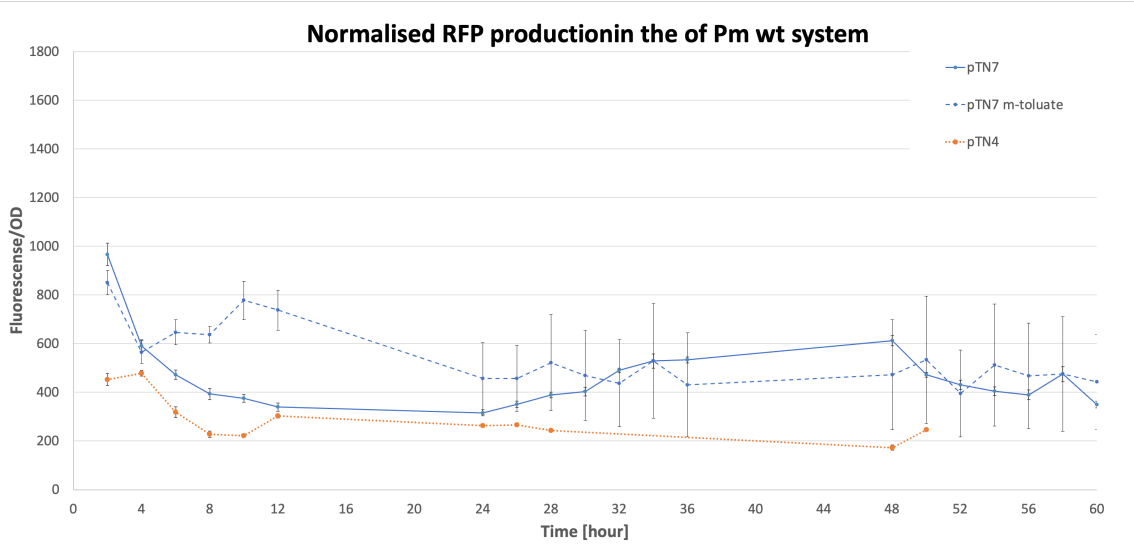


Figure 32: FI/OD measurements of the Pm wild type system, comparing pTN7 induced m-toluate 0.5 mM, and uninduced conditions. Illustrating the promoter activity and responsiveness to induction over a 60 hour time period

Plasmid pTN9, in Figure 33, demonstrates a similar pattern as pTN7, where the induced strain initially show higher relative fluorescence output than the uninduced strain and they both converge to hour 24. Notably, there is a peak in RPF expression at hour 10 for the induced strain of *M. algicola* containing pTN9 too. However, the pTN9 variation exhibits higher values for the first 12 hours compared to the pTN7 variation.

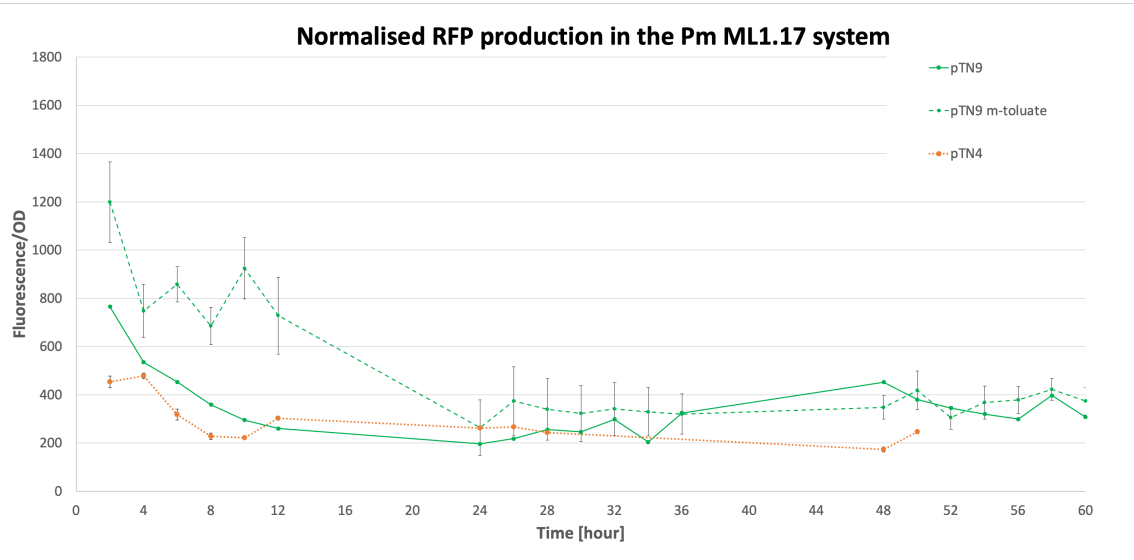


Figure 33: FI/OD measurements of the Pm ML1.17 system, comparing pTN9 induced m-toluate 0.5 mM, and uninduced conditions. Illustrating the promoter activity and responsiveness to induction over a 60 hour time period

M. algicola containing pTN11, shown Figure 34, seems to follow the same trends as the other Pm variation with a peak at hour 10. Conversley, both pTN7 and pTN9 exhibit their lowest values at 4 hours, whereas pTN11 shows its lowest value at 6 hours. This indicates that the PmG5 variant is a weaker promoter in *M. algicola*, taking longer for production to exceed degradation.

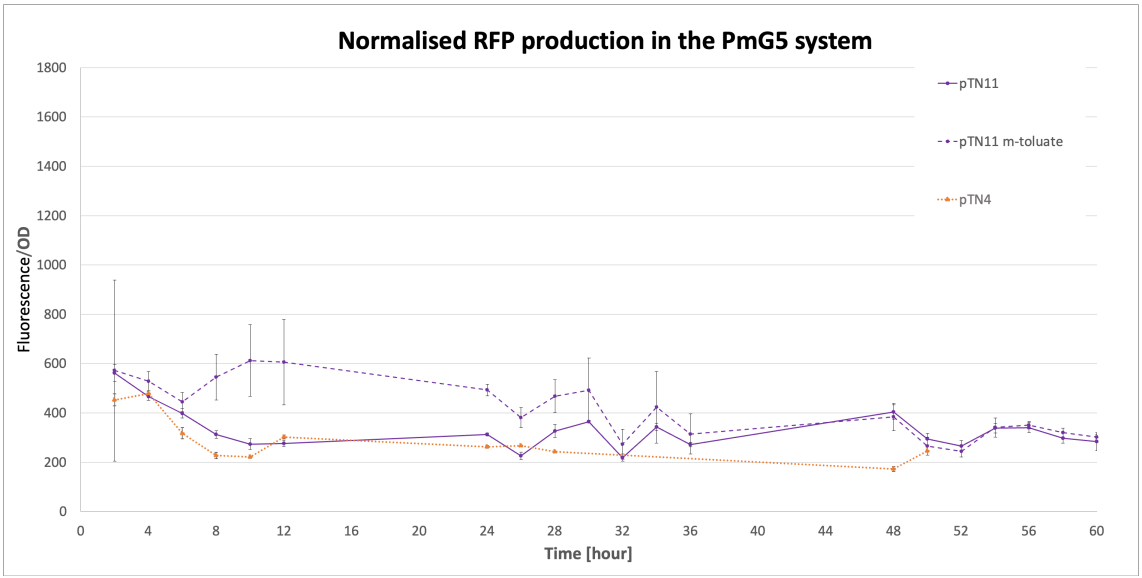


Figure 34: FI/OD measurements of the PmG5 system, comparing pTN11 induced m-toluate 0.5 mM, and uninduced conditions. Illustrating the promoter activity and responsiveness to induction over a 60 hour time period

The AraC-pBAD and Pm ML1.17 systems showed the highest and most sustained gene expression in *M. algicola*, indicating a potential for applications requiring stable and sustained protein production. The AntR-Pant, LacI-Ptrc , PmG5 , and Pm wt systems demonstrated more moderate expression levels. The RhaSR-pRha system showed minimal response to induction, suggesting limited utility for applications for strong and controlled gene expression.

4 Discussion

In the construction of genetic cassettes for the toolbox, it is essential to evaluate the various components. One aim of this thesis was to develop antibiotic resistance cassettes and assess their functionality in *M. algicola*. The other aim was to investigate the performance of different promoter systems in *M. algicola*. These results were then to be compared with studies conducted in other bacteria, providing insights into the responsiveness and ease of use of the constructed plasmids in the toolbox. The following sections will discuss each of these aims separately.

4.1 Evaluation of the antibiotic resistance cassettes

The primary objective of constructing plasmids from PCR-Blunt II-TOPO clones was to integrate antibiotic resistance genes that would serve as cassettes of selective markers in the toolbox. The construction of verified plasmids with various antibiotic resistance genes, Am^R, Km^R, Tc^R, and Sp^R, were successfully achieved. These cassettes and plasmids are now readily available for use in any gram-negative bacteria for testing their effectiveness in conferring resistance.

The plasmids were tested in *M. algicola*, and pTN3, pTN4, and pTN6, containing Tc^R, Am^R and Sp^R respectively, successfully conferred resistance to the respective antibiotics. This confirmed the effectiveness of these plasmids in providing *M. algicola* with the desired antibiotic resistance. However, the *M. algicola* strain with the pTN5 containing Km^R did not grow on LA Km plates, indicating that *M. algicola* does not express the kanamycin resistance gene effectively.

A potential reason for the lack of Km^R expressed in *M.algicola* might be a regulatory mechanism in *M.algicola* that prevent the expression of Km^R. The promoter driving Km^R might not be well recognized in *M. algicola*, or it could be subject to repression or silencing by specific regulatory proteins, RNA molecules, or genomic elements. Alternatively, improper folding of the Km^R protein could lead to an inability to express resistance. While the plasmid is taken up by the bacteria, the Km^R protein might not fold correctly, preventing it from functioning properly and conferring resistance. Another possibility is post-transcriptional regulation, where the Km^R mRNA is produced but gets rapidly degraded by mechanisms in *M. algicola*, preventing efficient translation into protein.

In response to the lack of Km tolerance observed in *M. algicola* with Km^R plasmids, the resistance gene was exchanged from kanamycin to apramycin in a series of plasmids. Hence, the efficiency of exchanging the selective markers was tested, confirming that the resistance markers can be easily replaced. This substitution exemplifies the flexibility of this genetic engineering strategy, showcasing the use of a versatile toolbox of traits designed to accommodate the characteristics of different microbial hosts. The successfully change of the selection marker resulted in the construction of pTN7-14. These plasmids were further used to test of different promoter systems and their strength in *M.algicola*.

4.2 Evaluation of different promoter systems

A set of 8 different promoters was tested in *M.algicola*, with all promoters/regulator cassettes inserted into the same backbone, pTN4. The consistent use of the same backbone allows for a systematic comparison of the different expression systems without confounding effects from different vectors, ensuring that any differences specifically are due to the promoter systems. This consistency helps identify bottlenecks in recombinant protein production and facilitates consistent evaluations of expression system efficiency[58].

As promoters are often optimised for specific host machinery, the different promoter systems are tested in various bacteria to observe and evaluate how they work for each bacteria. The Pm promoter might be more efficiently recognized by the RNA polymerase of for example *E. coli* compared to *P. fluorescens* or *M. algicola*. Different bacteria might vary in their ability to uptake and metabolise the synthetic inducers. Additionally, plasmid stability and copy number can vary

between species due to differences in replication systems and intracellular environments. High copy numbers or more stable plasmids higher expression levels may favour one species over another.

4.2.1 Impact of promoter system performance on bacterial growth

The production of the fluorescence protein could be a metabolic burden, which slows down the cell growth. As shown in Figure 35, the induced systems tends to have a lower growth rate to the uninduced strains. This difference in growth rates could suggest that the energy and resources required for the synthesis of RFP are redirected from those needed for cell growth.

Most of the induced strains show an increase in growth over time, eventually reaching values similar to their uninduced counterparts at 24 or 48 hours. This suggests that the majority of the induced plasmids do not hinder cell growth but rather delay it. However, there are two exceptions, where the Pm wt and AraC-pBAD systems still show significantly lower growth than their respective uninduced state even after 48 hours. Notably, the *M. algicola* strains containing the LacI-Ptrc and the RhaSR-pRha systems do not show significantly lower growth for the induced than the non-induced, indicating a low metabolic burden associated with these expression systems.

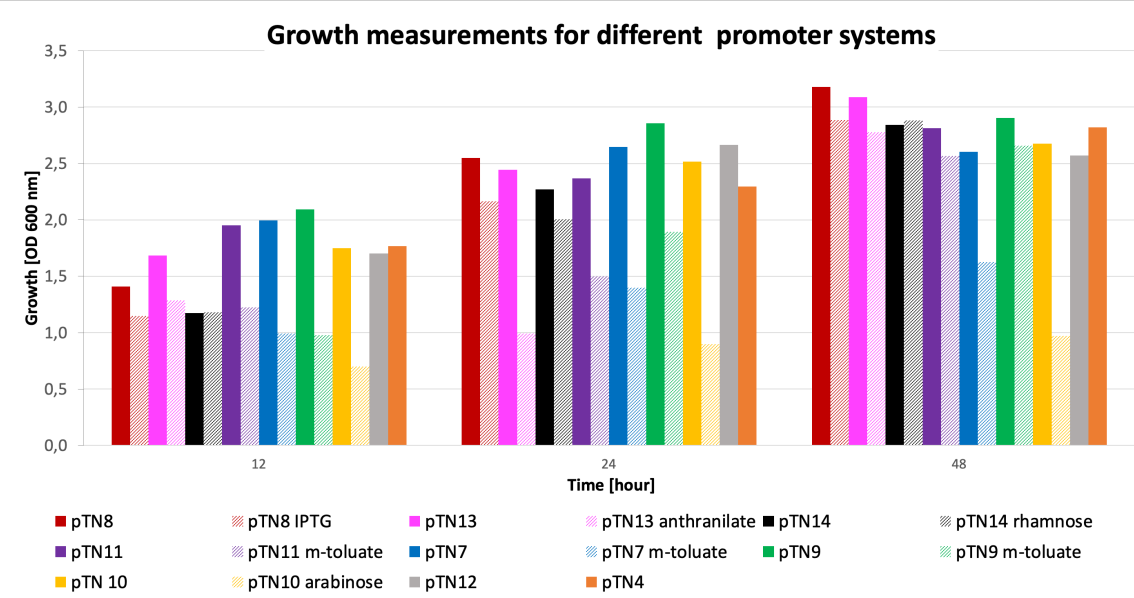


Figure 35: Optical Density measurements at 600 nm for *M. algicola* strains containing various plasmids. Measurements were taken at 12, 24, and 48 hours under both induced and non-induced conditions. The chart illustrates the growth dynamics, highlighting the differences in cell density between induced and non-induced strains.

From the barcharts in Figure 35 and 36, it is clear that most induced strains exhibit slower growth than their uninduced counterparts, suggesting a metabolic burden associated with the induction process. The FI/OD measurements show significantly higher values for the induced strains, with the differences becoming less pronounced by hour 48. Comparing the OD graphs with the FI/OD graphs, it is observed that generally the strains with the most effective induction exhibit the weakest growth. This correlation highlights the metabolic cost of gene expression in *M. algicola* under induced conditions. However, not all systems follow this pattern. For instance, the *M. algicola* strains containing the AntR-Pant (pTN13), pmG5 (pTN11), and pm ML1.17 (pTN9) systems show relatively high induced FI/OD values, indicating effective induction, yet their growth rates eventually catch up to uninduced strains by hour 48. This suggests that these systems, while imposing an initial metabolic burden that slows down growth, allow the induced strains to catch up as the uninduced strains reach the stationary phase. Consequently, most induced strains, requiring more time due to the metabolic load, eventually reach the same stationary plateau. Conversely, the Pm wt and AraC-pBAD system demonstrates significantly lower OD values for induced strains

at all time points, indicating a high and sustained metabolic burden.

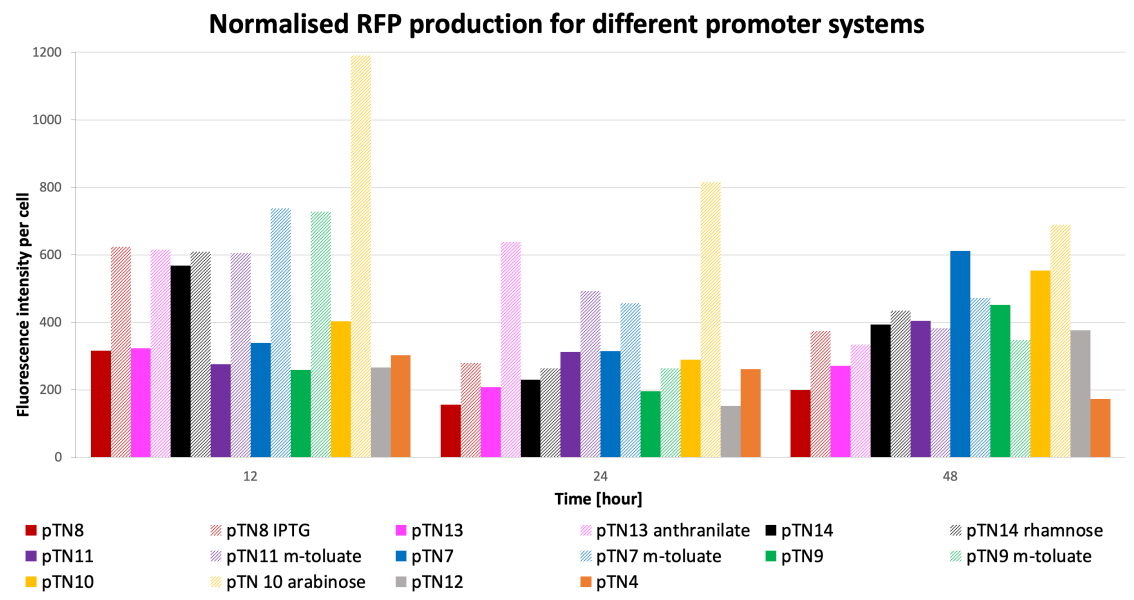


Figure 36: Fluorescence intensity normalised by per cell for *M. algicola* strains containing various plasmids. The chart illustrates the relative expression levels of RFP.

Notably, the growth restraint is higher compared to similar experiments conducted in *P. fluorescens*, as tested in previous master's thesis by Vold[2], Haaland[3], and Wesche[4]. The reasons for this are uncertain but might be due to the LB media used for *M. algicola* not being specifically optimised for this strain, potentially causing stress due to the media composition. Additionally, the potential negative effects of the inducers on *M. algicola* growth were not evaluated. Inducers, while necessary for activating gene expression for their respective promoter system, can themselves impose metabolic stress or toxicity on the bacteria. To assess these potential negative effects, it would be necessary to grow *M. algicola* wt with varying concentrations of each inducer to observe any inhibitory effects on growth in the absence of plasmids or alternatively using the control strain containing pTN4.

Understanding these trait for each promoter system in *M. algicola* is valuable for selecting an appropriate promoters, ensuring optimal gene expression under different experimental conditions, and helps identifying the most effective system for desired applications in the toolbox.

4.2.2 Comparison of the induced promoter systems

Measuring optical density and fluorescence intensity of each *M. algicola* strain containing plasmids with various promoter systems provides insights into the expression efficiency and strength of these promoters. OD measurement indicates cell growth, while FI measures the expression level of RFP through fluorescence. The ratio of FI to OD allows for normalisation of fluorescence to cell density, providing a more accurate assessment of promoter activity.

Comparing induced and non-induced samples reveals the responsiveness and regulation potential of the inducible promoters. Overall, the Pconstitutive system displays higher initial gene expression per cell, which significantly declines and then stabilizes at around 400 over time. Whereas, the control strain, pTN4, maintains consistently lower values, suggesting that the background fluorescence level for *M. algicola* at these conditions are around 200-250. Hence, the pTN4 is used as a reference to evaluate the strength of promoter systems.

The fluorescence per cell of all the induced systems, shown in Figure 36, compares the performance and characteristics of the different promoter systems in relation to each other. It is observed that

all the promoter systems in their induced form showed some level of RFP expression the first 12 hours compared to the control, pTN4. This indicates that the promoters were inducible, in different degree, and exhibited gene expression above the background levels of *M.algicola*.

All strains exhibit a high initial expression level, that sharply declines. This is most likely due to depletion of the expressed RFP from previous culture, before the dilution to OD = 0.1. It is important to note that low cell numbers in these initial measurements can lead to greater uncertainty in the measured values. All the systems reaches the point where production exceeds degradation of RFP between 4 and 6 hours. This timing indicates how effective the promoters are in quickly initiating gene expression, which is interesting for experiments requiring rapid protein production.

At the 24 hour measurement, the strains containing Ptrc-LacI, RhaSR-pRha, and Pm ML1.17 have fallen to the same level of RFP production as the control strain, pTN4, indicating that these systems lose their effectiveness over time. In contrast, strains with AntR-Pant, PmG5, Pm wt, and AraC-pBAD show higher levels of gene expression. Systems that decline to control levels may be useful in situations where transient expression is sufficient or preferred. In contrast, systems that maintain higher levels of expression are advantageous for continuous production needs.

These differences can be strategically used to select the suiting gene expression levels to the specific needs of a project. For example, selecting a variant with higher expression levels can be advantageous in applications requiring abundant protein production. Faster induction systems are beneficial when quick response times are essential, and precise control of gene expression is essential in experiments requiring tight regulation. By understanding and utilising the unique properties of each promoter system or variant, researchers can optimise gene expression systems for a variety of experimental or industrial applications.

4.2.3 Assessment and evaluation of promoter systems in *M. algicola* and *P. fluorescense*

When considering the promoter variants in Figure 36, it is clear that they differ in strength, which affect the transcription rates of the RFP gene, resulting in different levels of expression and induction dynamics in *M. algicola*. By comparing these systems in *M. algicola* and *P. fluorescens*, insights into species-specific differences in promoter strength, gene expression, and the efficiency of different promoter systems can be gained.

Comparing the Pm variations presented in Figure 37, reveals that the effect of induction, where expression exceeds degradation, occurs at hour 6. The Pm ML1.17 variation has the highest expression but also the largest drop in gene expression at hour 24. This suggests that although this variant is effective at quickly increasing gene expression, it may also be unstable over time, or there may be a saturation or depletion effect occurring. Pm wt demonstrates more stable induction, maintaining second highest values, whereas PmG5 shows the lowest FI/OD values. Thereafter, they all converge closely with their non-induced counterparts in a stationary phase, slightly above the background expression control in *M. algicola*. Isolating the Pm variations was done to enhance readability by preventing clutter from too many promoter systems, making the comparison more manageable and easier to follow.

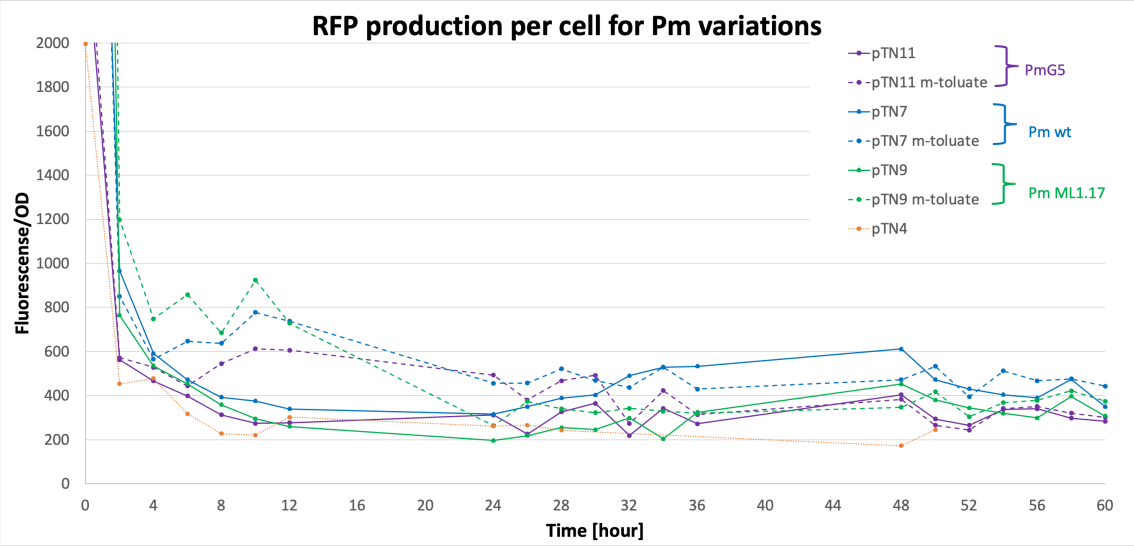


Figure 37: Comparison of the normalised fluorescence intensity per cell in the different Pm variations.

In a thesis conducted by Vold, the promoter strength of Pm variations Pm wt, PmG5 and Pm ML1.17 were tested in *Pseudomonas fluorescence* SBW25[2]. Her findings demonstrated that among the Pm variants, the Pm wt exhibited the highest expression levels. The Pm ML1.17 variant also maintained high expression levels, though slightly weaker than the wild-type. Contrary to expectations, the PmG5 variant, was found to be the weakest of the three, suggesting potential strain-dependent variability in promoter performance[2].

For comparison, in *M. algicola*, the induced Pm ML1.17 variation showed the highest expression levels, followed by the wild type. Both bacteria exhibited the lowest expression from the PmG5 variation. Additionally, the stationary phase was reached within 24 hours for all variations in both bacteria, indicating that the growth dynamics are similar. However, the efficiency and expression levels of the promoters varied between the bacterial species, and the promoter strengths were not consistent across species. This highlights the importance of testing promoter systems in different host strains to understand their performance and optimise their use in each specific applications.

For the use in conditional suicide plasmids to control the replication gene *trfA*, a system that provides reliable and controlled induction, allowing for precise regulation of gen expression, is desirable. Among the Pm systems tested, the ML1.17 variant in pTN9 would be recommended due to its high expression levels, and peak induction at hour 10. This variant makes the most suitable choice for to ensure that the *trfA* gene is sufficiently expressed to trigger the conditional suicide mechanism when needed. Further, as Wagle[1] is testing the dynamics of *trfA* expression, it would be beneficial to compare the promoter's effectiveness directly with the *trfA* gene.

The LacI-Ptrc system is repressed by the LacI protein until IPTG binds to LacI, releasing it from the operator and allowing transcription of the RFP gene. The AraC-pBAD system is repressed by AraC in the absence of arabinose, subsequently when arabinose binds to AraC, it activates the pBAD promoter, enhancing RFP transcription. The AntR-Pant system requires anthranilate to activate the AntR protein, which then promotes transcription from the Pant promoter, increasing RFP expression. The RhaSR-pRha system is regulated by the RhaS and RhaR proteins, which activate the pRha promoter in the presence of rhamnose, leading to increased transcription of the RFP gene. These expression systems differ in their regulatory mechanisms and response to inducers, and a comparison of the induced and uninduced systems are shown in Figure 38.

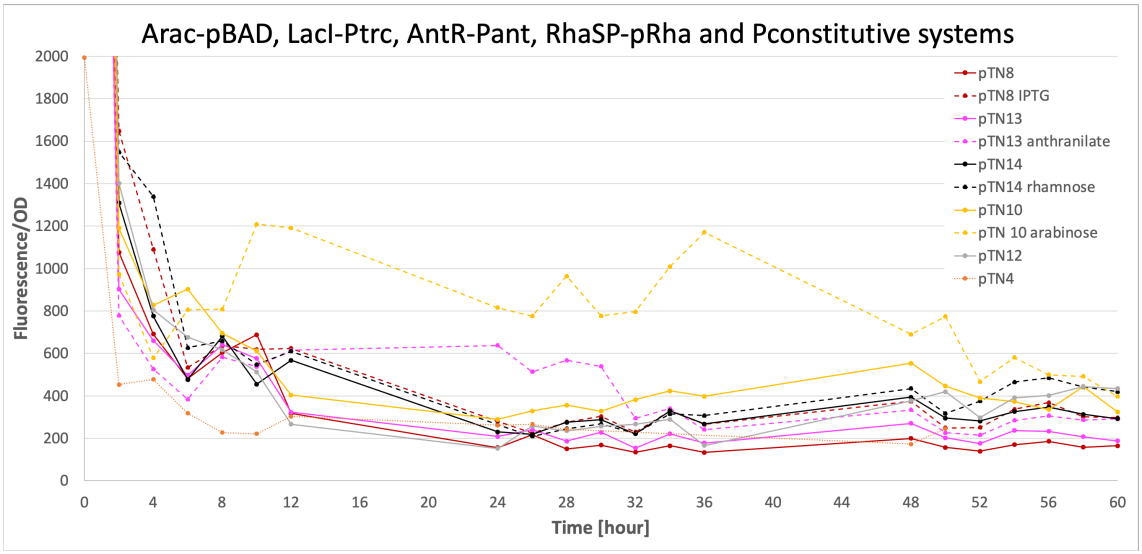


Figure 38: Normalised flourescense intensity per cell values of promoter systems compared over time. LacI-Ptrc (pTN8, red), AraC-pBAD (pTN10, yellow), AntR-Pant (pTN13, pink), and RhaSR-pRha (pTN14, black), constiutive (pTN12, grey) and control (pTN4, orange).

The AraC-pBAD system shows a significant response to induction and stabilises at a higher FI/OD ratio, indicating an effective promoter activity in *M. algicola*. In contrast, the AntR-Pant system, induced by anthranilate, exhibits more moderate induction and stabilises at a lower FI/OD ratio compared to AraC-pBAD. Despite this, the AntR-Pant system demonstrates a strong promoter activity compared to other systems tested in *M. algicola*.

The induced AntR-Pant system maintains a stationary gene expression, whereas the uninduced strain converges to background expression. This makes the AntR-Pant system in a tight promoter that can be used for a precises control of gene expression.

A previous study by Vold on the AraC-pBAD system in *P. fluorescens* revealed that both induced and uninduced conditions demonstrated similarly low fluorescence levels, suggesting that the pBAD system was not effectively functioning in *P. fluorescens*[2]. Conversely, in *M. algicola* the AraC-pBAD system showed clear RFP expression under induced conditions, indicating effective induction. These high levels of RFP expression remained relatively high for an extended period before eventually converging towards the background expression level. This suggests that the AraC-pBAD system in *M. algicola* can be effectively induced, maintaining high gene expression, and may have potential as a strong promoter.

The differences between *P. fluorescens* and *M. algicola* in response to the induction of the AraC-pBAD system underscores how cellular mechanisms work differently in various bacteria.*Marinobacter algicola*, as a marine bacteria, may have more efficient mechanisms for uptake of arabinose, or different regulatory pathways that enhance the functionality of the pBAD system, compared to *P. fluorescens*.This suggests that *M. algicola* can induce and maintain higher levels of gene expression using the AraC-pBAD system.

In Figure 36, it is observed that neither the induced LacI-Ptrc nor the RhaSR-pRha systems show a significant increase in RFP expression after the initial hours. Although these systems exhibit slightly higher fluorescence intensity, it is not pronounced. The low promoter activity could be due to weak promoters that inherently have a low activity in *M. algicola*. Factors such as pH, nutrient availability, and temperature affect the activity. However, since these conditions were identical for all tested systems, it is likely that the promoter strength is the limiting factor of lower RFP expression in *M. algicola*.

Vold tested the Ptrc-LacI promoter system in *P. fluorescens* SBW25, and found that the Ptrc-LacI promoter exhibited increased fluorescence of RFP when induced with IPTG, even when the cells had reached the stationary phase. The fluorescence of RFP under uninduced conditions was

low, around the control background level of *P. fluorescens*, indicating that the Ptrc-LacI promoter required induction to be functional in this host[2].

In this study, where the Ptrc-LacI system was tested in *M. algicola*, the induced strain initially showed significant increase in RFP expression, which eventually decreased to background levels. This trend contrasts with the induced system in *P. fluorescens*, which maintained increased expression for a longer period before declining. Additionally, the uninduced strain in *P. fluorescens* showed no gene expression, whereas in *M. algicola*, the uninduced strain also showed initial RFP production before stabilising at background level. This indicates that the Ptrc-LacI system functions as a tight promoter in *P. fluorescens*, which can be useful for precise control.

In Wesche's study on the RhaSR-pRha system in *P. fluorescens*, strains induced with rhamnose demonstrated lower growth rates and and increased fluorescence intensity compared to uninduced strains, highlighting effective promoter activity under induced conditions[4].

Contrary in this study, the RFP expression in *M. algicola* containing the RhaSR-pRha promoter system, under both induced and uninduced conditions, initially showed relatively high fluorescence expression levels. However, there was a sharp declin in relative fluorescense for both conditions, therafter the FI/OD values remained fluctuating at low expression level. This suggests that the RhaSR-pRha promoter exhibits a weak response to rhamnose induction, as the induced and uninduced strain maintains nearly the same FI/OD values. This weak response could be due to inefficient uptake of rhamnose in *M. algicola*, or the promoter may have low activity in this host. This indicates that the RhaSR-pRha system is not suitable for controlled gene expression in *M. algicola*, as it fails to distinguish between induced and uninduced states.

In Wesche's study of the AntR-Pant promoter system in *P. fluorescens*, early addition of anthranilate was found to inhibit growth, but showed strong gene expression, indicating an effective promoter activity[4]. In this study of the AntR-Pant systems in *M. algicola*, the uninduced strain initially showed higher fluorescence levels before the induced strain surpassed the it, maintaining significantly higher normalised fluorescence ratio for an extended time. Eventually the induced strain declined to low expression level similar to the uninduced counterpart.

Comparing these two experiments, the AntR-Pant promoter system appears to work more efficiently in *P. fluorescens*, where it achieves strong gene expression. In *M. algicola*, the promoter maintains gene expression over an extended period, indicating a more stable system. In *P. fluorescens*, the RFP expression declines after peaking, in contrast to *M. algicola*, where production reaches a stationary phase and remains elevated longer. This suggests that the AntR-Pant system may be more stabel in *M. algicola*. However, Wesche's experiment lasted 48 hours, and did not capture when RFP expression converged to background levels, but the system appeared to reach a stationary phase with relatively high gene expression, suggesting AntR-Pant is a strong promoter in *P. fluorescens*.

As the origins of AntR-Pant is native *P. fluorescens*, the regulatory elements are suited to its cellular environment and metabolic pathways, potentially making it favorable for *P. fluorescens* compared to *M. algicola*. However, as demonstrated in these comparisons, each system needs to be tested in the specific bacteria to determine which is best suited for the experiment's requirements. This underscores the value of these easily changeable cassettes in our genetic toolbox.

Other reasons for low expression to consider include the possibility that the concentration levels of inducers may have been too low to fully activate the promoters, resulting in lower increased expression. The inducers may also be degraded or metabolised too quickly, reducing their effective concentration. Since these promoter systems are not native to *M. algicola*, the cells might have difficulty taking up the inducer from the medium, resulting in less optimal intracellular concentrations and weaker gene expression.

For controlling the replication gene *trfA* in conditional suicide plasmids, a system that provides reliable and controlled induction for precise gene regulation is essential. The AraC-pBAD system, pTN10, appears to be the overall best choice for *trfA* expression in the conditional suicide plasmid. This system shows pronounced and the most stable induction, indicating it could be reliable for controlled gene expression. The response to arabinose inducer and the higher FI/OD ratio suggest

that it can provide the necessary expression levels to trigger the conditional suicide mechanism. Following the AraC-pBAD system, the ML1.17 variant in pTN9 is the second recommended option, due to its high expression levels and peak induction. This variant ensures that the *trfA* gene is sufficiently expressed to trigger the conditional suicide mechanism when needed. Additionally, as Wagle[1] is testing the dynamics of *trfA* expression, it would be beneficial to compare the promoter's effectiveness directly with the *trfA* gene.

5 Conclusion and further work

This thesis contributes to the broader objective of developing an accessible and versatile genetic toolbox that allows for controlled gene expression across a variety of bacterial species. The focus has been on constructing cassettes encoding different antibiotic resistance genes for the toolbox, enabling the selection and maintenance of plasmid-holding bacteria and ensuring compatibility with various species. The construction of plasmids pTN4, pTN5, and pTN6, conferring apramycin, kanamycin, and spectinomycin resistance respectively, was successful. These plasmids are derivatives of the previously engineered pTN3 plasmid, which confers tetracycline resistance.

The ease of changing cassettes was tested in the construction of pTN7-pTN14, where the Km^R gene were substituted with an Am^R gene in response to poor expression of Km^R shown in *M. algicola*. This confirmed the quick substitution utilising digestion enzymes, PstI and EcoRI, and ligation, demonstrating an effective process for customising the plasmids to adapt to the conditions of conjugating into *M. algicola*. This process leveraged the construction of vectors from the same backbone as those used in promoter-probe assays, and suggests that other antibiotic genes could also be used in this system.

Various promoter systems were tested in *M. algicola* to investigate their strength and efficacy in this species. The induced strains generally demonstrated a considerably higher normalised fluorescence ratio, with the exception of RhaSR pRha. Specifically, the AraC-pBAD promoter system in pTN10 was identified as most effective, showing the strongest gene expression and responsiveness to induction. The Pm wt and PmG5 system, pTN7 and pTN11 respectively, showed moderate promoter activity in induced strains. AntR-Pant in pTN13 exhibited moderate gene expression in induced strain and maintained a consistent stationary phase, identifying it as a potentially precise and well-regulated promoter in *M. algicola*. Promoters, LacI-Ptrc in pTN8, and RhaSR pRha in pTN14, showed low induction effects, indicating low efficiencies in *M. algicola* under the tested conditions.

The results of gene expression in *M.algicola* were compared with similar experiments done in *P. fluorescens* to assess the versatility of the promoter systems. The promoters performed well in *P. fluorescens* but did not exhibit the same efficiency in *M. algicola*, highlighting the importance of testing and optimising promoter systems for each specific bacterial host.

In further work one of the long-term goals is implementing homologous recombination, enabling the precise insertion of the genetic elements of the plasmids into the bacterial genome. This would aim to incorporate the desired plasmid genes into the host chromosome, providing a more stable and heritable sequence. By integrating the plasmid's genetic material into the genome, concerns about plasmid loss are eliminated, and control of expression by induction of these genes can be achieved. The inducible AraC-pBAD promoter system from this thesis could potentially control *trfA* expression, ensuring precise control of plasmid replication, while the AntR-Pant system is suitable for regulating toxic marker genes, balancing expression and host cell viability in *M. algicola*.

To gain more insight into which type of promoter systems are compatible with *M. algicola*, a thorough examination of various promoter systems would be beneficial. New systems could be tested and compared under different induction concentrations, times, and in specialised media to identify the a more effective promoter for use in *M.algicola*. Exploring multiple promoter systems will provide more options for controlling gene expression under specific conditions for *M. algicola*.

Additionally, testing the constructed plasmid systems across a broader range of bacterial strains is essential for understanding their characteristics and performance in diverse environments. This step will provide insights into the versatility and adaptability of the plasmids, ensuring that they can be effectively used in various strains and applications, ultimately achieving the goal of a versatile and flexible toolbox.

Further work on these areas will bring the development of our conditional suicide plasmids closer to achieving the goal of a flexible and versatile genetic toolbox.

Bibliography

[1] V. W. Wagle, 'In preparation, testing of promoter cassettes and the development of cassettes for regulation of replication with *trfA* in *Azotobacter chroococcum*', M.Tech thesis, Norwegian University of Science and Technology, 2024.

[2] S. Vold, 'Reporter genes for relative quantification of promoter strength and c-di-gmp in pseudomonas fluorescens', M.Tech thesis, Norwegian University of Science and Technology, 2021.

[3] H. Haaland, 'A study of c-di-gmp levels and alginate production in pseudonomas fluorescens sbw25', M.Tech thesis, Norwegian University of Science and Technology, 2022.

[4] A. F. Wesche, 'Karakterisering av promotorsystemene antr-pant og rhasr-prha for pseudomonas fluorescens og effekten av antrinilat på alginatproduksjon', M.Tech thesis, Norwegian University of Science and Technology, 2024.

[5] T. A. Nærby, 'To be and then to be gone developing generalized protocols for conditional suicide plasmids focusing on antibiotic resistance', *Department og Biotechnology, NTNU?*, 2023.

[6] H. Ertesvåg, 'Alginate-modifying enzymes: Biological roles and biotechnological uses', *Frontiers in Microbiology*, vol. 6, 2015, ISSN: 1664-302X. DOI: 10.3389/fmicb.2015.00523.

[7] S. H. Khan, 'Genome-editing technologies: concept, pros, and cons of various genome-editing techniques and bioethical concerns for clinical application', *Molecular Therapy - Nucleic Acids*, vol. 16, pp. 326–334, 2019, ISSN: 21622531. DOI: 10.1016/j.omtn.2019.02.027.

[8] A. Gawin, L. Tietze, O. A. Aarstad, F. L. Aachmann, T. Brautaset and H. Ertesvåg, 'Functional characterization of three *Azotobacter chroococcum* alginate-modifying enzymes related to the *Azotobacter vinelandii* textitAlgE mannuronan C-5-epimerase family', *Scientific Reports*, vol. 10, no. 1, p. 12 470, 2020, ISSN: 2045-2322. DOI: 10.1038/s41598-020-68789-3.

[9] J. H. Hu, K. M. Davis and D. R. Liu, 'Chemical biology approaches to genome editing: understanding, controlling, and delivering programmable nucleases', *Cell Chemical Biology*, vol. 23, no. 1, pp. 57–73, 2016, ISSN: 24519456. DOI: 10.1016/j.chembiol.2015.12.009.

[10] J. Van Der Oost and C. Patinios, 'The genome editing revolution', *Trends in Biotechnology*, vol. 41, no. 3, pp. 396–409, 2023, ISSN: 01677799. DOI: 10.1016/j.tibtech.2022.12.022.

[11] M. R. Green and J. Sambrook, 'A guide to cloning the products of polymerase chain reactions', *Cold Spring Harbor Protocols*, vol. 2021, no. 9, pdb.top101345, 2021, ISSN: 1940-3402, 1559-6095. DOI: 10.1101/pdb.top101345.

[12] X. Li, J. Jin, Z. Guo and L. Liu, 'Evolution of plasmid-construction', *International Journal of Biological Macromolecules*, vol. 209, pp. 1319–1326, 2022, ISSN: 01418130. DOI: 10.1016/j.ijbiomac.2022.04.094.

[13] S. Wright, 'Recombinant DNA Technology and Its Social Transformation, 1972-1982', *Osiris*, vol. 2, pp. 303–360, 1986, Publisher: [Saint Catherines Press, The University of Chicago Press, The History of Science Society], ISSN: 03697827, 19338287. DOI: 10.1086/368659.

[14] S. Marsin, A. Mathieu, T. Kortulewski, R. Guérois and J. P. Radicella, 'Unveiling novel recO distant orthologues involved in homologous recombination', *PLoS Genetics*, vol. 4, no. 8, I. Matic, Ed., e1000146, 2008, ISSN: 1553-7404. DOI: 10.1371/journal.pgen.1000146.

[15] G. R. Smith, 'Homologous recombination in procaryotes', *Microbiological Reviews*, vol. 52, no. 1, pp. 1–28, 1988, ISSN: 0146-0749. DOI: 10.1128/mr.52.1.1-28.1988.

[16] D. L. Court, J. A. Sawitzke and L. C. Thomason, 'Genetic engineering using homologous recombination', *Annual Review of Genetics*, vol. 36, no. 1, pp. 361–388, 2002, ISSN: 0066-4197, 1545-2948. DOI: 10.1146/annurev.genet.36.061102.093104.

[17] L. Krejci, V. Altmannova, M. Spirek and X. Zhao, 'Homologous recombination and its regulation', *Nucleic Acids Research*, vol. 40, no. 13, pp. 5795–5818, 2012, ISSN: 0305-1048, 1362-4962. DOI: 10.1093/nar/gks270.

[18] Ø. U. Holm, 'Karakterisering av mutanter i genene for sucroseporinene scry1 og scry2 fra *Azotobacter vinelandii*', M.Tech thesis, Norwegian University of Science and Technology, 2009.

[19] N. Savić and G. Schwank, 'Advances in therapeutic CRISPR/Cas9 genome editing', *Translational Research*, vol. 168, pp. 15–21, 2016, ISSN: 19315244. DOI: 10.1016/j.trsl.2015.09.008.

[20] C. Gager and A. L. Flores-Mireles, 'Blunted blades: New CRISPR-derived technologies to dissect microbial multi-drug resistance and biofilm formation', *mSphere*, vol. 9, no. 4, A. P. Mitchell, Ed., e00642–23, 2024, ISSN: 2379-5042. DOI: 10.1128/msphere.00642-23.

[21] K. Nishida and A. Kondo, 'CRISPR-derived genome editing technologies for metabolic engineering', *Metabolic Engineering*, vol. 63, pp. 141–147, 2021, ISSN: 10967176. DOI: 10.1016/j.ymben.2020.12.002.

[22] Y. G. Kim, J. Cha and S. Chandrasegaran, 'Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain.', en, *Proceedings of the National Academy of Sciences*, vol. 93, no. 3, pp. 1156–1160, 1996, ISSN: 0027-8424, 1091-6490. DOI: 10.1073/pnas.93.3.1156.

[23] T. Cathomen and J. Keith Joung, 'Zinc-finger nucleases: the next generation emerges', *Molecular Therapy*, vol. 16, no. 7, pp. 1200–1207, 2008, ISSN: 15250016. DOI: 10.1038/mt.2008.114.

[24] F. D. Urnov, E. J. Rebar, M. C. Holmes, H. S. Zhang and P. D. Gregory, 'Genome editing with engineered zinc finger nucleases', *Nature Reviews Genetics*, vol. 11, no. 9, pp. 636–646, 2010, ISSN: 1471-0056, 1471-0064. DOI: 10.1038/nrg2842.

[25] M. Bibikova, K. Beumer, J. K. Trautman and D. Carroll, 'Enhancing gene targeting with designed zinc finger nucleases', *Science*, vol. 300, no. 5620, pp. 764–764, 2003, ISSN: 0036-8075, 1095-9203. DOI: 10.1126/science.1079512.

[26] T. Li, S. Huang, W. Z. Jiang *et al.*, 'TAL nucleases (TALNs): Hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain', *Nucleic Acids Research*, vol. 39, no. 1, pp. 359–372, 2011, ISSN: 1362-4962, 0305-1048. DOI: 10.1093/nar/gkq704.

[27] F. R. Slater, M. J. Bailey, A. J. Tett and S. L. Turner, 'Progress towards understanding the fate of plasmids in bacterial communities: Fate of plasmids in bacterial communities', *FEMS Microbiology Ecology*, vol. 66, no. 1, pp. 3–13, 2008, ISSN: 01686496, 15746941. DOI: 10.1111/j.1574-6941.2008.00505.x.

[28] T. Imanaka and S. Aiba, 'A perspective on the application of genetic engineering: stability of recombinant plasmid', *Annals of the New York Academy of Sciences*, vol. 369, no. 1, pp. 1–14, 1981, ISSN: 0077-8923, 1749-6632. DOI: 10.1111/j.1749-6632.1981.tb14172.x.

[29] D. R. Gill, I. A. Pringle and S. C. Hyde, 'Progress and prospects: The design and production of plasmid vectors', en, *Gene Therapy*, vol. 16, no. 2, pp. 165–171, 2009, ISSN: 0969-7128, 1476-5462. DOI: 10.1038/gt.2008.183.

[30] O. Tolmachov, 'Designing plasmid vectors', in *Gene Therapy of Cancer*, W. Walther and U. S. Stein, Eds., vol. 542, Series Title: Methods in Molecular Biology™, Totowa, NJ: Humana Press, 2009, pp. 117–129, ISBN: 978-1-934115-85-5. DOI: 10.1007/978-1-59745-561-9_6.

[31] P. Karunakaran, D. T. Endresen, H. Ertesvåg, J. M. Blatny and S. Valla, 'A small derivative of the broad-host-range plasmid RK2 which can be switched from a replicating to a non-replicating state as a response to an externally added inducer', *FEMS Microbiology Letters*, vol. 180, no. 2, pp. 221–227, 1999, ISSN: 03781097, 15746968. DOI: 10.1111/j.1574-6968.1999.tb08799.x.

[32] K. Haugan, P. Karunakaran, A. Tøndervik and S. Valla, 'The host range of RK2 minimal replicon copy-up mutants is limited by species-specific differences in the maximum tolerable copy number', *Plasmid*, vol. 33, no. 1, pp. 27–39, 1995, ISSN: 0147619X. DOI: 10.1006/plas.1995.1004.

[33] C. Reading and M. Cole, 'Clavulanic acid: A beta-lactamase-inhibiting beta-lactam from *Streptomyces clavuligerus*', *Antimicrobial Agents and Chemotherapy*, vol. 11, no. 5, pp. 852–857, 1977, ISSN: 0066-4804, 1098-6596. DOI: 10.1128/AAC.11.5.852.

[34] D. M. Mills, V. Bajaj and C. A. Lee, 'A 40 kb chromosomal fragment encoding *Salmonella typhimurium* invasion genes is absent from the corresponding region of the *Escherichia coli* K-12 chromosome', *Molecular Microbiology*, vol. 15, no. 4, pp. 749–759, 2006, ISSN: 0950382X, 13652958. DOI: 10.1111/j.1365-2958.1995.tb02382.x.

[35] T. J. Schmidhauser and D. R. Helinski, 'Regions of broad-host-range plasmid RK2 involved in replication and stable maintenance in nine species of gram-negative bacteria', *Journal of Bacteriology*, vol. 164, no. 1, pp. 446–455, 1985, ISSN: 0021-9193, 1098-5530. DOI: 10.1128/jb.164.1.446-455.1985.

[36] K. Kolatka, S. Kubik, M. Rajewska and I. Konieczny, 'Replication and partitioning of the broad-host-range plasmid RK2', *Plasmid*, vol. 64, no. 3, pp. 119–134, 2010, ISSN: 0147619X. DOI: 10.1016/j.plasmid.2010.06.004.

[37] J. M. Blatny, T. Brautaset, H. C. Winther-Larsen, P. Karunakaran and S. Valla, 'Improved broad-host-range RK2 vectors sseful for high and low regulated gene expression levels in gram-negative bacteria', *Plasmid*, vol. 38, no. 1, pp. 35–51, 1997, ISSN: 0147619X. DOI: 10.1006/plas.1997.1294.

[38] J. Quandt and M. F. Hynes, 'Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria', *Gene*, vol. 127, no. 1, pp. 15–21, 1993, ISSN: 03781119. DOI: 10.1016/0378-1119(93)90611-6.

[39] T. Schweder, I. Schmidt, H. Herrmann, P. Neubauer, M. Hecker and K. Hofmann, 'An expression vector system providing plasmid stability and conditional suicide of plasmid-containing cells', *Applied Microbiology and Biotechnology*, vol. 38, no. 1, 1992, ISSN: 0175-7598, 1432-0614. DOI: 10.1007/BF00169425.

[40] K. Gerdes, P. B. Rasmussen and S. Molin, 'Unique type of plasmid maintenance function: Postsegregational killing of plasmid-free cells.', *Proceedings of the National Academy of Sciences*, vol. 83, no. 10, pp. 3116–3120, 1986, ISSN: 0027-8424, 1091-6490. DOI: 10.1073/pnas.83.10.3116.

[41] G. Jain and H. Ertesvåg, 'Improved site-specific mutagenesis in *Rhodococcus opacus* using a novel conditional suicide plasmid', *Applied Microbiology and Biotechnology*, vol. 106, no. 21, pp. 7129–7138, 2022, ISSN: 0175-7598, 1432-0614. DOI: 10.1007/s00253-022-12204-6.

[42] S. Mehrotra and V. Goyal, 'Agrobacterium-mediated alien gene transfer biofabricates designer plants', in *Genetically Modified Organisms in Food*, Elsevier, 2016, pp. 63–73, ISBN: 978-0-12-802259-7. DOI: 10.1016/B978-0-12-802259-7.00007-5.

[43] M. H. Saier and J. García-Lara, Eds., *The spirochetes: molecular and cellular biology* (JMMB symposium series v. 3). Horizon Scientific, 2001, OCLC: ocm47901873, ISBN: 978-1-898486-27-5.

[44] C. E. Vickers, S. F. Bydder, Y. Zhou and L. K. Nielsen, 'Dual gene expression cassette vectors with antibiotic selection markers for engineering in *Saccharomyces cerevisiae*', *Microbial Cell Factories*, vol. 12, no. 1, p. 96, 2013, ISSN: 1475-2859. DOI: 10.1186/1475-2859-12-96.

[45] F. R. Cianfanelli, O. Cunrath and D. Bumann, 'Efficient dual-negative selection for bacterial genome editing', en, *BMC Microbiology*, vol. 20, no. 1, p. 129, 2020, ISSN: 1471-2180. DOI: 10.1186/s12866-020-01819-2.

[46] R. Kundar and K. Gokarn, 'CRISPR-Cas System: A Tool to Eliminate Drug-Resistant Gram-Negative Bacteria', *Pharmaceuticals*, vol. 15, no. 12, p. 1498, 2022, ISSN: 1424-8247. DOI: 10.3390/ph15121498.

[47] M. S. Jurica and B. L. Stoddard, 'Homing endonucleases: Structure, function and evolution', *Cellular and Molecular Life Sciences (CMLS)*, vol. 55, no. 10, pp. 1304–1326, 1999, ISSN: 1420-682X, 1420-9071. DOI: 10.1007/s000180050372.

[48] Z. Chen, 'A highly sensitive selection method for directed evolution of homing endonucleases', *Nucleic Acids Research*, vol. 33, no. 18, e154–e154, 2005, ISSN: 0305-1048, 1362-4962. DOI: 10.1093/nar/gni148.

[49] J. Ferrando, O. Filluelo, D. R. Zeigler and P. Picart, 'Barriers to simultaneous multilocus integration in *Bacillus subtilis* tumble down: Development of a straightforward screening method for the colorimetric detection of one-step multiple gene insertion using the CRISPR-Cas9 system', *Microbial Cell Factories*, vol. 22, no. 1, p. 21, 2023, ISSN: 1475-2859. DOI: 10.1186/s12934-023-02032-2.

[50] M. Kolar, P. J. Punt, C. A. Van Den Hondel and H. Schwab, 'Transformation of penicillium chrysogenum using dominant selection markers and expression of an *Escherichia coli lacZ* fusion gene', en, *Gene*, vol. 62, no. 1, pp. 127–134, 1988, ISSN: 03781119. DOI: 10.1016/0378-1119(88)90586-0.

[51] A. Kalnins, K. Otto, U. Rüther and B. Müller-Hill, 'Sequence of the *lacZ* gene of *Escherichia coli*.', en, *The EMBO Journal*, vol. 2, no. 4, pp. 593–597, 1983, ISSN: 02614189. DOI: 10.1002/j.1460-2075.1983.tb01468.x.

[52] M. T. Martins, I. G. Rivera, D. L. Clark, M. H. Stewart, R. L. Wolfe and B. H. Olson, 'Distribution of *uidA* gene sequences in *Escherichia coli* isolates in water sources and comparison with the expression of beta-glucuronidase activity in 4-methylumbelliferyl-beta-D-glucuronide media', *Applied and Environmental Microbiology*, vol. 59, no. 7, pp. 2271–2276, 1993, ISSN: 0099-2240. DOI: 10.1128/aem.59.7.2271-2276.1993.

[53] A. Vacas, C. Sugden, Ó. Velasco-Rodriguez *et al.*, 'Construction of two mCherry plasmids (pXG-mCherry) for transgenic *Leishmania* : valuable tools for future molecular analysis', *Journal of Parasitology Research*, vol. 2017, pp. 1–11, 2017, ISSN: 2090-0023, 2090-0031. DOI: 10.1155/2017/1964531.

[54] D. L. Nelson and M. M. Cox, *Lehninger Biochemie* (Springer-Lehrbuch). Berlin, Heidelberg: Springer Berlin Heidelberg, 2001, ISBN: 978-3-662-08290-4.

[55] A. Gawin, S. Valla and T. Brautaset, 'The XylS/ *Pm* regulator/promoter system and its use in fundamental studies of bacterial gene expression, recombinant protein production and metabolic engineering', *Microbial Biotechnology*, vol. 10, no. 4, pp. 702–718, 2017, ISSN: 1751-7915, 1751-7915. DOI: 10.1111/1751-7915.12701.

[56] P. Meysman, J. Collado-Vides, E. Morett, R. Viola, K. Engelen and K. Laukens, 'Structural properties of prokaryotic promoter regions correlate with functional features', *PLoS ONE*, vol. 9, no. 2, D. Chatterji, Ed., e88717, 2014, ISSN: 1932-6203. DOI: 10.1371/journal.pone.0088717.

[57] M. B. Miller and B. L. Bassler, 'Quorum Sensing in Bacteria', *Annual Review of Microbiology*, vol. 55, no. 1, pp. 165–199, 2001, ISSN: 0066-4227, 1545-3251. DOI: 10.1146/annurev.micro.55.1.165.

[58] S. Balzer, V. Kucharova, J. Megerle, R. Lale, T. Brautaset and S. Valla, 'A comparative analysis of the properties of regulated promoter systems commonly used for recombinant gene expression in escherichia coli', *Microbial Cell Factories*, vol. 12, no. 1, p. 26, 2013, ISSN: 1475-2859. DOI: 10.1186/1475-2859-12-26.

[59] S.-T. Liang, M. Bipatnath, Y.-C. Xu *et al.*, 'Activities of constitutive promoters in Escherichia coli 1 1Edited by D. E. Draper', *Journal of Molecular Biology*, vol. 292, no. 1, pp. 19–37, 1999, ISSN: 00222836. DOI: 10.1006/jmbi.1999.3056.

[60] H. M. Tingstad, 'Use of homologous recombination to study alginate biosynthesis in *Azotobacter vinelandii*.', M.Tech thesis, Norwegian University of Science and Technology, 2021.

[61] M. T. Gallegos, S. Marqués and J. L. Ramos, 'Expression of the TOL plasmid xylS gene in Pseudomonas putida occurs from a alpha 70-dependent promoter or from alpha 70- and alpha 54-dependent tandem promoters according to the compound used for growth', *Journal of Bacteriology*, vol. 178, no. 8, pp. 2356–2361, 1996, ISSN: 0021-9193, 1098-5530. DOI: 10.1128/jb.178.8.2356-2361.1996.

[62] T. Brautaset, R. Lale and S. Valla, 'Positively regulated bacterial expression systems', *Microbial Biotechnology*, vol. 2, no. 1, pp. 15–30, 2009, ISSN: 1751-7915, 1751-7915. DOI: 10.1111/j.1751-7915.2008.00048.x.

[63] K. Bakkevig, H. Sletta, M. Gimmestad *et al.*, 'Role of the *Pseudomonas fluorescens* alginate lyase (AlgL) in clearing the periplasm of alginates not exported to the extracellular environment', *Journal of Bacteriology*, vol. 187, no. 24, pp. 8375–8384, 2005, ISSN: 0021-9193, 1098-5530. DOI: 10.1128/JB.187.24.8375-8384.2005.

[64] A. Khlebnikov and J. D. Keasling, 'Effect of *l acY* Expression on Homogeneity of Induction from the P $_{t\ ac}$ and P $_{trc}$ Promoters by Natural and Synthetic Inducers', *Biotechnology Progress*, vol. 18, no. 3, pp. 672–674, 2002, ISSN: 8756-7938, 1520-6033. DOI: 10.1021/bp010141k.

[65] A. Timmes, M. Rodgers and R. Schleif, 'Biochemical and physiological properties of the DNA binding domain of AraC protein', *Journal of Molecular Biology*, vol. 340, no. 4, pp. 731–738, 2004, ISSN: 00222836. DOI: 10.1016/j.jmb.2004.05.018.

[66] D. M. Retallack, T. C. Thomas, Y. Shao *et al.*, 'Identification of anthranilate and benzoate metabolic operons of pseudomonas fluorescens and functional characterization of their promoter regions', *Microbial Cell Factories*, vol. 5, no. 1, 2006, ISSN: 1475-2859. DOI: 10.1186/1475-2859-5-1.

[67] Y. Choi, H.-Y. Park, S. J. Park *et al.*, 'Growth phase-differential quorum sensing regulation of anthranilate metabolism in *Pseudomonas* aeruginosa', *Molecules and Cells*, vol. 32, no. 1, pp. 57–66, 2011, ISSN: 10168478. DOI: 10.1007/s10059-011-2322-6.

[68] S. M. Egan and R. F. Schleif, 'A Regulatory Cascade in the Induction of rhaBAD', *Journal of Molecular Biology*, vol. 234, no. 1, pp. 87–98, 1993, ISSN: 00222836. DOI: 10.1006/jmbi.1993.1565.

[69] S. M. Egan and R. F. Schleif, 'DNA-dependent renaturation of an insoluble DNA binding protein', *Journal of Molecular Biology*, vol. 243, no. 5, pp. 821–829, 1994, ISSN: 00222836. DOI: 10.1006/jmbi.1994.1684.

[70] J. M. Park, P. Vinuselvi and S. K. Lee, 'The mechanism of sugar-mediated catabolite repression of the propionate catabolic genes in Escherichia coli', *Gene*, vol. 504, no. 1, pp. 116–121, 2012, ISSN: 03781119. DOI: 10.1016/j.gene.2012.04.074.

[71] I. Serganova and R. G. Blasberg, 'Molecular Imaging with Reporter Genes: Has Its Promise Been Delivered?', *Journal of Nuclear Medicine*, vol. 60, no. 12, pp. 1665–1681, 2019, ISSN: 0161-5505, 2159-662X. DOI: 10.2967/jnumed.118.220004.

[72] K. D. Piatkevich and V. V. Verkhusha, 'Guide to red fluorescent proteins and biosensors for flow cytometry', in *Methods in Cell Biology*, vol. 102, Elsevier, 2011, pp. 431–461, ISBN: 978-0-12-374912-3. DOI: 10.1016/B978-0-12-374912-3.00017-1.

[73] E. Hebisch, J. Knebel, J. Landsberg, E. Frey and M. Leisner, 'High variation of fluorescence protein maturation times in closely related escherichia coli strains', *PLoS ONE*, vol. 8, no. 10, A. Hofmann, Ed., e75991, 2013, ISSN: 1932-6203. DOI: 10.1371/journal.pone.0075991.

[74] E. M. Ransom, C. D. Ellermeier and D. S. Weiss, 'Use of mCherry red fluorescent protein for studies of protein localization and gene expression in clostridium difficile', *Applied and Environmental Microbiology*, vol. 81, no. 5, M. Kivisaar, Ed., pp. 1652–1660, 2015, ISSN: 0099-2240, 1098-5336. DOI: 10.1128/AEM.03446-14.

[75] N. C. Shaner, R. E. Campbell, P. A. Steinbach, B. N. G. Giepmans, A. E. Palmer and R. Y. Tsien, 'Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein', *Nature Biotechnology*, vol. 22, no. 12, pp. 1567–1572, 2004, ISSN: 1087-0156, 1546-1696. DOI: 10.1038/nbt1037.

[76] E. M. Merzlyak, J. Goedhart, D. Shcherbo *et al.*, 'Bright monomeric red fluorescent protein with an extended fluorescence lifetime', *Nature Methods*, vol. 4, no. 7, pp. 555–557, 2007, ISSN: 1548-7091, 1548-7105. DOI: 10.1038/nmeth1062.

[77] Y. Lee, V. Weerawongwiwat, J.-H. Kim *et al.*, '*Marinobacter arenosus sp.* nov., a halotolerant bacterium isolated from a tidal flat', *Archives of Microbiology*, vol. 204, no. 2, p. 155, 2022, ISSN: 0302-8933, 1432-072X. DOI: 10.1007/s00203-021-02696-7.

[78] D. H. Green, J. P. Bowman, E. A. Smith, T. Gutierrez and C. J. S. Bolch, 'Marinobacter algicola sp. nov., isolated from laboratory cultures of paralytic shellfish toxin-producing dinoflagellates', *International Journal of Systematic and Evolutionary Microbiology*, vol. 56, no. 3, pp. 523–527, 2006, ISSN: 1466-5026, 1466-5034. DOI: 10.1099/ijs.0.63447-0.

[79] S. Rughöft, N. Jehmlich, T. Gutierrez and S. Kleindienst, 'Comparative proteomics of Marinobacter sp. TT1 reveals corexit impacts on hydrocarbon metabolism, chemotactic motility, and biofilm formation', *Microorganisms*, vol. 9, no. 1, p. 3, 2020, ISSN: 2076-2607. DOI: 10.3390/microorganisms9010003.

[80] K. H. Instefjord, 'Marinobacter algicola - en ny alginatprodusent?', M.Tech thesis, Norwegian University of Science and Technology, 2013.

[81] R. Simon, U. Priefer and A. Pühler, 'A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria', *Bio/Technology*, vol. 1, no. 9, pp. 784–791, 1983, ISSN: 0733-222X. DOI: 10.1038/nbt1183-784.

[82] D. Hanahan, 'Studies on transformation of Escherichia coli with plasmids', *Journal of Molecular Biology*, vol. 166, no. 4, pp. 557–580, 1983, ISSN: 00222836. DOI: 10.1016/S0022-2836(83)80284-8.

[83] A. Harms, M. Liesch, J. Körner, M. Québatte, P. Engel and C. Dehio, 'A bacterial toxin-antitoxin module is the origin of inter-bacterial and inter-kingdom effectors of Bartonella', *PLOS Genetics*, vol. 13, no. 10, P. H. Viollier, Ed., e1007077, 2017, ISSN: 1553-7404. DOI: 10.1371/journal.pgen.1007077.

[84] *Zero blunt topo® pcr cloning kit*, Invitrogen, 2012.

[85] D. Whaley, K. Damyar, R. P. Witek, A. Mendoza, M. Alexander and J. R. Lakey, 'Cryopreservation: An overview of principles and cell-specific considerations', *Cell Transplantation*, vol. 30, p. 096 368 972 199 961, 2021, ISSN: 0963-6897, 1555-3892. DOI: 10.1177/0963689721999617.

[86] S. Panja, S. Saha, B. Jana and T. Basu, 'Role of membrane potential on artificial transformation of E. coli with plasmid DNA', *Journal of Biotechnology*, vol. 127, no. 1, pp. 14–20, 2006, ISSN: 01681656. DOI: 10.1016/j.jbiotec.2006.06.008.

[87] X. Tang, Y. Nakata, H.-O. Li *et al.*, 'The optimization of preparations of competent cells for transformation of *E.coli*', *Nucleic Acids Research*, vol. 22, no. 14, pp. 2857–2858, 1994, ISSN: 0305-1048, 1362-4962. DOI: 10.1093/nar/22.14.2857.

[88] A. Y. Chang, V. W. Chau, J. A. Landas and Y. Pang, 'Preparation of calcium competent escherichia coli and heat-shock transformation', *Journal of Emerging Investigators Methods*, vol. 1, pp. 22–25, 2017, Undergraduate Methods Paper.

[89] I. Liu, M. Liu and K. Shergill, 'The effect of spheroplast formation on the transformation efficiency in escherichia coli dh5', *Journal of Experimental Microbiology and Immunology*, vol. 9, pp. 81–85, 2006.

[90] I. M. Van Die, H. E. N. Bergmans and W. P. M. Hoekstra, 'Transformation in Escherichia coli: studies on the role of the heat shock in induction of competence', *Microbiology*, vol. 129, no. 3, pp. 663–670, 1983, ISSN: 1350-0872, 1465-2080. DOI: 10.1099/00221287-129-3-663.

[91] E. Lanka and B. M. Wilkins, 'DNA prosessing reactions in bacterial conjugation', *Annual Review of Biochemistry*, vol. 64, no. 1, pp. 141–169, 1995, ISSN: 0066-4154, 1545-4509. DOI: 10.1146/annurev.bi.64.070195.001041.

[92] M. Llosa, F. X. Gomis-Rüth, M. Coll and F. D. L. Cruz, 'Bacterial conjugation: A two-step mechanism for DNA transport', *Molecular Microbiology*, vol. 45, no. 1, pp. 1–8, 2002, ISSN: 0950-382X, 1365-2958. DOI: 10.1046/j.1365-2958.2002.03014.x.

[93] M. Llosa and F. De La Cruz, 'Bacterial conjugation: A potential tool for genomic engineering', *Research in Microbiology*, vol. 156, no. 1, pp. 1–6, 2005, ISSN: 09232508. DOI: 10.1016/j.resmic.2004.07.008.

[94] H. Birnboim and J. Doly, 'A rapid alkaline extraction procedure for screening recombinant plasmid DNA', *Nucleic Acids Research*, vol. 7, no. 6, pp. 1513–1523, 1979, ISSN: 1362-4962, 0305-1048. DOI: 10.1093/nar/7.6.1513.

[95] S. Ehrt and D. Schnappinger, 'Isolation of plasmids from E. coli by alkaline lysis', in *E. coli Plasmid Vectors*, vol. 235, New Jersey: Humana Press, 2003, pp. 75–78, ISBN: 978-1-59259-409-2. DOI: 10.1385/1-59259-409-3:75.

[96] R. J. Reece, *Analysis of genes and genomes*. Chichester, West Sussex, England ; Hoboken, NJ: John Wiley & Sons, 2004, ISBN: 978-0-470-84380-2.

[97] M. Marko, R. Chipperfield and H. Birnboim, 'A procedure for the large-scale isolation of highly purified plasmid DNA using alkaline extraction and binding to glass powder', *Analytical Biochemistry*, vol. 121, no. 2, pp. 382–387, 1982, ISSN: 00032697. DOI: 10.1016/0003-2697(82)90497-3.

[98] *Purelink™ hipure plasmid dna purification kits user guide*, Available from: http://www.thermofisher.com, Thermo Fisher Scientific, Waltham, MA, USA, 2023.

[99] Thermo Fisher Scientific, *Rna and dna quantification brochure*, Accessed: 05-March-2024, 2021. [Online]. Available: https://assets.thermofisher.com/TFS-Assets/BID/brochures/rna-and-dna-quantification-brochure.pdf.

[100] A. Pingoud, 'Structure and function of type II restriction endonucleases', *Nucleic Acids Research*, vol. 29, no. 18, pp. 3705–3727, 2001, ISSN: 13624962. DOI: 10.1093/nar/29.18.3705.

[101] Benchling. 'Biology Software'. Retrieved from https://www.benchling.com. (2024), [Online]. Available: https://www.benchling.com/.

[102] P. Y. Lee, J. Costumbrado, C.-Y. Hsu and Y. H. Kim, 'Agarose gel electrophoresis for the separation of DNA fragments', eng, *Journal of Visualized Experiments: JoVE*, no. 62, 2012, ISSN: 1940-087X. DOI: 10.3791/3923.

[103] D. P. Clark, N. J. Pazdernik and M. R. McGehee, *Molecular biology* (Academic cell), eng, Third edition. London San Diego Cambridge Oxford: Academic Press, an imprint of Elsevier, 2019.

[104] *Monarch pcr and dna cleanup kit protocol*, New England BioLabs.

[105] K. Shi, T. E. Bohl, J. Park *et al.*, 'T4 DNA ligase structure reveals a prototypical ATP-dependent ligase with a unique mode of sliding clamp interaction', *Nucleic Acids Research*, vol. 46, no. 19, pp. 10 474–10 488, 2018, ISSN: 0305-1048, 1362-4962. DOI: 10.1093/nar/gky776.

[106] W. Gaastra and K. Hansen, 'Ligation of DNA with T4 DNA ligase', in *Nucleic Acids*, vol. 2, New Jersey: Humana Press, 1984, pp. 225–230, ISBN: 978-0-89603-064-0. DOI: 10.1385/0-89603-064-4:225.

[107] B. Patel, 'Simple, fast, and efficient cloning of PCR products with TOPO® cloning vectors', *BioTechniques*, vol. 46, no. 7, p. 559, 2009, ISSN: 0736-6205, 1940-9818. DOI: 10.2144/000113200.

[108] J. J. Champoux, 'DNA Topoisomerases: Structure, Function, and Mechanism', *Annual Review of Biochemistry*, vol. 70, no. 1, pp. 369–413, 2001, ISSN: 0066-4154, 1545-4509. DOI: 10.1146/annurev.biochem.70.1.369.

[109] S. Shuman, 'Recombination mediated by vaccinia virus DNA topoisomerase I in Escherichia coli is sequence specific.', *Proceedings of the National Academy of Sciences*, vol. 88, no. 22, pp. 10 104–10 108, 1991, ISSN: 0027-8424, 1091-6490. DOI: 10.1073/pnas.88.22.10104.

[110] S. Shuman, 'Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA topoisomerase.', *Journal of Biological Chemistry*, vol. 269, no. 51, pp. 32 678–32 684, 1994, ISSN: 00219258. DOI: 10.1016/S0021-9258(18)31688-0.

[111] Thermo Fisher Scientific, *Figure from zero blunt™ topo™ pcr cloning kit, with pcr™-blunt ii-topo™ vector, one shot™ top10 chemically competent e. coli, and purelink™ quick plasmid miniprep kit*, Catalog No. K280002, 2023.

[112] K. Mullis, 'Principles of the polymerase chain reaction', *Journal of Molecular Biology*, vol. 5(2), pp. 123–135, 1990.

[113] National Human Genome Research Institute. 'Polymerase chain reaction fact sheet'. Courtesy: National Human Genome Research Institute. (2023), [Online]. Available: https://www.genome.gov/about-genomics/fact-sheets/Polymerase-Chain-Reaction-Fact-Sheet.

[114] M. Smith, *Polymerase chain reaction*, National Human Genome Research Institute, Accessed: 04-March-2024, 2024. [Online]. Available: https://www.genome.gov/genetics-glossary/Polymerase-Chain-Reaction.

[115] F. Sanger, S. Nicklen and A. R. Coulson, 'DNA sequencing with chain-terminating inhibitors', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 74, no. 12, pp. 5463–5467, 1977, ISSN: 0027-8424. DOI: 10.1073/pnas.74.12.5463.

[116] M. L. Metzker, 'Emerging technologies in DNA sequencing', *Genome Research*, vol. 15, no. 12, pp. 1767–1776, 2005, ISSN: 1088-9051. DOI: 10.1101/gr.3770505.

[117] 'Sanger sequencing'. Accessed: 05-February-2024. (2024), [Online]. Available: https://eurofinsgenomics.eu/en/eurofinsgenomics/material-and-methods/sanger-sequencing/.

[118] S. Bruder, E. J. Moldenhauer, R. D. Lemke, R. Ledesma-Amaro and J. Kabisch, 'Drop-in biofuel production using fatty acid photodecarboxylase from Chlorella variabilis in the oleaginous yeast Yarrowia lipolytica', *Biotechnology for Biofuels*, vol. 12, no. 1, p. 202, 2019, ISSN: 1754-6834. DOI: 10.1186/s13068-019-1542-4.

[119] J. Hennemann, R. S. Iwasaki, T. N. Grund, R. P. Diensthuber, F. Richter and A. Möglich, 'Optogenetic Control by Pulsed Illumination', en, *ChemBioChem*, vol. 19, no. 12, pp. 1296–1304, 2018, ISSN: 1439-4227, 1439-7633. DOI: 10.1002/cbic.201800030.

[120] A. G. Allen, K. Barthelson and M. Lardelli, 'pHAPE: A plasmid for production of DNA size marker ladders for gel electrophoresis', *Biology Methods and Protocols*, vol. 8, 2023, ISSN: 2396-8923. DOI: 10.1093/biomethods/bpad015.

Appendix

A Solutions and medium

Agarose solution
8 g/l LE agarose
TAE buffer
50 μl GelGreen
TAE buffer was added to the LE agarose and heated in the microwave until dissolved. Then GelGreen was added, and the solution was stored at 60°C.

Ligation mix
14 μl insert DNA
3 μl vector DNA
2 μl 10x ligase buffer (T4 ligase)
1 μl T4 ligase
Ligate from 3 h to ON at 16°C.

Luria Agar (LA)
5 g/l NaCl
5 g/l Yeast extract
10 g/l Tryptone
15 g/l bacteriological agar
Autoclaved at 121°C for 20 min
Antibiotics added post-autoclaving

Luria Broth (LB)
5 g/l NaCl
5 g/l Yeast extract
10 g/l Tryptone
Autoclaved at 121°C for 20 min.

PCR reaction mixture
10 μl 5x NEB Q5 Reaction Buffer
1 μl dNTP mix (10 mM)
2.5 μl Forward Primer (10 μM)
2.5 μl Reverse Primer (10 μM)
1 μl Template DNA
10 μl 5X Q5 High GC Enhancer
0.5 μl NEB Q5 High-fidelity DNA polymerase
22.5 μl Autoclaved RO-water
NEB Q5 High-fidelity DNA polymerase is added lastly, just before strating the reaction. Primers used in this project are presented in Table 9.

Psi Medium
0.5% yeast extract (5 g/l)
2% tryptone (20 g/l)
0.5% MgSO4 (5 g/l)
pH was adjusted to 7.6 with 1 M KOH, and solution were autoclaved at 121°C for 20 min.

Restriction cut mix:
100 - 200 ng DNA
2 μl buffer μl
0.5 enzyme
Water added until solution is 19.5 μl
Incubate at 37°C for 1 h or ON.

Super Optimal Broth (SOC)
20 g/l Tryptone
5 g/l Yeast extract
0.5 g/l NaCl
2.5 mM KCl
3.6 g/l Glucose
5.08 g MgCl2
The SOC media was sterilized by filtration, and stored in 1.5 ml tubes at -20°C ready for use.

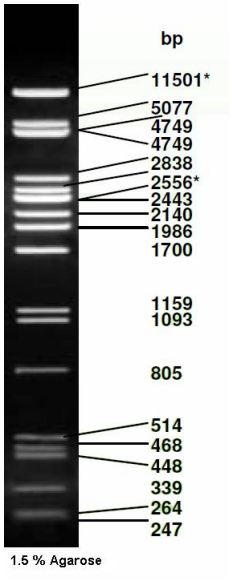
Standard ladder:
16.7 μl λ-DNA
10 μl buffer
70 μl RO-water
2 μl enzyme (PstI or HindIII)
Incubate at 37°C for 1 h or ON.

Transformation Buffer 1 (TFB1)
0.588 g potassium acetate
2.42 g rubidium chloride
0.294 g calcium chloride
2.0 g manganese(II) chloride
30 ml glycerol
The pH adjusted to 5.8 with diluted acetic acid (10%)
The solution sterilized by filtration

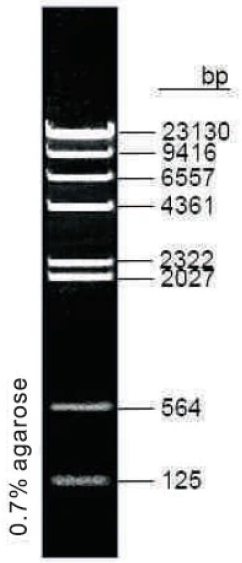
Transformation Buffer 2 (TFB2)
0.21 g MOPS
1.1 g Calcium chloride
0.121 g Rubidium chloride
15 ml Glycerol
The pH adjusted to 6.5 with diluted NaOH
The solution sterilized by filtration

B λDNA standard ladders

In this thesis, two different λ DNA ladders are utilized as reference markers to accurately determine DNA fragment sizes in gel electrophoresis. These ladders are derived from λ phage, a virus that infects bacteria. The λ DNA ladder is produced by digesting lambda phage DNA with specific restriction enzymes, such as PstI and HindIII, which were used in this project. These enzymes, as illustrated in Figure 39, cut the DNA at specific recognition sites. The resulting fragments of different sizes, serves as markers to determine the size of unknown DNA fragments during gel electrophoresis[120].



(a) Gel electrophoresis with λ PstI DNA standard.



(b) Gel electrophoresis with λ HindIII DNA standard.

Figure 39: λ DNA ladders, HindIII and PstI, were used as reference markers in gel electrophoresis. Composition and method is listed in Appendix A

C Genetic materials

Primer	Sequence (5' to 3')
TcSbf	AGT GGG CAG ATC TTC GAA TG
TcSgrDI	TAA GAT CGT CGC TAC ATG GCT CTG CTG TAG TG
KmSbf	CTT CCT GCA GGG AAA GCC ACG TTG TGT CTC
KmSgrDI	TAA CAT CGT CGA CGC CCG TCA AGT CAG CGT AAT G
AmNsiSbf	ATG CAT CCT GCA GGT TGC AAC AGT GCC GTT GAT CGT G
AmSgrDI	CGT CGA CGT TGT AAA ACG ACG GCC AGT G
SpF	ACG TAG CCG AAG CTG CAC TG
SpSgrDI	GTA ACT CGT CGA CGG CGT CGG CTT GAA CGA ATT G
m13 Forward	GTA AAA CGA CGG CCA G
m13 reverse	CAG GAA ACA GCT ATG AC

Table 9: Primer sequences used in this project

Restriction enzymes	**Recognition sequences**
DraI	5'-TTTAAA-3' 3'-AAATTT-5'
DraIII	5'-CACNNNGTG-3' 3'-GTGNNNCAC-5'
EcoRI	5'-GAATTC-3' 3'-CTTAAG-5'
HindIII	5'-AAGCTT-3' 3'-TTCGAA-5'
NcoI	5'-CCATGG-3' 3'-GGTACC-5'
NsiI	5'-ATGCAT-3' 3'-TACGTA-5'
PstI	5'-CTGCAG-3' 3'-GACGTC-5'
SalI	5'-GTCGAC-3' 3'-CAGCTG-5'
Sbf1	5'-CCTGCAGG-3' 3'-GGACGTCC5'
SgrDI	5'-CGTCGACG-3' 3'-GCAGCTGC5'
XhoI	5'-CTCGAG-3' 3'-GAGCTC-5'
XmaI	5'-CCCGGG-3' 3'-GGGCCC5'

Table 10: Overview of recognition sites and cutting patters of all restriction enzymes used in this study. The cutting patters for each enzyme are indicated in red colour.

D Setup of growth and fluorescence plates

The different setup of 96 well plates are illustrated in Figure 40 where the wells are labeled according to the plasmid conjugated into *M. algicola*. All plates were cultivated at 30°C to ensure optimal growth conditions for *M. algicola*. The "blank" wells contained LB medium with Am.

Plate 1

Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	**pTN7**	**pTN7**	**pTN7**	Blank	**pTN10**	**pTN10**	**pTN10**	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	**pTN9**	**pTN9**	**pTN9**	Blank	**pTN12**	**pTN12**	**pTN12**	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	**pTN11**	**pTN11**	**pTN11**	Blank	**pTN14**	**pTN14**	**pTN14**	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Plate 2

Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	**pTN7 m-tol**	**pTN7 m-tol**	**pTN7 m-tol**	Blank	**pTN10 arab**	**pTN10 arab**	**pTN10 arab**	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	**pTN9 m-tol**	**pTN9 m-tol**	**pTN9 m-tol**	Blank	**pTN12**	**pTN12**	**pTN12**	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	**pTN11 m-tol**	**pTN11 m-tol**	**pTN11 m-tol**	Blank	**pTN14 rham**	**pTN14 rham**	**pTN14 rham**	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Plate 3

Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	**pTN8**	**pTN8**	**pTN8**	Blank	**pTN8 IPTG**	**pTN8 IPTG**	**pTN8 IPTG**	Blank	**pTN11**	**pTN11**	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	**pTN11**	Blank	Blank
Blank	**pTN13**	**pTN13**	**pTN13**	Blank	**pTN13 antr**	**pTN13 antr**	**pTN13 antr**	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	**pTN11 mtol**	**pTN11 mtol**	Blank
Blank	**pTN14**	**pTN14**	**pTN14**	Blank	**pTN14 rham**	**pTN14 rham**	**pTN14 rham**	Blank	**pTN11 mtol**	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Plate 4

Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	**pTN4**	**pTN4**	**pTN4**	Blank	**pTN12**	**pTN12**	**pTN12**	Blank	**pTN8**	**pTN8**	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	**pTN8**	Blank	Blank
Blank	**pTN7**	**pTN7**	**pTN7**	Blank	**pTN7 m-tol**	**pTN7 m-tol**	**pTN7 m-tol**	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	**pTN8 IPTG**	**pTN8 IPTG**	Blank
Blank	**pTN9**	**pTN9**	**pTN9**	Blank	**pTN9 m-tol**	**pTN9 m-tol**	**pTN9 m-tol**	Blank	**pTN8 IPTG**	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Figure 40: Illustration of plate setup for measuring growth and fluorescence intensity in 96-well plates.

E Raw data for growth and fluorescence analysis

OD_{600} measurements

The following tables present the OD measurements for the growth analysis of the different plasmids conjugated in *M. algicola*. These measurements were taken in three parallels at regular intervals to observe bacterial growth, with the plate setup shown in Appendix D.

	OD_600 measurments																													
	09.00	11.00	13.00	15.00	17.00	19.00	21.00	09.00	11.00	13.00	15.00	17.00	19.00	21.00	09.00	11.00	13.00	15.00	17.00	19.00	21.00									
	28.mai	0	2	4	6	8	10	12	24	26	28	30	32	34	36	48	50	52	54	56	58	60								
pTN8		0,12	0,43	0,96	1,17	1,52	1,06	1,39	2,60	2,71	2,75	2,81	2,83	2,97	2,90	3,17	3,14	3,23	3,23	3,29	3,25	3,33								
pTN8		0,12	0,53	1,09	1,15	1,16	1,04	1,63	2,79	2,91	3,20	3,20	3,34	3,33	3,38	3,50	3,50	3,50	3,50	3,50	3,50	3,50								
pTN8		0,12	0,69	1,14	1,07	1,12	1,07	1,21	2,26	2,43	2,43	2,56	2,52	2,63	2,61	2,87	2,86	2,91	2,86	3,00	2,94	3,01								
pTN8 IPTG		0,12	0,34	0,74	1,18	1,21	1,09	1,10	2,09	2,34	2,33	2,44	2,51	2,58	2,52	2,84	2,75	2,87	2,76	2,98	2,85	2,98								
pTN8 IPTG		0,12	0,29	0,66	1,04	1,21	1,13	1,14	2,09	2,34	2,32	2,46	2,50	2,59	2,53	2,85	2,80	2,83	2,76	2,92	2,78	2,92								
pTN8 IPTG		0,12	0,32	0,70	1,01	1,17	1,15	1,20	2,32	2,60	2,53	2,69	2,61	2,71	2,61	2,96	2,84	2,92	2,84	2,96	2,93	2,98								
pTN13		0,13	0,79	1,18	1,01	1,12	1,14	1,60	2,67	2,95	3,46	3,43	3,50	3,35	3,50	3,50	3,50	3,50	3,50	3,50	3,50	3,50								
pTN13		0,13	0,56	1,05	1,10	1,08	1,02	2,25	2,36	2,58	2,47	2,59	2,54	2,64	2,60	2,89	2,82	2,94	2,81	3,01	2,92	3,05								
pTN13		0,13	0,55	1,05	1,13	1,16	1,07	1,21	2,30	2,51	2,48	2,60	2,52	2,61	2,58	2,88	2,88	2,97	2,84	2,95	2,92	3,02								
pTN13 antranilat		0,13	0,53	1,31	1,42	1,41	1,33	1,28	0,92	0,96	1,03	1,32	1,51	1,83	1,95	2,89	2,84	2,91	2,86	2,96	2,83	2,90								
pTN13 antranilat		0,12	0,55	1,32	1,46	1,40	1,28	1,29	1,00	1,07	1,02	1,14	1,24	1,57	1,77	2,73	2,68	2,81	2,75	2,79	2,68	2,72								
pTN13 antranilat		0,13	0,52	1,28	1,46	1,41	1,30	1,28	1,05	1,11	1,02	1,12	1,07	1,20	1,60	2,71	2,68	2,79	2,74	2,87	2,79	2,80								
pTN14		0,13	0,49	0,98	1,14	1,08	2,04	1,15	2,31	2,55	2,54	2,68	2,57	2,73	2,62	2,85	2,91	2,94	2,91	2,95	2,88	2,98								
pTN14		0,13	0,42	0,89	1,07	1,11	1,08	1,17	2,26	2,50	2,52	2,62	2,55	2,68	2,60	2,87	2,88	2,93	2,86	3,03	2,90	3,02								
pTN14		0,13	0,40	0,87	1,18	1,18	1,11	1,20	2,24	2,49	2,43	2,57	2,52	2,63	2,59	2,80	2,80	2,83	2,80	3,01	2,87	2,92								
pTN14 rhanmose		0,13	0,27	0,48	0,79	1,03	1,06	1,16	1,91	2,20	2,31	2,47	2,56	2,68	2,64	2,77	2,73	2,74	2,65	2,71	2,68	2,74								
pTN14 rhanmose		0,13	0,27	0,44	0,73	0,99	1,04	1,14	1,88	2,15	2,23	2,42	2,51	2,64	2,60	2,79	2,75	2,72	2,63	2,73	2,67	2,73								
pTN14 rhanmose		0,14	0,34	0,67	1,01	1,22	1,20	1,25	2,24	2,70	2,78	2,88	2,98	3,04	3,02	3,09	3,05	2,97	2,92	2,99	2,91	2,94								
pTN11		0,32	0,81	1,23	1,34	1,59	1,74	1,90	2,35	2,49	2,38	2,53	2,48	2,52	2,44	2,77	2,72	2,79	2,78	2,97	2,87	3,03								
pTN11		0,32	0,78	1,22	1,39	1,70	1,82	1,96	2,39	2,48	2,40	2,56	2,57	2,61	2,51	2,81	2,78	2,82	2,74	2,93	2,87	2,96								
pTN11		0,32	0,75	1,23	1,39	1,67	1,81	1,99	2,37	2,53	2,41	2,51	2,48	2,52	2,45	2,87	2,86	2,90	2,85	2,97	2,93	3,00								
pTN11 m-tol		0,32	0,95	1,23	1,03	0,96	0,80	0,76	1,46	1,27	1,20	0,99	1,13	0,86	1,18	2,25	2,40	2,60	2,55	2,73	2,66	2,72								
pTN11 m-tol		0,30	0,07	1,19	1,23	1,37	1,31	1,42	1,50	1,62	1,66	1,93	1,93	2,11	2,20	2,74	2,75	2,82	2,72	2,91	2,79	2,82								
pTN11 m-tol		0,31	1,03	1,25	1,26	1,39	1,41	1,50	1,53	1,60	1,54	1,73	1,81	1,96	2,07	2,72	2,74	2,82	2,75	2,93	2,81	2,83								

pTN7 m-tol	0,24	0,82	0,82	1,04	0,94	0,97	0,95	1,20	1,21	1,15	1,16	1,18	1,14	1,29	1,29	1,28	1,50	1,46	1,66	1,65	1,92		
pTN7 m-tol	0,25	0,83	0,85	1,13	1,06	1,12	1,15	1,95	2,10	2,09	2,23	2,33	2,39	2,58	2,70	2,66	2,83	2,75	2,85	2,79	2,84		
pTN7 m-tol	0,25	0,93	0,96	1,09	1,01	0,96	0,89	1,04	1,13	0,95	0,93	0,94	0,96	0,81	0,90	0,87	1,03	0,89	1,02	0,86	1,03		
pTN7	0,22	0,82	1,14	1,51	1,63	1,83	1,97	2,64	2,71	2,68	2,55	2,56	2,52	2,65	2,63	2,61	2,63	2,70	2,62	2,67	2,98		
pTN7	0,26	0,87	1,13	1,50	1,70	1,85	1,99	2,66	2,67	2,64	2,58	2,55	2,54	2,60	2,62	2,58	2,53	2,66	2,59	2,63	2,84		
pTN7	0,25	0,89	1,21	1,56	1,75	1,91	2,03	2,64	2,65	2,62	2,55	2,54	2,51	2,57	2,56	2,68	2,64	2,73	2,71	2,73	2,88		
pTN9 m-tol	0,14	0,64	0,63	0,83	0,92	0,85	0,93	2,35	2,44	2,49	2,53	2,60	2,58	2,70	2,73	2,73	3,00	2,84	3,07	2,92	3,95		
pTN9 m-tol	0,14	0,69	0,69	0,84	0,97	0,95	1,21	2,42	2,50	2,52	2,60	2,61	2,59	2,70	2,68	2,70	2,95	2,80	2,94	2,86	3,00		
pTN9 m-tol	0,14	0,85	0,85	0,79	0,75	0,75	0,80	0,92	0,91	1,00	1,02	1,17	1,26	1,46	2,56	2,77	3,12	3,02	3,18	3,16	3,36		
pTN9	0,15	0,73	0,85	1,10	1,50	1,82	2,03	2,74	2,87	2,85	2,84	2,80	2,73	2,77	2,71	2,71	2,75	2,86	2,81	2,80	2,99		
pTN9	0,15	0,73	0,81	1,08	1,40	1,79	2,02	2,69	2,86	2,82	2,82	2,79	2,73	2,79	2,71	2,76	2,74	2,86	2,83	2,86	2,92		
pTN9	0,15	1,59	1,47	1,44	1,80	2,14	2,23	3,14	3,47	3,27	3,28	3,40	3,37	3,32	3,28	3,19	3,22	3,50	3,46	3,41	3,50		
pTN10 arabinose	0,15	0,80	0,75	0,87	0,83	0,68	0,72	0,95	0,96	0,83	0,79	0,83	0,76	0,59	0,75	0,84	1,04	1,00	1,34	1,33	1,65		
pTN10 arabinose	0,16	0,91	0,84	0,97	0,75	0,69	0,67	0,83	0,80	0,83	0,88	0,89	0,86	0,63	0,82	0,73	0,82	0,72	0,77	0,70	0,76		
pTN10 arabinose	0,15	0,85	0,83	0,80	0,70	0,71	0,71	0,91	0,81	0,83	0,81	0,81	0,78	0,72	1,34	1,35	1,68	1,72	2,00	2,07	2,46		
pTN10	0,15	0,72	1,02	1,04	1,18	1,38	1,73	2,50	2,60	2,62	2,61	2,64	2,64	2,71	2,68	2,67	2,63	2,77	2,79	2,80	2,97		
pTN10	0,15	0,76	0,97	1,07	1,21	1,44	1,80	2,52	2,64	2,65	2,65	2,66	2,65	2,76	2,68	2,69	2,62	2,85	2,77	2,77	2,98		
pTN10	0,16	0,75	0,98	1,09	1,23	1,43	1,72	2,53	2,71	2,68	2,68	2,70	2,68	2,78	2,67	2,69	2,65	2,79	2,80	2,86	3,03		
pTN12	0,14	0,62	0,97	1,13	1,28	1,55	1,72	2,71	2,70	2,56	2,58	2,56	2,51	2,55	2,59	2,66	2,88	2,70	2,93	2,70	2,80		
pTN12	0,14	0,73	1,04	1,31	1,25	1,57	1,65	2,62	2,63	2,52	2,56	2,57	2,56	2,72	2,67	2,68	2,95	2,81	2,91	2,78	2,92		
pTN12	0,14	0,61	1,00	1,32	1,39	1,61	1,74	2,67	2,65	2,55	2,58	2,59	2,54	2,63	2,47	2,68	2,87	2,72	2,76	2,77	2,85		
pTN4	0,30	0,82	0,96	1,22	1,49	1,65	1,75	2,30	2,30	2,37					2,83	2,71							
pTN4	0,32	0,83	0,94	1,31	1,53	1,68	1,78	2,29	2,34	2,42					2,78	2,75							
pTN4	0,31	0,80	0,95	1,21	1,52	1,62	1,77	2,30	2,29	2,38					2,85	2,68							

Figure 41: Raw data of the OD measurements done every other hour for each strain. The colours indicate which strains were cultivated on the same 96-plates.

Fluorescence intensity measurements

The tables below show the fluorescence intensity measurements for the analysis of gene expression in *M. algicola* containing different plasmids. These measurements were taken in three parallels and normalized to OD to account for variations in cell density, which is calculated in Section E. The plate setup is shown in Appendix D.

	FI measurments																													
	09.00	11.00		13.00		15.00		17.00		19.00		21.00		09.00		11.00		13.00		15.00		17.00		19.00		21.00				
	28.mai	0	2	4	6	8	10	12	24	26	28	30	32	34	36	48	50	52	54	56	58	60								
pTN8		1119	583	835	526	768	790	362	277	520	350	363	327	393	372	482	477	451	464	577	545	502								
pTN8		1082	582	642	564	759	744	315	330	646	234	284	219	272	204	341	287	263	330	300	251	333								
pTN8		1008	609	728	537	764	653	662	591	574	673	797	626	811	614	1080	727	634	838	944	740	792								
pTN8 IPTG		1109	487	679	680	789	750	720	642	537	674	790	561	801	663	1051	696	669	866	1111	857	819								
pTN8 IPTG		1084	522	726	483	778	703	716	621	577	663	737	602	831	691	987	733	698	950	1036	826	867								
pTN8 IPTG		1053	567	879	559	718	627	711	558	527	651	773	580	970	688	1205	664	793	996	1120	900	950								
pTN13		1027	635	629	559	757	609	384	395	837	240	317	220	261	234	309	305	241	236	242	220	221								
pTN13		967	544	786	514	735	635	666	601	599	652	824	573	789	641	1106	782	681	958	939	837	826								
pTN13		1089	537	751	537	659	622	584	536	493	681	824	526	848	671	1096	777	730	976	1021	886	746								
pTN13 antranilat		1069	458	703	538	835	731	779	606	519	533	569	370	525	452	922	650	644	772	955	819	931								
pTN13 antranilat		1020	446	668	566	804	688	810	599	573	618	659	350	504	398	962	607	559	788	898	845	803								
pTN13 antranilat		1070	345	683	564	814	684	782	695	521	591	705	404	537	432	898	597	626	817	783	714	720								
pTN14		1116	603	715	528	753	701	655	537	564	713	788	576	902	703	1101	903	843	941	1080	897	851								
pTN14		1039	569	696	550	770	614	671	478	544	692	745	536	785	685	1118	808	734	915	980	884	777								
pTN14		1154	541	712	539	772	608	672	551	539	657	734	579	967	703	1140	822	868	940	1062	926	977								
pTN14 rhanmose		1025	467	756	524	710	606	775	485	482	581	690	592	872	773	1284	913	1062	1214	1390	1250	1156								
pTN14 rhanmose		1054	462	695	521	677	642	729	551	482	555	650	578	814	845	1273	879	1008	1256	1353	1172	1173								
pTN14 rhanmose		954	440	672	538	738	561	657	551	512	660	736	627	945	918	1204	910	1093	1337	1342	1228	1194								
pTN11		786	415	575	514	513	441	527	734	618	699	922	511	827	645	1029	743	669	870	948	830	722								
pTN11		836	467	547	568	496	494	572	758	528	800	937	539	892	695	1219	898	806	993	981	811	842								
pTN11		806	436	593	559	543	533	522	727	550	853	914	597	904	674	1163	816	787	966	1089	942	986								
pTN11 m-tol		747	421	707	520	720	787	832	738	564	714	763	414	704	541	826	580	594	777	967	820	804								
pTN11 m-tol		940	347	638	513	678	670	688	700	576	636	710	471	663	544	1094	744	731	1013	1000	921	856								
pTN11 m-tol		819	406	594	535	627	700	707	779	567	703	812	447	719	630	1033	775	685	946	1038	906	865								

pTN7 m-tol	1353	723	488	763	644	830	744	647	663	700	696	642	796	752	710	726	567	685	663	667	664
pTN7 m-tol	1386	751	472	638	624	711	714	512	677	631	686	654	660	557	772	956	817	1046	1108	1077	1109
pTN7 m-tol	1435	729	530	708	649	833	741	755	687	854	642	649	922	701	824	883	729	878	810	776	786
pTN7	1326	871	661	683	621	738	653	789	889	1033	967	1238	1264	1349	1533	1217	1082	1043	971	1156	1003
pTN7	1398	811	701	746	698	685	723	860	965	993	1062	1284	1437	1410	1590	1249	1181	1155	1092	1319	1059
pTN7	1343	815	698	727	675	676	656	856	961	1064	1066	1233	1297	1411	1658	1251	1089	1072	1021	1337	980
pTN9 m-tol	1552	801	577	686	624	730	692	431	746	612	644	736	630	686	999	1245	974	1155	1245	1287	1249
pTN9 m-tol	1665	889	483	647	586	735	610	403	732	626	701	732	787	768	1024	1341	1064	1242	1306	1395	1466
pTN9 m-tol	2576	919	559	779	597	893	836	667	711	805	637	717	704	743	747	837	726	792	925	1093	1144
pTN9	1467	891	651	567	674	637	640	658	765	996	959	1108	1272	1165	1545	1328	1148	1188	1140	1477	1259
pTN9	1411	934	680	707	665	740	675	685	842	987	931	1154	134	1275	1768	1424	1280	1255	1027	1566	1138
pTN9	1558	510	343	366	349	324	315	338	396	300	310	421	393	439	624	540	573	507	551	553	500
pTN10 arabinose	1571	908	448	721	583	901	825	662	689	875	625	674	852	823	704	789	595	691	669	756	649
pTN10 arabinose	1409	776	458	663	620	822	904	698	638	756	652	669	764	735	678	863	564	719	763	668	678
pTN10 arabinose	1628	809	494	749	636	797	771	839	669	771	645	677	806	711	625	616	492	592	623	587	606
pTN10	1467	957	846	925	902	838	685	778	853	921	902	1064	1122	1082	1421	1266	1072	1072	943	1380	985
pTN10	1419	857	826	1044	749	854	729	719	870	961	840	965	1163	1091	1476	1189	987	1059	940	1204	1006
pTN10	1483	847	784	923	865	906	705	685	894	951	864	1027	1089	1111	1551	1141	1018	1003	925	1152	920
pTN12	1610	941	811	895	761	753	504	380	657	590	646	726	713	518	1042	1098	971	990	1137	1226	1178
pTN12	1527	896	828	802	840	871	442	425	685	593	678	625	746	412	877	1042	753	1062	1200	1191	1156
pTN12	1625	895	784	843	817	799	414	412	716	614	657	707	742	381	990	1221	862	1166	1117	1246	1393
pTN4	602	415	469	372	321	353	540	588	636	591					448	689					
pTN4	579	378	490	421	360	363	514	613	626	586					505	692					
pTN4	668	314	406	397	350	379	551	606	585	562					510	621					

Figure 42: Raw data of the FI measurements done every other hour for each strain. The colours indicate which strains were cultivated on the same 96-plates.

Calculation of FI/OD, means and standard deviation

The standard deviation is calculated using the formula:

$$\sigma = \sqrt{\frac{\sum(x_i - \mu)^2}{N - 1}}$$

Where x_i are the individual measurements, μ is the mean of the measurements, and N is the number of measurements, is this thesis N= 3.

In Excel, the function `STDEV.P` was used to calculate the standard deviation directly for each time point.

OD mean	0	2	4	6	8	10	12	24	26	28	30	32	34	36	48	50	52	54	56	58	60
pTN8	0,119	0,549	1,064	1,129	1,264	1,060	1,409	2,550	2,682	2,795	2,858	2,895	2,975	2,964	3,177	3,166	3,212	3,195	3,261	3,229	3,279
pTN8 IPTG	0,122	0,319	0,698	1,076	1,196	1,121	1,147	2,166	2,427	2,393	2,528	2,538	2,627	2,557	2,885	2,797	2,874	2,786	2,955	2,851	2,960
pTN13	0,132	0,634	1,094	1,079	1,118	1,079	1,685	2,445	2,682	2,800	2,874	2,852	2,863	2,894	3,089	3,067	3,136	3,053	3,153	3,114	3,189
pTN13 anthranilat	0,128	0,535	1,302	1,448	1,404	1,302	1,284	0,993	1,048	1,023	1,195	1,274	1,531	1,775	2,775	2,732	2,835	2,783	2,872	2,767	2,806
pTN14	0,128	0,436	0,912	1,131	1,123	1,409	1,173	2,270	2,512	2,498	2,622	2,543	2,680	2,605	2,842	2,862	2,899	2,857	2,996	2,886	2,974
pTN14 rhamnose	0,131	0,295	0,528	0,842	1,076	1,100	1,181	2,007	2,349	2,442	2,588	2,682	2,785	2,753	2,883	2,839	2,812	2,731	2,811	2,756	2,801
pTN11	0,321	0,782	1,225	1,373	1,653	1,788	1,952	2,369	2,502	2,400	2,533	2,509	2,551	2,465	2,813	2,786	2,834	2,789	2,956	2,889	2,998
pTN11 m-toulate	0,312	0,684	1,223	1,176	1,238	1,174	1,226	1,498	1,495	1,464	1,548	1,625	1,642	1,819	2,567	2,630	2,747	2,672	2,856	2,751	2,787
pTN7	0,244	0,861	1,161	1,523	1,691	1,863	1,995	2,646	2,678	2,647	2,560	2,552	2,522	2,608	2,605	2,621	2,597	2,695	2,637	2,677	2,899
pTN7 m-tol	0,245	0,863	0,879	1,087	1,003	1,017	0,994	1,397	1,478	1,396	1,440	1,484	1,497	1,558	1,628	1,604	1,784	1,699	1,841	1,765	1,928
pTN9	0,146	1,018	1,043	1,207	1,569	1,920	2,092	2,857	3,063	2,983	2,982	2,997	2,942	2,959	2,902	2,886	2,904	3,074	3,033	3,022	3,138
pTN9 m-tol	0,143	0,726	0,722	0,821	0,879	0,851	0,979	1,894	1,952	2,002	2,050	2,130	2,145	2,289	2,659	2,732	3,021	2,888	3,063	2,980	3,436
pTn 10	0,152	0,745	0,989	1,068	1,207	1,415	1,749	2,517	2,650	2,651	2,647	2,668	2,657	2,751	2,677	2,684	2,633	2,802	2,786	2,810	2,992
pTN10 arabnise	0,152	0,853	0,806	0,882	0,758	0,695	0,700	0,899	0,858	0,830	0,825	0,846	0,800	0,646	0,970	0,976	1,179	1,149	1,371	1,364	1,625
pTN12	0,140	0,650	1,004	1,251	1,306	1,577	1,702	2,664	2,658	2,544	2,573	2,572	2,536	2,633	2,572	2,672	2,899	2,743	2,868	2,746	2,858

FI Mean	0	2	4	6	8	10	12	24	26	28	30	32	34	36	48	50	52	54	56	58	60
pTN8	1070	591	735	542	764	729	446	399	580	419	481	391	492	397	634	497	449	544	607	512	542
pTN8 IPTG	1082	525	761	574	762	693	716	607	547	663	767	581	867	681	1081	698	720	937	1089	861	879
pTN13	1028	572	722	537	717	622	545	511	643	524	655	440	633	515	837	621	551	723	734	648	598
pTN13 anthranilat	1053	416	685	556	818	701	790	633	538	581	644	375	522	427	927	618	610	792	879	793	818
pTN14	1103	571	708	539	765	641	666	522	549	687	756	564	885	697	1120	844	815	932	1041	902	868
pTN14 rhamnose	1011	456	708	528	708	603	720	529	492	599	692	599	877	845	1254	901	1054	1269	1362	1217	1174
pTN11	809	439	572	547	517	489	540	740	565	784	924	549	874	671	1137	819	754	943	1006	861	850
pTN11 m-toulate	835	391	646	523	675	719	742	739	569	684	762	444	695	572	984	700	670	912	1002	882	842
pTN7	1356	832	687	719	665	700	677	835	938	1030	1032	1252	1333	1390	1594	1239	1117	1090	1028	1271	1014
pTN7 m-tol	1391	734	497	703	639	791	733	638	676	728	675	648	793	670	769	855	704	870	860	840	853
pTN9	1479	778	558	547	563	567	543	560	668	761	733	894	600	960	1312	1097	1000	983	906	1199	966
pTN9 m-tol	1931	870	540	704	602	786	713	500	730	681	661	728	707	732	923	1141	921	1063	1159	1258	1286
pTn 10	1456	887	819	964	839	866	706	727	872	944	869	1019	1125	1095	1483	1199	1026	1045	936	1245	970
pTN10 arabnise	1536	831	467	711	613	840	833	733	665	801	641	673	807	756	669	756	550	667	685	670	644
pTN12	1587	911	808	847	806	808	453	406	686	599	660	686	734	437	970	1120	862	1073	1151	1221	1242

Figure 43: Calculated mean of the data gathered from fluorescence intensity and optical density values for each strain every other hour.

SD_OD	0	2	4	6	8	10	12	24	26	28	30	32	34	36	48	50	52	54	56	58	60
pTN8	0,0017	0,1079	0,0748	0,0400	0,1784	0,0120	0,1727	0,2195	0,1954	0,3149	0,2636	0,3406	0,2870	0,3198	0,2590	0,2617	0,2390	0,2641	0,2066	0,2294	0,2014
pTN8 IPTG	0,0012	0,0194	0,0335	0,0747	0,0165	0,0241	0,0393	0,1056	0,1219	0,0992	0,1109	0,0510	0,0600	0,0407	0,0552	0,0380	0,0359	0,0399	0,0239	0,0613	0,0305
pTN13	0,0000	0,1095	0,0603	0,0521	0,0323	0,0480	0,4314	0,1612	0,1944	0,4641	0,3953	0,4580	0,3416	0,4286	0,2907	0,3073	0,2578	0,3165	0,2467	0,2732	0,2201
pTN13 anthranilat	0,0029	0,0122	0,0150	0,0192	0,0036	0,0184	0,0012	0,0527	0,0644	0,0045	0,0910	0,1794	0,2572	0,1404	0,0779	0,0769	0,0522	0,0569	0,0671	0,0633	0,0734
pTN14	0,0022	0,0393	0,0511	0,0480	0,0391	0,4450	0,0180	0,0275	0,0256	0,0465	0,0426	0,0205	0,0426	0,0145	0,0313	0,0483	0,0497	0,0445	0,0335	0,0140	0,0400
pTN14 rhamnose	0,0037	0,0335	0,0997	0,1216	0,0997	0,0728	0,0470	0,1641	0,2469	0,2435	0,2072	0,2105	0,1797	0,1902	0,1442	0,1463	0,1134	0,1316	0,1298	0,1114	0,0977
pTN11	0,0019	0,0274	0,0025	0,0228	0,0489	0,0366	0,0376	0,0139	0,0215	0,0126	0,0221	0,0418	0,0394	0,0312	0,0420	0,0561	0,0459	0,0486	0,0191	0,0311	0,0282
pTN11 m-toulate	0,0078	0,4357	0,0263	0,1015	0,1974	0,2662	0,3337	0,0309	0,1605	0,1957	0,4050	0,3507	0,5596	0,4518	0,2267	0,1664	0,1035	0,0885	0,0916	0,0683	0,0502
pTN7	0,0149	0,0293	0,0359	0,0287	0,0515	0,0332	0,0290	0,0123	0,0257	0,0240	0,0141	0,0095	0,0102	0,0315	0,0314	0,0448	0,0494	0,0303	0,0496	0,0403	0,0577
pTN7 m-tol	0,0033	0,0483	0,0597	0,0338	0,0511	0,0741	0,1100	0,3974	0,4390	0,4965	0,5653	0,6085	0,6335	0,7503	0,7710	0,7651	0,7614	0,7802	0,7569	0,7931	0,7382
pTN9	0,0014	0,4052	0,3039	0,1644	0,1708	0,1590	0,0969	0,2034	0,2841	0,2054	0,2114	0,2874	0,3036	0,2544	0,2699	0,2162	0,2252	0,3015	0,2999	0,2720	0,2577
pTN9 m-tol	0,0005	0,0924	0,0913	0,0256	0,0974	0,0833	0,1730	0,6925	0,7367	0,7058	0,7307	0,6772	0,6239	0,5850	0,0709	0,0271	0,0721	0,0971	0,0985	0,1267	0,3910
pTn 10	0,0037	0,0192	0,0193	0,0213	0,0212	0,0267	0,0351	0,0131	0,0460	0,0249	0,0280	0,0267	0,0158	0,0318	0,0034	0,0096	0,0131	0,0365	0,0128	0,0362	0,0251
pTN10 arabnise	0,0025	0,0433	0,0378	0,0691	0,0537	0,0120	0,0247	0,0491	0,0750	0,0029	0,0381	0,0357	0,0406	0,0520	0,2597	0,2702	0,3618	0,4220	0,5042	0,5603	0,6976
pTN12	0,0012	0,0531	0,0287	0,0866	0,0569	0,0271	0,0386	0,0364	0,0280	0,0197	0,0071	0,0110	0,0229	0,0696	0,0818	0,0120	0,0368	0,0512	0,0738	0,0347	0,0495

ST_FI	0	2	4	6	8	10	12	24	26	28	30	32	34	36	48	50	52	54	56	58	60
pTN8	46,1	12,5	78,9	16,0	3,7	56,9	153,7	137,2	51,6	185,7	225,5	172,1	230,9	168,3	320,3	180,2	151,5	215,0	263,8	201,0	189,5
pTN8 IPTG	22,9	32,7	85,4	81,1	31,2	50,7	3,7	35,7	21,6	9,4	22,1	16,8	73,6	12,6	91,5	28,2	53,0	53,8	37,7	30,3	54,1
pTN13	49,8	44,6	67,3	18,4	42,0	10,6	118,4	86,0	143,8	201,4	239,0	156,5	263,9	199,3	373,4	223,7	219,9	344,7	349,5	303,1	268,3
pTN13 anthranilat	23,3	50,7	14,3	12,8	12,9	21,3	14,0	43,7	25,0	35,5	56,5	22,3	13,6	22,3	26,4	23,0	36,6	18,6	71,5	56,6	86,8
pTN14	47,8	25,4	8,3	9,0	8,5	42,5	7,8	31,6	10,8	23,1	23,3	19,6	75,3	8,5	16,0	41,9	58,2	12,0	43,5	17,6	82,6
pTN14 rhamnose	42,0	11,7	35,4	7,4	24,9	33,1	48,6	31,1	14,1	44,6	35,1	20,6	53,6	59,2	35,4	15,4	35,1	51,0	20,5	32,8	15,5
pTN11	20,5	21,4	18,9	23,6	19,4	37,7	22,5	13,3	38,3	63,9	9,5	35,8	33,8	20,5	79,7	63,3	60,6	52,8	60,2	57,8	107,9
pTN11 m-toulate	79,6	31,9	46,5	9,2	38,0	49,6	63,9	32,3	5,1	34,5	41,7	23,4	23,7	41,3	114,7	85,6	56,9	99,3	29,0	44,5	26,9
pTN7	30,7	27,4	18,2	26,4	32,3	27,4	32,3	32,6	34,9	29,1	45,8	23,0	75,0	29,0	51,1	15,6	45,1	47,5	49,6	81,4	33,2
pTN7 m-tol	33,7	12,0	24,5	51,2	10,8	56,8	13,5	99,4	9,8	93,2	23,5	4,9	107,0	82,6	46,6	96,0	103,5	147,5	185,1	173,4	187,7
pTN9	60,6	190,6	152,5	140,0	151,1	176,9	162,1	157,6	194,7	326,0	299,6	335,2	487,0	370,9	495,2	396,0	306,9	337,9	255,2	458,0	333,0
pTN9 m-tol	458,4	50,1	40,7	55,4	16,0	75,7	93,4	118,4	14,4	87,9	28,7	8,2	64,1	34,3	125,1	218,5	142,9	194,9	167,1	124,9	134,1
pTn 10	27,2	49,7	25,8	56,6	65,2	29,0	18,0	38,4	16,8	17,0	25,5	40,8	30,3	12,1	53,3	51,5	35,1	29,9	7,9	97,6	36,6
pTN10 arabnise	92,8	56,1	19,8	35,8	22,2	44,3	54,6	76,4	21,0	52,9	11,4	3,3	35,9	48,1	32,9	103,5	43,1	54,5	58,3	69,0	29,6
pTN12	43,1	21,5	18,1	38,1	33,2	48,6	37,6	18,9	24,1	10,7	13,3	43,8	14,7	58,7	68,9	74,8	89,0	72,2	35,4	22,7	106,9

Figure 44: Calculated standard deviations of the fluorescence intensity and optical density values for each strain every other hour.

Further, to calculate the standard diviation of the FI/OD relation, SD_R, the following equation was used:

$$SD_R = \left|\frac{\bar{FI}}{\bar{OD}}\right| \sqrt{\left(\frac{SD_{FI}}{\bar{FI}}\right)^2 + \left(\frac{SD_{OD}}{\bar{OD}}\right)^2}$$

Where:
$\bar{FI}$ = Mean value of the FI measurements.
$\bar{OD}$ = Mean value of the OD measurements.
SD_{FI} = Standard deviation of the FI measurements.
SD_{OD} = Standard deviation of the OD measurements.

This formula quantifies the uncertainty of the ratio $\frac{FI}{OD}$ based on the individual uncertainties of FI and OD, and the calsulations are shown in Figure 45.

SD_FI/OD	0	2	4	6	8	10	12	24	26	28	30	32	34	36	48	50	52	54	56	58	60
pTN8	407,2	212,9	88,7	22,1	85,4	54,3	115,8	55,5	24,9	68,6	80,4	61,5	79,2	58,6	102,1	58,4	48,3	68,7	81,7	63,3	58,7
pTN8 IPTG	209,2	143,7	133,0	84,0	27,5	47,1	21,6	21,4	14,4	12,1	15,9	8,0	29,0	6,5	32,5	10,6	18,7	19,9	13,1	12,5	18,5
pTN13	377,3	170,8	71,5	29,4	41,9	27,4	108,5	37,8	56,4	78,4	88,9	60,2	95,9	73,7	123,5	75,7	71,6	115,6	112,3	99,0	85,1
pTN13 anthranilat	260,3	96,3	12,6	10,2	9,3	18,0	10,9	55,5	39,6	34,8	62,6	45,0	58,0	22,8	13,4	10,6	13,5	8,9	25,9	21,5	31,9
pTN14	401,0	131,5	44,4	21,8	24,9	146,8	11,0	14,2	4,8	10,6	10,0	7,9	28,6	3,6	7,1	15,5	20,6	6,6	15,0	6,3	28,0
pTN14 rhamnose	388,4	180,3	261,4	91,0	65,3	47,2	47,8	26,6	22,8	30,5	25,3	19,1	28,0	30,2	25,0	17,2	19,6	29,2	23,5	21,5	15,6
pTN11	65,6	33,7	15,5	18,4	15,0	21,8	12,7	5,9	15,4	26,7	4,9	14,7	14,3	9,0	29,0	23,5	21,8	19,8	20,5	20,3	36,1
pTN11 m-toulate	263,8	367,0	39,7	39,1	92,2	145,2	173,0	23,8	41,0	66,7	131,4	60,7	145,0	81,3	56,1	36,6	22,7	38,8	15,2	18,0	11,1
pTN7	361,9	45,7	24,1	19,5	22,5	16,1	16,9	12,4	13,5	11,5	18,0	9,2	29,8	12,9	21,0	10,0	19,2	18,2	20,2	31,2	13,4
pTN7 m-tol	157,0	49,6	47,4	51,2	34,2	79,5	82,8	148,1	136,0	197,3	184,7	179,2	235,1	213,8	225,5	261,3	178,2	250,6	216,9	235,3	195,4
pTN9	426,4	357,3	213,7	131,4	104,0	95,3	78,4	56,9	66,7	110,7	101,9	115,5	166,9	128,4	175,7	140,1	109,0	114,3	89,2	155,7	109,1
pTN9 m-tol	3198,5	167,3	110,2	72,6	78,0	126,9	160,1	115,0	141,2	127,7	115,8	108,8	100,4	83,1	48,0	80,1	47,9	68,6	55,9	45,6	57,8
pTn 10	296,0	73,3	30,7	56,0	55,3	23,6	13,1	15,3	8,5	7,2	10,3	15,8	11,7	6,4	19,9	19,2	13,5	11,7	3,2	35,2	12,5
pTN10 arabnise	633,9	82,2	36,6	75,0	64,4	67,1	88,7	96,0	72,1	63,8	38,4	33,8	68,2	120,1	187,9	239,1	147,9	218,6	188,6	208,2	171,3
pTN12	323,2	119,1	29,2	55,8	37,0	32,0	22,9	7,4	9,5	4,6	5,2	17,1	6,4	22,7	29,3	28,0	30,9	27,3	16,1	10,0	38,2

Figure 45: Calculated standard diviationsof the normalised fluorescense values for each strain.

F Constructed plasmid maps

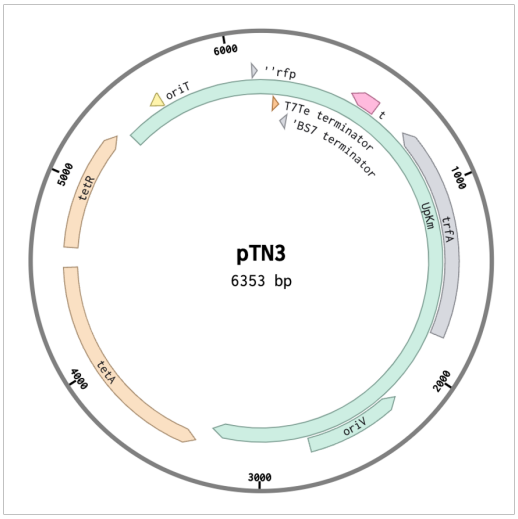


Figure 46: pTN3 plasmid constructed in previous work[5], derived from pSV2 and tetracycline resistant and used as backbone for plasmids constructed in this thesis.

Figure 47: pTN4 constructed from pTN3 backbone and an insertion of an apramycin resistance gene.

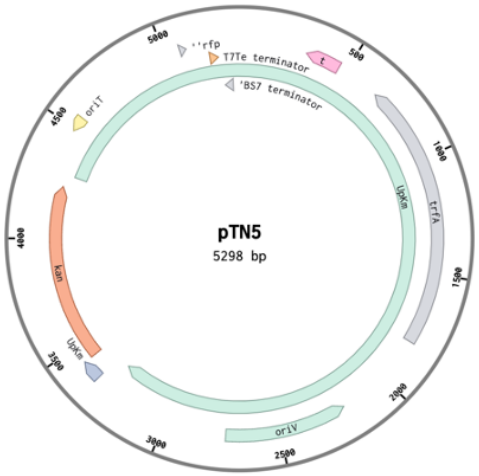


Figure 48: pTN5 constructed from pTN3 backbone and an insertion of a kanamycin resistance gene.

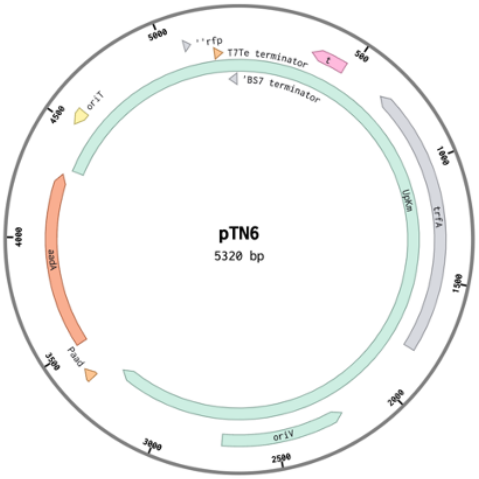


Figure 49: pTN6 constructed from pTN3 backbone and an insertion of a spectinomycin resistance gene.

Figure 50: pTN7 derived from pSV2 and pTN4.

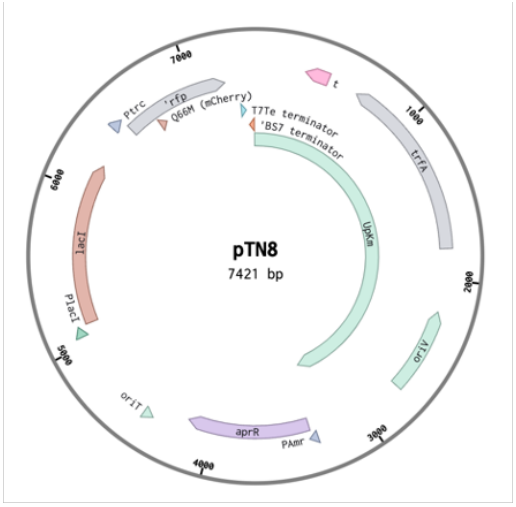


Figure 51: pTN8 derived from pSV3 and pTN4.

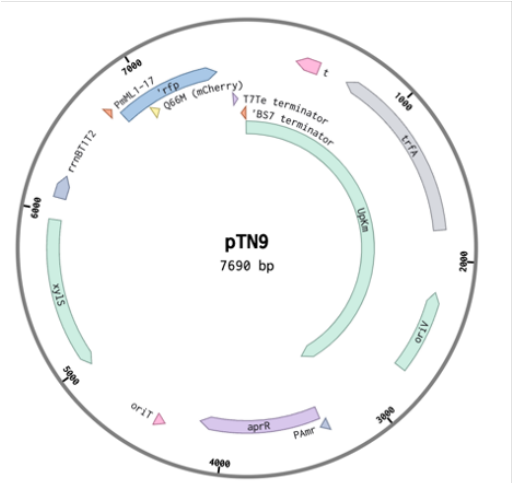


Figure 52: pTN9 derived from pSV4 and pTN4.

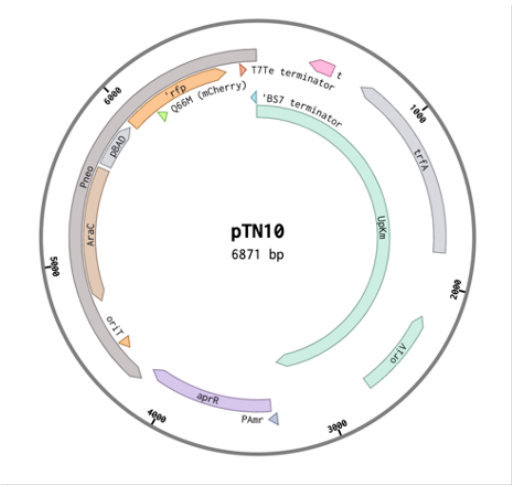


Figure 53: pTN10 derived from pSV5 and pTN4.

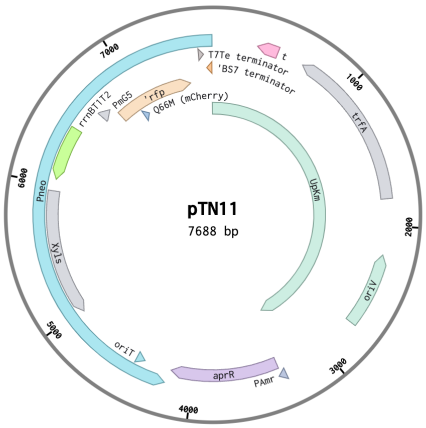


Figure 54: pTN11 derived from pSV6 and pTN4.

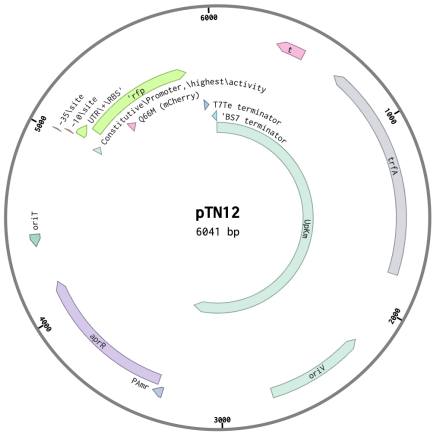


Figure 55: pTN12 derived from pHH108 and pTN4.

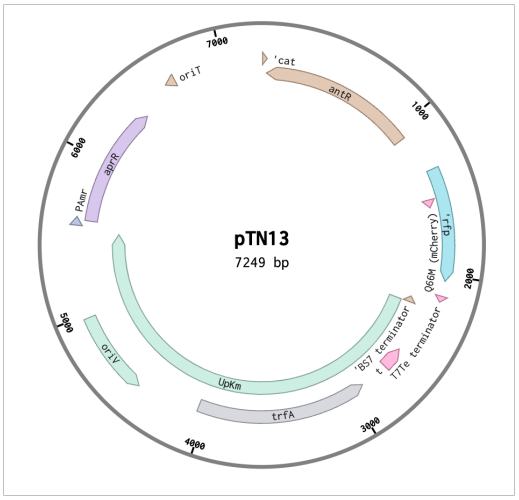


Figure 56: pTN13 derived from pAFW4 and pTN4.

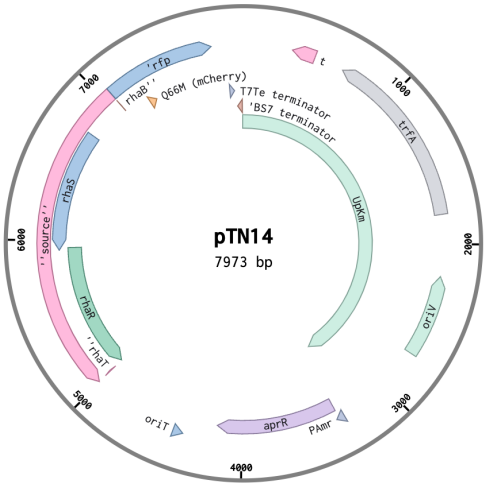


Figure 57: pTN14 derived from pAFW5 and pTN4.

G Alligned sequencing results of plasmids

Sequencing results for the constructed plasmids are presented below, where the first line shows the template DNA. Subsequent lines display the sequences obtained from different primers used for the specific analysis, which are detailed in Appendix C. For the pTN5$_T$, two samples were included in the alignment so that at least one always displays the correct base pair within the Km gene. Further the sequencing of pTN5 confirms the correct sequencing without any mismatches or mutations of base pairs were present within the resistance gene.

The genes of interest for the plasmids span the following base ranges: pTN4 (Am^R) from 3352 to 4167, pTN5$_T$ (Km^R) from 3285 to 4100, pTN5 (Km^R) from 3427 to 4242, and pTN6 (Sp^R) from 1029 to 1820.

```
Page 1 GGV903_42339039_42339039 (GGV903_42339039_42339039.ab1), GGV906_42339060_42339060 (GG...

             1                                                                                82
pTN4         GATCCTCTAGCTAGAGTCAGCTTTATGCTTGTAAACCGTTTTGTGAAAAAATTTTTAAAATAAAAAAGGGGACCTCTAGGGT
GGV910_42... --------------------------------------------------------------------------------

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

             83                                                                               164
pTN4         CCCCAATTAATTAGTAATATAATCTATTAAAGGTCATTCAAAAGGTCATCCACCGGATCAGCTTAGTAAAGCCCTCGCTAGA
GGV910_42... --------------------------------------------------------------------------------

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

             165                                                                              246
pTN4         TTTTAATGCGGATGTTGCGATTACTTCGCCAACTATTGCGATAACAAGAAAAAGCCAGCCTTTCATGATATATCTCCCAATT
GGV910_42... --------------------------------------------------------------------------------

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

             247                                                                              328
pTN4         TGTGTAGGGCTTATTATGCACGCTTAAAAATAATAAAAGCAGACTTGACCTGATAGTTTGGCTGTGAGCAATTATGTGCTTA
GGV910_42... --------------------------------------------------------------------------------

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

             329                                                                              410
pTN4         GTGCATCTAACGCTTGAGTTAAGCCGCGCCGCGAAGCGGCGTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCA
GGV910_42... --------------------------------------------------------------------------------

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

             411                                                                              492
pTN4         AGGATCGGGCCTTGATGTTACCCGAGAGCTTGGCACCCAGCCTGCGCGAGCAGGGGAATTGATCCGGTGGATGACCTTTTGA
GGV910_42... --------------------------------------------------------------------------------

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

             493                                                                              574
pTN4         ATGACCTTTAATAGATTATATTACTAATTAATTGGGGACCCTAGAGGTCCCCTTTTTTATTTTAAAAATTTTTTCACAAAAC
GGV910_42... --------------------------------------------------------------------------------

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

             575                                                                              656
pTN4         GGTTTACAAGCATAAAGCTGACCCTCTAGCAAGCTTGCGATGCAGGTGGCTGCTGAACCCCCAGCCGGAACTGACCCCACAA
GGV910_42... --------------------------------------------------------------------------------

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

             657                                                                              738
pTN4         GGCCCTAGCGTTTGCAATGCACCAGGTCATCATTGACCCAGGCGTGTTCCACCAGGCCGCTGCCTCGCAACTCTTCGCAGGC
GGV910_42... --------------------------------------------------------------------------------

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

             739                                                                              820
pTN4         TTCGCCGACCTGCTCGCGCCACTTCTTCACGCGGGTGGAATCCGATCCGCACATGAGGCGGAAGGTTTCCAGCTTGAGCGGG
GGV910_42... --------------------------------------------------------------------------------

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

             821                                                                              902
pTN4         TACGGCTCCCGGTGCGAGCTGAAATAGTCGAACATCCGTCGGGCCGTCGGCGACAGCTTGCGGTACTTCTCCCATGTGAATT
GGV910_42... --------------------------------------------------------------------------------

.....................................................................................
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             903                                                                 984
pTN4         TCGTGTAGTGGTCGCCAGCAAACAGCACGACGATTTCCTCGTCGATCAGGACCTGGCAACGGGACGTTTTCTTGCCACGGTC
GGV910_42... ---------------------------------------------------------------------------------

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

             985                                                                1066
pTN4         CAGGACGCGGAAGCGGTGCAGCAGCGACACCGATTCCAGGTGCCCAACGCGGTCGGACGTGAAGCCCATCGCCGTCGCCTGT
GGV910_42... ---------------------------------------------------------------------------------

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

             1067                                                               1148
pTN4         AGGCGCGACAGGCATTCCTCGGCCTTCGTGTAATACCGGCCATTGATCGACCAGCCCAGGTCCTGGCAAAGCTCGTAGAACG
GGV910_42... ---------------------------------------------------------------------------------

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

             1149                                                               1230
pTN4         TGAAGGTGATCGGCTCGCCGATAGGGGTGCGCTTCGCGTACTCCAACACCTGCTGCCACACCAGTTCGTCATCGTCGGCCCG
GGV910_42... ---------------------------------------------------------------------------------

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

             1231                                                               1312
pTN4         CAGCTCGACGCCGGTGTAGGTGATCTTCACGTCCTTGTTGACGTGGAAAATGACCTTGTTTTGCAGCGCCTCGCGCGGGATT
GGV910_42... ---------------------------------------------------------------------------------

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

             1313                                                               1394
pTN4         TTCTTGTTGCGCGTGGTGAACAGGGCAGAGCGGGCCGTGTCGTTTGGCATCGCTCGCATCGTGTCCGGCCACGGCGCAATAT
GGV910_42... ---------------------------------------------------------------------------------

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

             1395                                                               1476
pTN4         CGAACAAGGAAAGCTGCATTTCCTTGATCTGCTGCTTCGTGTGTTTCAGCAACGCGGCCTGCTTGGCCTCGCTGACCTGTTT
GGV910_42... ---------------------------------------------------------------------------------

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

             1477                                                               1558
pTN4         TGCCAGGTCCTCGCCGGCGGTTTTTCGCTTCTTGGTCGTCATAGTTCCTCGCGTGTCGATGGTCATCGACTTCGCCAAACCT
GGV910_42... ---------------------------------------------------------------------------------

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

             1559                                                               1640
pTN4         GCCGCCTCCTGTTCGAGACGACGCGAACGCTCCACGGCGGCCGATGGCGCGGGCAGGGCAGGGGGAGCCAGTTGCACGCTGT
GGV910_42... ---------------------------------------------------------------------------------

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

             1641                                                               1722
pTN4         CGCGCTCGATCTTGGCCGTAGCTTGCTGGACCATCGAGCCGACGGACTGGAAGGTTTCGCGGGGCGCACGCATGACGGTGCG
GGV910_42... ---------------------------------------------------------------------------------

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

             1723                                                               1804
pTN4         GCTTGCGATGGTTTCGGCATCCTCGGCGGAAAACCCCGCGTCGATCAGTTCTTGCCTGTATGCCTTCCGGTCAAACGTCCGA
GGV910_42... ---------------------------------------------------------------------------------

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            1805                                                                                1886
pTN4        TTCATTCACCCTCCTTGCGGGATTGCCCCGGAATTAATTCCCCGGATCGATCCGTCGATCTTGATCCCCTGCGCCATCAGAT
GGV910_42... --------------------------------------------------------------------------------

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            1887                                                                                1968
pTN4        CCTTGGCGGCAAGAAAGCCATCCAGTTTACTTTGCAGGGCTTCCCAACCTTACCAGAGGGCGCCCCAGCTGGCAATTCCGGT
GGV910_42... --------------------------------------------------------------------------------

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

            1969                                                                                2050
pTN4        TCGCTTGCTGTCCATAAAACCGCCCAGTCTAGCTATCGCCATGTAAGCCCACTGCAAGCTACCTGCTTTCTCTTTGCGCTTG
GGV910_42... --------------------------------------------------------------------------------

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

            2051                                                                                2132
pTN4        CGTTTTCCCTTGTCCAGATAGCCCAGTAGCTGACATTCATCCGGGGTCAGCACCGTTTCTGCGGACTGGCTTTCTACGTGGC
GGV910_42... --------------------------------------------------------------------------------

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

            2133                                                                                2214
pTN4        TGCCATTTTTGGGGTGAGGTCGTTCGCGGCCGAGGGGCGCAGCCCCTGGGGGGATGGGGTGCCGCGTTAGCGGGCCGGGAGG
GGV910_42... --------------------------------------------------------------------------------

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

            2215                                                                                2296
pTN4        GTTCGAGAAGGGGGGGCACCCCCCTTCGGCGTGCGCGGTCACGCGCCAGGGCGCAGCCCTGGTTAAAAACAAGGTTTATAAA
GGV910_42... --------------------------------------------------------------------------------

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

            2297                                                                                2378
pTN4        TATTGGTTTAAAAGCAGGTTAAAAGACAGGTTAGCGGTGGCCGAAAAACGGGCGGAAACCCTTGCAAATGCTGGATTTTCTG
GGV910_42... --------------------------------------------------------------------------------

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

            2379                                                                                2460
pTN4        CCTGTGGACAGCCCCTCAAATGTCAATAGGTGCGCCCCTCATCTGTCATCACTCTGCCCCTCAAGTGTCAAGGATCGCGCCC
GGV910_42... --------------------------------------------------------------------------------

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

            2461                                                                                2542
pTN4        CTCATCTGTCAGTAGTCGCGCCCCTCAAGTGTCAATACCGCAGGGCACTTATCCCCAGGCTTGTCCACATCATCTGTGGGAA
GGV910_42... --------------------------------------------------------------------------------

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

            2543                                                                                2624
pTN4        ACTCGCGTAAAATCAGGCGTTTTCGCCGATTTGCGAGGCTGGCCAGCTCCACGTCGCCGGCCGAAATCGAGCCTGCCCCTCA
GGV910_42... --------------------------------------------------------------------------------

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

            2625                                                                                2706
pTN4        TCTGTCAACGCCGCGCCGGGTGAGTCGGCCCCTCAAGTGTCAACGTCCGCCCCTCATCTGTCAGTGAGGGCCAAGTTTTCCG
GGV910_42... --------------------------------------------------------------------------------

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Page 4 GGV903_42339039_42339039 (GGV903_42339039_42339039.ab1), GGV906_42339060_42339060 (GG...

             2707                                                                      2788
pTN4         CGTGGTATCCACAACGCCGGCGGCCCTACATGGCTCTGCTGTAGTGAGTGGGTTGCGCTCCGGCAGCGGTCCTGATCCCCCG
GGV910_42... --------------------------------------------------------------------------------

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

             2789                                                                      2870
pTN4         CAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGG
GGV910_42... --------------------------------------------------------------------------------

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

             2871                                                                      2952
pTN4         ATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTA
GGV910_42... --------------------------------------------------------------------------------

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

             2953                                                                      3034
pTN4         TATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATC
GGV910_42... --------------------------------------------------------------------------------

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

             3035                                                                      3116
pTN4         CATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCG
GGV910_42... --------------------------------------------------------------------------------

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

             3117                                                                      3198
pTN4         CGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAA
GGV910_42... ----------------------------------------------------------------------------AAA

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             3199                                                                      3280
pTN4         CTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCTGCAT
GGV910_42... GGAATGGGTGAACCTATAGCAATACTCAAGCTATGCATCAAGCTTGGTACCG-AG--CTCGGATCCACTAGTAACGGCCGCC

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             3281                                                                      3362
pTN4         CGCGCGCACCGTACGTCTCGAGGAATTCCTGCAGGTTGCAACAGTGCCGTTGATCGTGCTATGATCGACTGATGTCATCAGC
GGV910_42... AGTGTGCTGGAATTCGCCCTTATGCATCCTGCAGGTTGCAACAGTGCCGTTGATCGTGCTATGATCGACTGATGTCATCAGC

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             3363                                                                      3444
pTN4         GGTGGAGTGCAATGTCGTGCAATACGAATGGCGAAAAGCCGAGCTCATCGGTCAGCTTCTCAACCTTGGGGTTACCCCCGGC
GGV910_42... GGTGGAGTGCAATGTCGTGCAATACGAATGGCGAAAAGCCGAGCTCATCGGTCAGCTTCTCAACCTTGGGGTTACCCCCGGC

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             3445                                                                      3526
pTN4         GGTGTGCTGCTGGTCCACAGCTCCTTCCGTAGCGTCCGGCCCCTCGAAGATGGGCCACTTGGACTGATCGAGGCCCTGCGTG
GGV910_42... GGTGTGCTGCTGGTCCACAGCTCCTTCCGTAGCGTCCGGCCCCTCGAAGATGGGCCACTTGGACTGATCGAGGCCCTGCGTG

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             3527                                                                      3608
pTN4         CTGCGCTGGGTCCGGGAGGGACGCTCGTCATGCCCTCGTGGTCAGGTCTGGACGACGAGCCGTTCGATCCTGCCACGTCGCC
GGV910_42... CTGCGCTGGGTCCGGGAGGGACGCTCGTCATGCCCTCGTGGTCAGGTCTGGACGACGAGCCGTTCGATCCTGCCACGTCGCC

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Page 5 GGV903_42339039_42339039 (GGV903_42339039_42339039.ab1), GGV906_42339060_42339060 (GG...

            3609                                                                        3690
pTN4        CGTTACACCGGACCTTGGAGTTGTCTCTGACACATTCTGGCGCCTGCCAAATGTAAAGCGCAGCGCCCATCCATTTGCCTTT
GGV910_42... CGTTACACCGGACCTTGGAGTTGTCTCTGACACATTCTGGCGCCTGCCAAATGTAAAGCGCAGCGCCCATCCATTTGCCTTT

.....

            3691                                                                        3772
pTN4        GCGGCAGCGGGGCCACAGGCAGAGCAGATCATCTCTGATCCATTGCCCCTGCCACCTCACTCGCCTGCAAGCCCGGTCGCCC
GGV910_42... GCGGCAGCGGGGCCACAGGCAGAGCAGATCATCTCTGATCCATTGCCCCTGCCACCTCACTCGCCTGCAAGCCCGGTCGCCC

.....

            3773                                                                        3854
pTN4        GTGTCCATGAACTCGATGGGCAGGTACTTCTCCTCGGCGTGGGACACGATGCCAACACGACGCTGCATCTTGCCGAGTTGAT
GGV910_42... GTGTCCATGAACTCGATGGGCAGGTACTTCTCCTCGGCGTGGGACACGATGCCAACACGACGCTGCATCTTGCCGAGTTGAT

.....

            3855                                                                        3936
pTN4        GGCAAAGGTTCCCTATGGGGTGCCGAGACACTGCACCATTCTTCAGGATGGCAAGTTGGTACGCGTCGATTATCTCGAGAAT
GGV910_42... GGCAAAGGTTCCCTATGGGGTGCCGAGACACTGCACCATTCTTCAGGATGGCAAGTTGGTACGCGTCGATTATCTCGAGAAT

.....

            3937                                                                        4018
pTN4        GACCACTGCTGTGAGCGCTTTGCCTTGGCGGACAGGTGGCTCAAGGAGAAGAGCCTTCAGAAGGAAGGTCCAGTCGGTCATG
GGV910_42... GACCACTGCTGTGAGCGCTTTGCCTTGGCGGACAGGTGGCTCAAGGAGAAGAGCCTTCAGAAGGAAGGTCCAGTCGGTCATG

.....

            4019                                                                        4100
pTN4        CCTTTGCTCGGTTGATCCGCTCCCGCGACATTGTGGCGACAGCCCTGGGTCAACTGGGCCGAGATCCGTTGATCTTCCTGCA
GGV910_42... CCTTTGCTCGGTTGATCCGCTCCCGCGACATTGTGGCGACAGCCCTGGGTCAACTGGGCCGAGATCCGTTGATCTTCCTGCA

.....

            4101                                                                        4182
pTN4        TCCGCCAGAGGGCGGGATGCGAAGAATGCGATGCCGCTCGCCAGTCGATTGGCTGAGCTCGAATTAATTCACTGGCCGTCGT
GGV910_42... TCCGCCAGAG-GCGGGATGCGAAGAATGCGATGCCGCTCGCCAGTCGATTGGCTGAGCTCGAATTAATTCACTGGCCGTCGT

.....

            4183                                                                        4264
pTN4        TTTACAACGTCGACGGATCTTTTCCGCTGCATAACCCTGCTTCGGGGTCATTATAGCGATTTTTTCGGTATATCCATCCTTT
GGV910_42... TTTACAACGTCGACGAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCA--TCTAGAGGGCCCAA

.....

            4265                                                                        4346
pTN4        TTCGCACGATATACAGGATTTTGCCAAAGGGTTCGTGTAGACTTTCCTTGGTGTATCCAACGGCGTCAGCCGGGCAGGATAG
GGV910_42... TTCGCCCTATAGTGAGTCGTATTACAATTCACTGGC----------CGTCGT-TTTACAACGTCGTGACTGGGAAAAC---C

.....

            4347                                                                        4428
pTN4        GTGAAGTAGGCCCACCCGCGAGCGGGTGTTCCTTCTTCACTGTCCCTTATTCGCACCTGGCGGTGCTCAACGGGAATCCTGC
GGV910_42... CTGGCGTTACC-------CAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCAGCTGG-CGTAATAGCGAAAAGGCCGCAC

.....

            4429                                                                        4510
pTN4        TCTGCGAGGCTGGCCGATAAGCTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCT
GGV910_42... CGATCGCCCTTCCAACAGTTGCGCAGCCATACGTACGGCAGTTTAAGGTTTACCCTATAAAAAAGAAGCCGTTTCGTCTGTT

.....
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             4511                                                                4592
pTN4         TTCGTCTTCAAGAATTAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGC
GGV910_42... TGGGAAGTCCAAGGGAATTTTTGACCCCCGGGCCACGAGGGGACCCCGGGCCGGGCCCTTGGGGCAAAAAACTCCCGGAATT

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

             4593                                                                4674
pTN4         CTTGCAGCACATCCCCCTTTCGCCAGCAGATCCACATCCTTGAAGGCCGCAGCGACGAGCAGAAGGAAACCCTCATTCGGGA
GGV910_42... TCCCGGGGGGGAATGGGAAAAAAAGGGGAAAAACCCAAAGGGCGGGGGGCCCCTT----------------------------

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

             4675                                                                4756
pTN4         AGTCAGCGAGGCCATCTCGCGCTCCCTGGATGCGCCGCTGACCAGCGTGCGAGTGATTATCACGGAGATGGCCAAGGGCCAC
GGV910_42... ---------------------------------------------------------------------------------

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

             4757                                                                4838
pTN4         TTCGGCATCGGCGGCGAACTGGCCAGCAAGGTCAGACGCTGAAGTGGAGATGCCCAAGGGCACTTCGGGTCGAGGAACCCGA
GGV910_42... ---------------------------------------------------------------------------------

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

             4839                                                                4920
pTN4         CCTGCATTGGGACGCGGCCACGGAGAGCGCGGGCAAACGCCGGCACTATAGCCAGTGGAGTTTGTAAAACGCTATTTCAGAG
GGV910_42... ---------------------------------------------------------------------------------

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

             4921                                                                5002
pTN4         CTTGGAGAGTGTCTAAGAAAGCCGGGCGATGCCAACCGGTGGCATGGATGAGCTGTACAAATAATAATAACCAGGCATCAAA
GGV910_42... ---------------------------------------------------------------------------------

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

             5003                                                                5084
pTN4         TAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACAC
GGV910_42... ---------------------------------------------------------------------------------

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

             5085                                                                5166
pTN4         TGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTAACTGCTCCCGGTCTCACTAGAAAATAAAAACAGCCGTGCCTCTTTCTGG
GGV910_42... ---------------------------------------------------------------------------------

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

             5167                5190
pTN4         CAACGGCTGTTTTTGCGGCCGCTG
GGV910_42... ------------------------

.....................................................................................
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Page 1 GGV894_42338940_42338940 (GGV894_42338940_42338940.ab1), GGV895_42338957_42338957 (GG...

             1                                                                                  82
pTN5_T [1... CTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAAT
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             83                                                                                164
pTN5_T [1... ACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGCCCAGGAACCGTAAAAAG
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             165                                                                               246
pTN5_T [1... GCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAA
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             247                                                                               328
pTN5_T [1... ACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTAC
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             329                                                                               410
pTN5_T [1... CGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAG
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             411                                                                               492
pTN5_T [1... GTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTG
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             493                                                                               574
pTN5_T [1... AGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGG
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             575                                                                               656
pTN5_T [1... TGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCA
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             657                                                                               738
pTN5_T [1... GTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGC
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

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             739                                                                                820
pTN5_T [1... AGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAA
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             821                                                                                902
pTN5_T [1... CTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAGC
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             903                                                                                984
pTN5_T [1... ACGTGTCAGTCCTGCTCCTCGGCCACGAAGTGCACGCAGTTGCCGGCCGGGTCGCGCAGGGCGAACTCCCGCCCCCACGGCT
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             985                                                                               1066
pTN5_T [1... GCTCGCCGATCTCGGTCATGGCCGGCCCGGAGGCGTCCCGGAAGTTCGTGGACACGACCTCCGACCACTCGGCGTACAGCTC
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             1067                                                                              1148
pTN5_T [1... GTCCAGGCCGCGCACCCACACCCAGGCCAGGGTGTTGTCCGGCACCACCTGGTCCTGGACCGCGCTGATGAACAGGGTCACG
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             1149                                                                              1230
pTN5_T [1... TCGTCCCGGACCACACCGGCGAAGTCGTCCTCCACGAAGTCCCGGGAGAACCCGAGCCGGTCGGTCCAGAACTCGACCGCTC
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             1231                                                                              1312
pTN5_T [1... CGGCGACGTCGCGCGCGGTGAGCACCGGAACGGCACTGGTCAACTTGGCCATGGTGGCCCTCCTCACGTGCTATTATTGAAG
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             1313                                                                              1394
pTN5_T [1... CATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACA
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             1395                                                                              1476
pTN5_T [1... TTTCCCCGAAAAGTGCCACCTGTATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGAAATTGTA
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

................................................................................
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             1477                                                                1558
pTN5_T [1... AGCGTTAATAATTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGC
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             1559                                                                1640
pTN5_T [1... ACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCG
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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

             1641                                                                1722
pTN5_T [1... CCACACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCATCGCCATG
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             1723                                                                1804
pTN5_T [1... GGTCACGACGAGATCCTCGCCGTCGGGCATGCTCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCTGATGCTCT
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             1805                                                                1886
pTN5_T [1... TCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGA
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             1887                                                                1968
pTN5_T [1... ATGGGCAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTG
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             1969                                                                2050
pTN5_T [1... AGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCT
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             2051                                                                2132
pTN5_T [1... GCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTCTTGCAGTTCATTCAGGGCACCGGACAGGTCGG
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             2133                                                                2214
pTN5_T [1... TCTTGACAAAAAGAACCGGGCGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCCCA
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             2215                                                                                2296
pTN5_T [1... GTCATAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATCATGCGAAACGATCCT
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             2297                                                                                2378
pTN5_T [1... CATCCTGTCTCTTGATCAGAGCTTGATCCCCTGCGCCATCAGATCCTTGGCGGCGAGAAAGCCATCCAGTTTACTTTGCAGG
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             2379                                                                                2460
pTN5_T [1... GCTTCCCAACCTTACCAGAGGGCGCCCCAGCTGGCAATTCCGGTTCGCTTGCTGTCCATAAAACCGCCCAGTCTAGCTATCG
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             2461                                                                                2542
pTN5_T [1... CCATGTAAGCCCACTGCAAGCTACCTGCTTTCTCTTTGCGCTTGCGTTTTCCCTTGTCCAGATAGCCCAGTAGCTGACATTC
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             2543                                                                                2624
pTN5_T [1... ATCCGGGGTCAGCACCGTTTCTGCGGACTGGCTTTCTACGTGAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGC
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             2625                                                                                2706
pTN5_T [1... CTGACATTTATATTCCCCAGAACATCAGGTTAATGGCGTTTTTGATGTCATTTTCGCGGTGGCTGAGATCAGCCACTTCTTC
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             2707                                                                                2788
pTN5_T [1... CCCGATAACGGAGACCGGCACACTGGCCATATCGGTGGTCATCATGCGCCAGCTTTCATCCCCGATATGCACCACCGGGTAA
GGV894_42... --------------------------------------------------------------------------------
GGV913_42... --------------------------------------------------------------------------------

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             2789                                                                                2870
pTN5_T [1... AGTTCACGGGAGACTTTATCTGACAGCAGACGTGCACTGGCCAGGGGGATCACCATCCGTCGCCCCGGCGTGTCAATAATAT
GGV894_42... ------------------------------------------------AGGGGGGAGAAAGATGGGTTCCTTTCCGTTT
GGV913_42... ----------------AGGGGGAAGAAGAGGGCCTCGCTGCATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGG

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             2871                                                                                2952
pTN5_T [1... ---CACTCTGTACATCCACAAACAGACGATAACGGCTCTCTCTTTTATAGGTGTAAACCTTAAACTGCCGTACGTATAGGCT
GGV894_42... GTTGGCTTGACCGTCCTATGTTTGTCGCCCCCGTCACACCCATCGAAGGAGTCCCAGGATCCCAGCCTTTCAGGTGGAAGTA
GGV913_42... TTTCTTAGAGCTTATCGGCCAGCCTCGCAGAGCAGGATTCCCG----------------TTGAGC-ACCGCCAGGTGCGAAT

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            2953                                                                    3034
pTN5_T [1... GCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGA
GGV894_42... GTGTACCCTTAACACCGAACACCCGAATCGCCGGTGGGCCTACTTCGCCCATCCTG--CCCGGCTGACGCCCTTGGATACAC
GGV913_42... AAGGGACAGTGAAGAAGGAACACCCGCTCGCGGGTGGGCCTACTTCACCTATCCTG--CCCGGCTGACGCCGTTGGATACAC

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            3035                                                                    3116
pTN5_T [1... TTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATA-----CGACTCACT
GGV894_42... CAAGGAAAGTCTACACGAACCCTTTGACAA----CATCCTGCATATCGTGCGAAAAAGGATGGATATACCGAAAAAATCATA
GGV913_42... CAAGGAAAGTCTACACGAACCCTTTGGCAA----AATCCTGTATATCGTGCGAAAAAGGATGGATATACCGAAAAAATCGCT

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            3117                                                                    3198
pTN5_T [1... ATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGGGAAAGCCACGTTGTGTC
GGV894_42... ATAATGACCCC----GAAGCAGGGTTATGCAGCGGAAAAGATCCGTCGACCTGCAGGGGGGGGGGGAAAGCCACGTTGTGTC
GGV913_42... ATAATGACCCC----GAAGCAGGGTTATGCAGCGGAAAAGATCCGTCGACCTGCAGGGGGGGGGGGAAAGCCACGTTGTGTC

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            3199                                                                    3280
pTN5_T [1... TCAAAATCTCTGATGTTACATTGCACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAAT
GGV894_42... TCAAAATCTCTGATGTTACATTGCACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAAT
GGV913_42... TCAAAATCTCTGATGTTACATTGCACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAAT

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            3281                                                                    3362
pTN5_T [1... ACAAGGGGTGTTATGAGCCATATTCAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTAT
GGV894_42... ACAAGGGGTGTTATGAGCCATATTCAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTAT
GGV913_42... ACAAGGGGTGTTATGAGCCATATTCAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTAT

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            3363                                                                    3444
pTN5_T [1... ATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCAGA
GGV894_42... ATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCAGA
GGV913_42... ATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCAGA

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            3445                                                                    3526
pTN5_T [1... GTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTT
GGV894_42... GTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTT
GGV913_42... GTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTT

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            3527                                                                    3608
pTN5_T [1... ATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGGAAAACAG
GGV894_42... ATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGGAAAACAG
GGV913_42... ATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGGAAAACAG

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            3609                                                                    3690
pTN5_T [1... CATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTC
GGV894_42... CATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTC
GGV913_42... CATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTC

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             3691                                                                3772
pTN5_T [1... GATTCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTG
GGV894_42... GATTCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGGTTG
GGV913_42... GATTCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTG

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             3773                                                                3854
pTN5_T [1... GTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCAT
GGV894_42... GTTGATGCGAGTGATTTTGATGACGAGCGTCATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCAT
GGV913_42... GTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCAT

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             3855                                                                3936
pTN5_T [1... TCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTAT
GGV894_42... TCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGAAATT-AAAAGGTTGTAT
GGV913_42... TCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTAT

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             3937                                                                4018
pTN5_T [1... TGATGTTGGACGAGTCGGAATCGC-AGACCGATACCAGGATCTTGCCATCCTATGGAACTGCCTCGGTGAGTTTTCTCCTTC
GGV894_42... TGATGTTGGACGAGTCGGAATCCCAAGACCGATACCGGGATCTTGCATTCTTAGGAAACTGCCTCGGGGAGTTTTCTCCTTC
GGV913_42... TGATGTTGGACGAATCGGAATCGC-AAAC-GATACCAGGATCTTGCCATCCTATGGAACTGCCTCGGTGAATTTTCCCCTTC

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             4019                                                                4100
pTN5_T [1... ATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGATGAG
GGV894_42... TTTACAAGAACGGCCTTTTTCCAAAATATGGCTATTGTAAACCCTGATTTGAAAAGATTGGACGTTCCTTTGAGGCTCGGGG
GGV913_42... ATACGAAACGGCTTTTCAAAAATGGGATGATAACCTGAATGAAAAAATGCAG---------TTCATTGAGGCCCAAG---AA

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             4101                                                                4182
pTN5_T [1... TTTTTCTAATCAGAATTGGTTAATTGGTTGTAACACTGGCAGAGCATTACGCTGACTTGACGGGCGTCGACGAAGGGCGAAT
GGV894_42... AATTTTCTAATCGGAATTGGTTAATTGGGTTTTAAACCGGGGGAAAAATTTAACCTTAATTTTAGGGGGGGGGGGTTTTTT
GGV913_42... TTTT---CAACC-AAATGGTTAATGGTTGAACCTGGCAGAAATTACCTTACTTGCGGGGCGGGGTCTGGTTAAAAAACAAAT

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             4183                                                                4264
pTN5_T [1... TCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGATGCATAGCTTGAGTATTCTATAGTGTCAC
GGV894_42... TGAAAAATTCAATATTTTTTTGAAGTTG-----------------------------------------------------
GGV913_42... TTTTGAATTGAGAAAAATACCCCTTTCCCCAACCCAAAGTTTCGGGGAAAAAAATATAACACCCCGGGCCCCCCACAAAAT

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             4265                                                                4346
pTN5_T [1... CTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAG
GGV894_42... ----------------------------------------------------------------------------------
GGV913_42... TCTACCCCGGGGCCTCCTCTTGGGGGGGGAGGGGAGCGGGGGGGGAAACACCATCTTTGAAAGACCCCCCC----------

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             4347                                                                4428
pTN5_T [1... CCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTT
GGV894_42... ----------------------------------------------------------------------------------
GGV913_42... ----------------------------------------------------------------------------------

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Page 7 GGV894_42338940_42338940 (GGV894_42338940_42338940.ab1), GGV895_42338957_42338957 (GG...

             4429                                                                        4508
pTN5_T [1... CCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCT
GGV894_42... ------------------------------------------------------------------------------
GGV913_42... ------------------------------------------------------------------------------

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            1                                                                                 82
pTN5        GATCCTCTAGCTAGAGTCAGCTTTATGCTTGTAAACCGTTTTGTGAAAAAATTTTTAAAATAAAAAAGGGGACCTCTAGGGT
GGV909_42... ---------------------------------------------------------------------------------

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            83                                                                               164
pTN5        CCCCAATTAATTAGTAATATAATCTATTAAAGGTCATTCAAAAGGTCATCCACCGGATCAGCTTAGTAAAGCCCTCGCTAGA
GGV909_42... ---------------------------------------------------------------------------------

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            165                                                                              246
pTN5        TTTTAATGCGGATGTTGCGATTACTTCGCCAACTATTGCGATAACAAGAAAAAGCCAGCCTTTCATGATATATCTCCCAATT
GGV909_42... ---------------------------------------------------------------------------------

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            247                                                                              328
pTN5        TGTGTAGGGCTTATTATGCACGCTTAAAAATAATAAAAGCAGACTTGACCTGATAGTTTGGCTGTGAGCAATTATGTGCTTA
GGV909_42... ---------------------------------------------------------------------------------

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            329                                                                              410
pTN5        GTGCATCTAACGCTTGAGTTAAGCCGCGCCGCGAAGCGGCGTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCA
GGV909_42... ---------------------------------------------------------------------------------

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            411                                                                              492
pTN5        AGGATCGGGCCTTGATGTTACCCGAGAGCTTGGCACCCAGCCTGCGCGAGCAGGGGAATTGATCCGGTGGATGACCTTTTGA
GGV909_42... ---------------------------------------------------------------------------------

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            493                                                                              574
pTN5        ATGACCTTTAATAGATTATATTACTAATTAATTGGGGACCCTAGAGGTCCCCTTTTTTATTTTAAAAATTTTTTCACAAAAC
GGV909_42... ---------------------------------------------------------------------------------

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            575                                                                              656
pTN5        GGTTTACAAGCATAAAGCTGACCCTCTAGCAAGCTTGCGATGCAGGTGGCTGCTGAACCCCCAGCCGGAACTGACCCCACAA
GGV909_42... ---------------------------------------------------------------------------------

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            657                                                                              738
pTN5        GGCCCTAGCGTTTGCAATGCACCAGGTCATCATTGACCCAGGCGTGTTCCACCAGGCCGCTGCCTCGCAACTCTTCGCAGGC
GGV909_42... ---------------------------------------------------------------------------------

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            739                                                                              820
pTN5        TTCGCCGACCTGCTCGCGCCACTTCTTCACGCGGGTGGAATCCGATCCGCACATGAGGCGGAAGGTTTCCAGCTTGAGCGGG
GGV909_42... ---------------------------------------------------------------------------------

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            821                                                                              902
pTN5        TACGGCTCCCGGTGCGAGCTGAAATAGTCGAACATCCGTCGGGCCGTCGGCGACAGCTTGCGGTACTTCTCCCATGTGAATT
GGV909_42... ---------------------------------------------------------------------------------

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             903                                                                 984
pTN5         TCGTGTAGTGGTCGCCAGCAAACAGCACGACGATTTCCTCGTCGATCAGGACCTGGCAACGGGACGTTTTCTTGCCACGGTC
GGV909_42... --------------------------------------------------------------------------------

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             985                                                                1066
pTN5         CAGGACGCGGAAGCGGTGCAGCAGCGACACCGATTCCAGGTGCCCAACGCGGTCGGACGTGAAGCCCATCGCCGTCGCCTGT
GGV909_42... --------------------------------------------------------------------------------

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             1067                                                               1148
pTN5         AGGCGCGACAGGCATTCCTCGGCCTTCGTGTAATACCGGCCATTGATCGACCAGCCCAGGTCCTGGCAAAGCTCGTAGAACG
GV909_42... --------------------------------------------------------------------------------

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             1149                                                               1230
pTN5         TGAAGGTGATCGGCTCGCCGATAGGGGTGCGCTTCGCGTACTCCAACACCTGCTGCCACACCAGTTCGTCATCGTCGGCCCG
GGV909_42... --------------------------------------------------------------------------------

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             1231                                                               1312
pTN5         CAGCTCGACGCCGGTGTAGGTGATCTTCACGTCCTTGTTGACGTGGAAAATGACCTTGTTTTGCAGCGCCTCGCGCGGGATT
GGV909_42... --------------------------------------------------------------------------------

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             1313                                                               1394
pTN5         TTCTTGTTGCGCGTGGTGAACAGGGCAGAGCGGGCCGTGTCGTTTGGCATCGCTCGCATCGTGTCCGGCCACGGCGCAATAT
GGV909_42... --------------------------------------------------------------------------------

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             1395                                                               1476
pTN5         CGAACAAGGAAAGCTGCATTTCCTTGATCTGCTGCTTCGTGTGTTTCAGCAACGCGGCCTGCTTGGCCTCGCTGACCTGTTT
GGV909_42... --------------------------------------------------------------------------------

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             1477                                                               1558
pTN5         TGCCAGGTCCTCGCCGGCGGTTTTTCGCTTCTTGGTCGTCATAGTTCCTCGCGTGTCGATGGTCATCGACTTCGCCAAACCT
GGV909_42... --------------------------------------------------------------------------------

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             1559                                                               1640
pTN5         GCCGCCTCCTGTTCGAGACGACGCGAACGCTCCACGGCGGCCGATGGCGCGGGCAGGGCAGGGGGAGCCAGTTGCACGCTGT
GGV909_42... --------------------------------------------------------------------------------

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             1641                                                               1722
pTN5         CGCGCTCGATCTTGGCCGTAGCTTGCTGGACCATCGAGCCGACGGACTGGAAGGTTTCGCGGGGCGCACGCATGACGGTGCG
GGV909_42... --------------------------------------------------------------------------------

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             1723                                                               1804
pTN5         GCTTGCGATGGTTTCGGCATCCTCGGCGGAAAACCCCGCGTCGATCAGTTCTTGCCTGTATGCCTTCCGGTCAAACGTCCGA
GGV909_42... --------------------------------------------------------------------------------

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            1805                                                                1886
pTN5        TTCATTCACCCTCCTTGCGGGATTGCCCCGGAATTAATTCCCCGGATCGATCCGTCGATCTTGATCCCCTGCGCCATCAGAT
GGV909_42... ------------------------------------------------------------------------------

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            1887                                                                1968
pTN5        CCTTGGCGGCAAGAAAGCCATCCAGTTTACTTTGCAGGGCTTCCCAACCTTACCAGAGGGCGCCCCAGCTGGCAATTCCGGT
GGV909_42... ------------------------------------------------------------------------------

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            1969                                                                2050
pTN5        TCGCTTGCTGTCCATAAAACCGCCCAGTCTAGCTATCGCCATGTAAGCCCACTGCAAGCTACCTGCTTTCTCTTTGCGCTTG
GGV909_42... ------------------------------------------------------------------------------

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            2051                                                                2132
pTN5        CGTTTTCCCTTGTCCAGATAGCCCAGTAGCTGACATTCATCCGGGGTCAGCACCGTTTCTGCGGACTGGCTTTCTACGTGGC
GGV909_42... ------------------------------------------------------------------------------

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            2133                                                                2214
pTN5        TGCCATTTTTGGGGTGAGGTCGTTCGCGGCCGAGGGGCGCAGCCCCTGGGGGGATGGGGTGCCGCGTTAGCGGGCCGGGAGG
GGV909_42... ------------------------------------------------------------------------------

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            2215                                                                2296
pTN5        GTTCGAGAAGGGGGGGCACCCCCCTTCGGCGTGCGCGGTCACGCGCCAGGGCGCAGCCCTGGTTAAAAACAAGGTTTATAAA
GGV909_42... ------------------------------------------------------------------------------

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            2297                                                                2378
pTN5        TATTGGTTTAAAAGCAGGTTAAAAGACAGGTTAGCGGTGGCCGAAAAACGGGCGGAAACCCTTGCAAATGCTGGATTTTCTG
GGV909_42... ------------------------------------------------------------------------------

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            2379                                                                2460
pTN5        CCTGTGGACAGCCCCTCAAATGTCAATAGGTGCGCCCCTCATCTGTCATCACTCTGCCCCTCAAGTGTCAAGGATCGCGCCC
GGV909_42... ------------------------------------------------------------------------------

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            2461                                                                2542
pTN5        CTCATCTGTCAGTAGTCGCGCCCCTCAAGTGTCAATACCGCAGGGCACTTATCCCCAGGCTTGTCCACATCATCTGTGGGAA
GGV909_42... ------------------------------------------------------------------------------

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            2543                                                                2624
pTN5        ACTCGCGTAAAATCAGGCGTTTTCGCCGATTTGCGAGGCTGGCCAGCTCCACGTCGCCGGCCGAAATCGAGCCTGCCCCTCA
GGV909_42... ------------------------------------------------------------------------------

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            2625                                                                2706
pTN5        TCTGTCAACGCCGCGCCGGGTGAGTCGGCCCCTCAAGTGTCAACGTCCGCCCCTCATCTGTCAGTGAGGGCCAAGTTTTCCG
GGV909_42... ------------------------------------------------------------------------------

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            2707                                                                        2788
pTN5        CGTGGTATCCACAACGCCGGCGGCCCTACATGGCTCTGCTGTAGTGAGTGGGTTGCGCTCCGGCAGCGGTCCTGATCCCCCG
GGV909_42... ------------------------------------------------------------------------------------

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            2789                                                                        2870
pTN5        CAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGG
GGV909_42... ------------------------------------------------------------------------------------

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            2871                                                                        2952
pTN5        ATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTA
GGV909_42... ----------------------------------------------------AAAGAGGGCCTCGTGAATACGTCCT-----

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            2953                                                                        3034
pTN5        TATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATC
GGV909_42... ------------------------------ATTTTTATAGGTTAATGTCAT---------GATAATAATGGTTTCTTAGAG

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            3035                                                                        3116
pTN5        CATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCT--GCAATGATAC
GGV909_42... CTTATCGGCCAGCCTCGCAGAGCAGGATTC----------CCGTTGAGC--ACCGCCAGGTGCGAATAAGGGACAGTGAAGA

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            3117                                                                        3198
pTN5        CGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGC
GGV909_42... AGGAACACCCGCTCGCGGGTGGGCCTACTTCACCTATCCTGCCCGGCTGACGCCGTTGGATACACCAAGGAAAGTCTACACG

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            3199                                                                        3280
pTN5        AACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGC-----CAGTTAATAGTTTGC
GGV909_42... AAC--CCTTTGGCAAAATCCTGTATATCGTGCGAAAAAGGATGGATATACCGAAAAAATCGCTATAATGACCCCGAAGCAGG

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            3281                                                                        3362
pTN5        GCTGCATCGCGCGCACCGTACGTCTCGAGGAATTCCTGCAGGGAAAGCCACGTTGTGTCTCAAAATCTCTGATGTTACATTG
GGV909_42... GTTATGCAGCGGAAAAGATCCGTCGACCTGCAGGGGGGGGGGGAAAGCCACGTTGTGTCTCAAAATCTCTGATGTTACATTG

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            3363                                                                        3444
pTN5        CACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTATGAGCCATAT
GGV909_42... CACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTATGAGCCATAT

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            3445                                                                        3526
pTN5        TCAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGAT
GGV909_42... TCAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGAT

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            3527                                                                        3608
pTN5        AATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAG
GGV909_42... AATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAG

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            3609                                                                3690
pTN5        GTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCATCAAGCA
GGV909_42... GTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCATCAAGCA

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            3691                                                                3772
pTN5        TTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGGAAAACAGCATTCCAGGTATTAGAAGAATAT
GGV909_42... TTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGGAAAACAGCATTCCAGGTATTAGAAGAATAT

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            3773                                                                3854
pTN5        CCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCTT
GGV909_42... CCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCTT

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            3855                                                                3936
pTN5        TTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATGA
GGV909_42... TTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATGA

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            3937                                                                4018
pTN5        CGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCATTCTCACCGGATTCAGTCGTCACT
GGV909_42... CGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCATTCTCACCGGATTCAGTCGTCACT

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            4019                                                                4100
pTN5        CATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCG
GGV909_42... CATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCG

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            4101                                                                4182
pTN5        CAGACCGATACCAGGATCTTGCCATCCTATGGAACTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAA
GGV909_42... CAGACCGATACCAGGATCTTGCCATCCTATGGAACTGCCTCGGGGAATTTTCTCTTCTTACAAAACGGTTTTTCAAAATATG

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            4183                                                                4264
pTN5        ATATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAGAATTGGTTAAT
GGV909_42... GGATTGATAATCCTGAATGAAAAATTGCAGTTCATTGAGGCCCATGAATTTTCAATCAGAATGGTTAATGGTTGAAACTGGC

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            4265                                                                4346
pTN5        TGGTTGTAACACTGGCAGAGCATTACGCTGACTTGACGGGCGTCGACGGATCTTTTCCGCTGCATAACCCTGCTTCGGGGTC
GGV909_42... AGAACTTACCTGATTGCCGGACGCGGTTTGTGAAATAACAAATTTTGGAAATGAGACAAAACCCCTTTCCCCAAACAAAACG

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            4347                                                                4428
pTN5        ATTATAGCGATTTTTTCGGTATATCCATCCTTTTTCGCACGATATACAGGATTTTGCCAAAGGGTTCGTGTAGACTTTCCTT
GGV909_42... TTCGGGGAAAAAAATATAAAACCATGGGCCCCCAAAAATTCCCCGGGCCCCCTTTTGGGGGGGGGAAGGGGGGAAAAAACT

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            4429                                                                4510
pTN5        GGTGTATCCAACGGCGTCAGCCGGGCAGGATAGGTGAAGTAGGCCCACCCGCGAGCGGGTGTTCCTTCTTCACTGTCCCTTA
GGV909_42... TT--------------------------------------------------------------------------------

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             4511                                                                                4592
pTN5         TTCGCACCTGGCGGTGCTCAACGGGAATCCTGCTCTGCGAGGCTGGCCGATAAGCTCTAAGAAACCATTATTATCATGACAT
GGV909_42... --------------------------------------------------------------------------------

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             4593                                                                                4674
pTN5         TAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTAATTCACTGGCCGTCGTTTTACAACGTCGTGAC
GGV909_42... --------------------------------------------------------------------------------

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             4675                                                                                4756
pTN5         TGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCAGATCCACATCCTTGAAGGCCG
GGV909_42... --------------------------------------------------------------------------------

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             4757                                                                                4838
pTN5         CAGCGACGAGCAGAAGGAAACCCTCATTCGGGAAGTCAGCGAGGCCATCTCGCGCTCCCTGGATGCGCCGCTGACCAGCGTG
GGV909_42... --------------------------------------------------------------------------------

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             4839                                                                                4920
pTN5         CGAGTGATTATCACGGAGATGGCCAAGGGCCACTTCGGCATCGGCGGCGAACTGGCCAGCAAGGTCAGACGCTGAAGTGGAG
GGV909_42... --------------------------------------------------------------------------------

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             4921                                                                                5002
pTN5         ATGCCCAAGGGCACTTCGGGTCGAGGAACCCGACCTGCATTGGGACGCGGCCACGGAGAGCGCGGGCAAACGCCGGCACTAT
GGV909_42... --------------------------------------------------------------------------------

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             5003                                                                                5084
pTN5         AGCCAGTGGAGTTTGTAAAACGCTATTTCAGAGCTTGGAGAGTGTCTAAGAAAGCCGGGCGATGCCAACCGGTGGCATGGAT
GGV909_42... --------------------------------------------------------------------------------

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             5085                                                                                5166
pTN5         GAGCTGTACAAATAATAATAACCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTG
GGV909_42... --------------------------------------------------------------------------------

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             5167                                                                                5248
pTN5         TTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTAACTGCTCCCGGTCTCA
GGV909_42... --------------------------------------------------------------------------------

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             5249                                                  5305
pTN5         CTAGAAAATAAAAACAGCCGTGCCTCTTTCTGGCAACGGCTGTTTTTGCGGCCGCTG
GGV909_42... ---------------------------------------------------------

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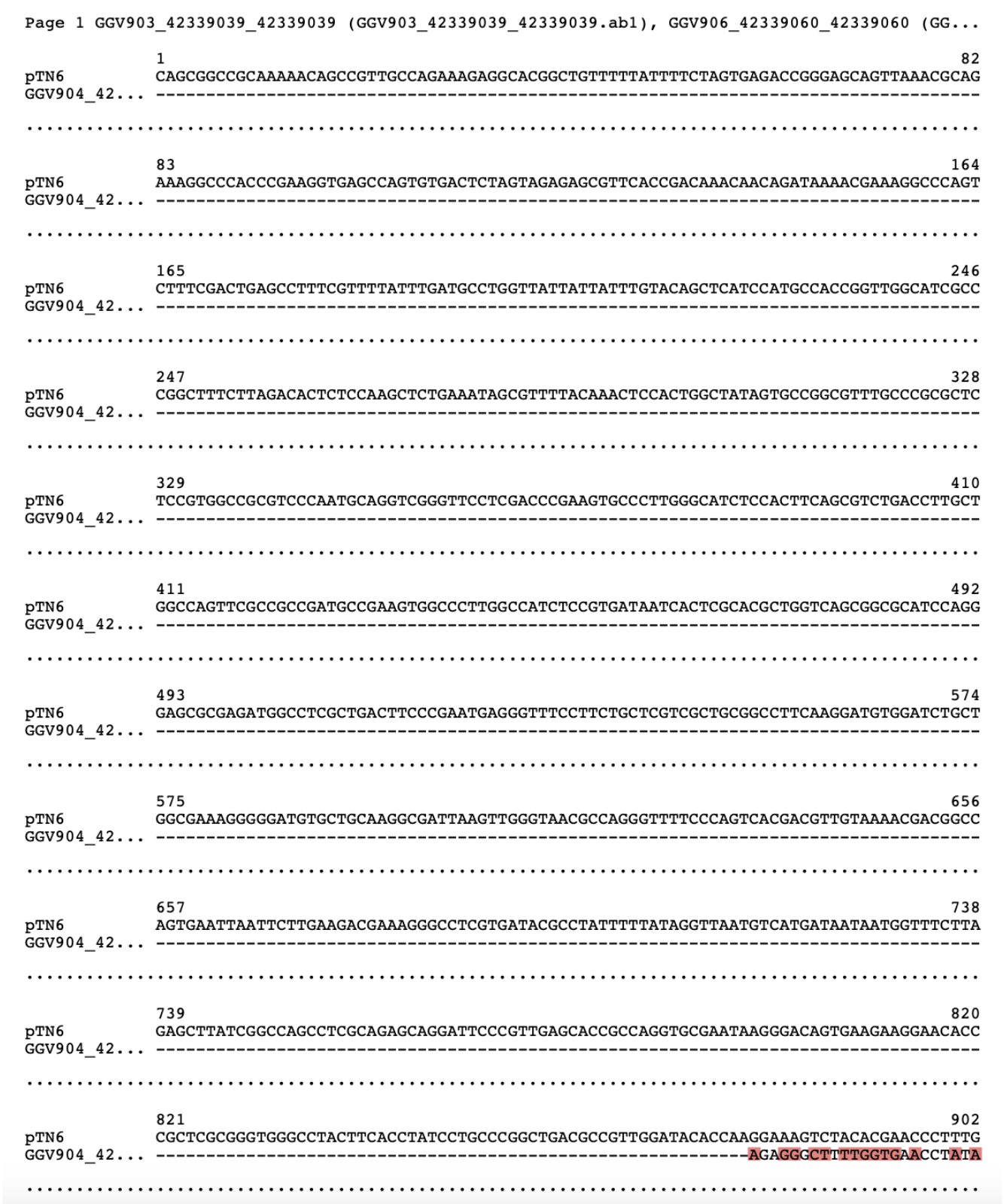
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pTN6 CGGCTTTCTTAGACACTCTCCAAGCTCTGAAATAGCGTTTTACAAACTCCACTGGCTATAGTGCCGGCGTTTGCCCGCGCTC
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pTN6 GAGCTTATCGGCCAGCCTCGCAGAGCAGGATTCCCGTTGAGCACCGCCAGGTGCGAATAAGGGACAGTGAAGAAGGAACACC
GGV904_42...
821 902
pTN6 CGCTCGCGGGTGGGCCTACTTCACCTATCCTGCCCGGCTGACGCCGTTGGATACACCAAGGAAAGTCTACACGAACCCTTTG
GGV904_42... ----------AGAGGGCTTTTGGTGAACCTATA

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            903                                                                 984
pTN6        GCAAAATCCTGTATATCGTGCGAAAAAGGATGGA--TATACCGAAAAAATCGCTATAATGACCCCGAAGCAGGGTTATGCAG
GGV904_42... GAATACTCAAG------CTATGCATCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCG

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            985                                                                1066
pTN6        CGGAAAAGATCCGTCGACGGCGTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCAC
GGV904_42... CCCTTGTAACTCGTCGACGGCGTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCAC

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            1067                                                               1148
pTN6        GTAGTGGACAAATTCTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCCTGTCTAGCTTCA
GGV904_42... GTAGTGGACAAATTCTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCCTGTCTAGCTTCA

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            1149                                                               1230
pTN6        AGTATGACGGGCTGATACTGGGCCGGCAGGCGCTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTA
GGV904_42... AGTATGACGGGCTGATACTGGGCCGGCAGGCGCTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTA

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            1231                                                               1312
pTN6        CTGCGCTGTACCAAATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCGT
GGV904_42... CTGCGCTGTACCAAATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCGT

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            1313                                                               1394
pTN6        TAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCTCCGCCGCTGGACCTACCAAGGCA
GGV904_42... TAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCTCCGCCGCTGGACCTACCAAGGCA

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            1395                                                               1476
pTN6        ACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCAGATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGT
GGV904_42... ACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCAGATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGT

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            1477                                                               1558
pTN6        CATTGCGCTGCCATTCTCCAAATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAACAATGGT
GGV904_42... CATTGCGCTGCCATTCTCCAAATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAACAATGGT

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            1559                                                               1640
pTN6        GACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTT
GGV904_42... GACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTT

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            1641                                                               1722
pTN6        GTTTCATCAAGCCTTACGGTCACCGTAACCAGCAAATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGT
GGV904_42... GTTTCATCAAGCCTTACGGTCACCGTAACCAGCAAATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGT

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            1723                                                               1804
pTN6        ACAAATGTACGGCCAGCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTCTGATAGTTGAGTCGATACTTCGGC
GGV904_42... ACAAATGTACGGCCAGCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTCTGATAGTTGAGTCGATACTTCGGC

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             1805                                                                1886
pTN6         GATCACCGCTTCCCTCATGATGTTTAACTTTGTTTTAGGGCGACTGCCCTGCTGCGTAACATCGTTGCTGCTCCATAACATC
GGV904_42... GATCACCGCTTCCCTCATGATGTTTAACTTTGTTTTAGGGCGACTGCCCTGCTGCGTAACATCGTTGCTGCTCCATAACATC

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             1887                                                                1968
pTN6         AAACATCGACCCACGGCGTAACGCGCTTGCTGCTTGGATGCCCGAGGCATAGACTGTACCCCAAAAAAACAGTCATAACAAG
GGV904_42... AAACATCGACCCACGGCGTAACGCGCTTGCTGCTTGGATGCCCGAGGCATAGACTGTACCCCAAAAAAACAGTCATAACAAG

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             1969                                                                2050
pTN6         CCATGAAAACCGCCACTGCGCCGTTACCACCGCTGCGTTCCTGCAGGAATTCCTCGAGACGTACGG---TGCGCGCGATGCA
GGV904_42... CCATGAAAACCGCCACTGCGCCGTTACACCGCTGCGTCCTGCAGCTACGTTCGTAATCAAGCACTTCCTTTTGCTTGACGCA

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             2051                                                                2132
pTN6         GCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGG--AGGCGGATAAAGTTG
GGV904_42... GGTGT-CGAAGGAACTCGCCAACGCCTCCTTAGTTTCACCAAACGTCCAATGTAAGAAGCCGAATCTAGGCTATTCATTTAC

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             2133                                                                2214
pTN6         CAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGG
GGV904_42... TACCCATTGGCCTGGGATCGTCAGCCACCCGCCACAATTCAGGGGAATTTGGAAATCCTCACTGGGGGCCTCGAGAGGCTTA

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             2215                                                                2296
pTN6         TATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGAT
GGV904_42... AGGGCCATTCCCCAATGGGTCATAAATTCTGGGCCTTTTTAACCGAAGGAAAAGGGGTCCAATACTGGGACCCC--------

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             2297                                                                2378
pTN6         GAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATAC
GGV904_42... --------------------------------------------------------------------------------

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             2379                                                                2460
pTN6         TTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCC
GGV904_42... --------------------------------------------------------------------------------

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             2461                                                                2542
pTN6         TTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGG
GGV904_42... --------------------------------------------------------------------------------

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             2543                                                                2624
pTN6         GGGATCAGGACCGCTGCCGGAGCGCAACCCACTCACTACAGCAGAGCCATGTAGGGCCGCCGGCGTTGTGGATACCACGCGG
GGV904_42... --------------------------------------------------------------------------------

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             2625                                                                2706
pTN6         AAAACTTGGCCCTCACTGACAGATGAGGGGCGGACGTTGACACTTGAGGGGCCGACTCACCCGGCGCGGCGTTGACAGATGA
GGV904_42... --------------------------------------------------------------------------------

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             2707                                                                                2788
pTN6         GGGGCAGGCTCGATTTCGGCCGGCGACGTGGAGCTGGCCAGCCTCGCAAATCGGCGAAAACGCCTGATTTTACGCGAGTTTC
GGV904_42... --------------------------------------------------------------------------------

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             2789                                                                                2870
pTN6         CCACAGATGATGTGGACAAGCCTGGGGATAAGTGCCCTGCGGTATTGACACTTGAGGGGCGCGACTACTGACAGATGAGGGG
GGV904_42... --------------------------------------------------------------------------------

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             2871                                                                                2952
pTN6         CGCGATCCTTGACACTTGAGGGGCAGAGTGATGACAGATGAGGGGCGCACCTATTGACATTTGAGGGGCTGTCCACAGGCAG
GGV904_42... --------------------------------------------------------------------------------

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             2953                                                                                3034
pTN6         AAAATCCAGCATTTGCAAGGGTTTCCGCCCGTTTTTCGGCCACCGCTAACCTGTCTTTTAACCTGCTTTTAAACCAATATTT
GGV904_42... --------------------------------------------------------------------------------

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             3035                                                                                3116
pTN6         ATAAACCTTGTTTTTAACCAGGGCTGCGCCCTGGCGCGTGACCGCGCACGCCGAAGGGGGGTGCCCCCCCTTCTCGAACCCT
GGV904_42... --------------------------------------------------------------------------------

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             3117                                                                                3198
pTN6         CCCGGCCCGCTAACGCGGCACCCCATCCCCCCAGGGGCTGCGCCCCTCGGCCGCGAACGACCTCACCCCAAAAATGGCAGCC
GGV904_42... --------------------------------------------------------------------------------

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             3199                                                                                3280
pTN6         ACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAA
GGV904_42... --------------------------------------------------------------------------------

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             3281                                                                                3362
pTN6         GCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCAAGCGAACC
GGV904_42... --------------------------------------------------------------------------------

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             3363                                                                                3444
pTN6         GGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAGGATC
GGV904_42... --------------------------------------------------------------------------------

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             3445                                                                                3526
pTN6         TGATGGCGCAGGGGATCAAGATCGACGGATCGATCCGGGGAATTAATTCCGGGGCAATCCCGCAAGGAGGGTGAATGAATCG
GGV904_42... --------------------------------------------------------------------------------

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             3527                                                                                3608
pTN6         GACGTTTGACCGGAAGGCATACAGGCAAGAACTGATCGACGCGGGGTTTTCCGCCGAGGATGCCGAAACCATCGCAAGCCGC
GGV904_42... --------------------------------------------------------------------------------

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            3609                                                                       3690
pTN6        ACCGTCATGCGTGCGCCCCGCGAAACCTTCCAGTCCGTCGGCTCGATGGTCCAGCAAGCTACGGCCAAGATCGAGCGCGACA
GGV904_42... ------------------------------------------------------------------------------

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            3691                                                                       3772
pTN6        GCGTGCAACTGGCTCCCCCTGCCCTGCCCGCGCCATCGGCCGCCGTGGAGCGTTCGCGTCGTCTCGAACAGGAGGCGGCAGG
GGV904_42... ------------------------------------------------------------------------------

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            3773                                                                       3854
pTN6        TTTGGCGAAGTCGATGACCATCGACACGCGAGGAACTATGACGACCAAGAAGCGAAAAACCGCCGGCGAGGACCTGGCAAAA
GGV904_42... ------------------------------------------------------------------------------

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            3855                                                                       3936
pTN6        CAGGTCAGCGAGGCCAAGCAGGCCGCGTTGCTGAAACACACGAAGCAGCAGATCAAGGAAATGCAGCTTTCCTTGTTCGATA
GGV904_42... ------------------------------------------------------------------------------

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            3937                                                                       4018
pTN6        TTGCGCCGTGGCCGGACACGATGCGAGCGATGCCAAACGACACGGCCCGCTCTGCCCTGTTCACCACGCGCAACAAGAAAAT
GGV904_42... ------------------------------------------------------------------------------

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            4019                                                                       4100
pTN6        CCCGCGCGAGGCGCTGCAAAACAAGGTCATTTTCCACGTCAACAAGGACGTGAAGATCACCTACACCGGCGTCGAGCTGCGG
GGV904_42... ------------------------------------------------------------------------------

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

            4101                                                                       4182
pTN6        GCCGACGATGACGAACTGGTGTGGCAGCAGGTGTTGGAGTACGCGAAGCGCACCCCTATCGGCGAGCCGATCACCTTCACGT
GGV904_42... ------------------------------------------------------------------------------

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

            4183                                                                       4264
pTN6        TCTACGAGCTTTGCCAGGACCTGGGCTGGTCGATCAATGGCCGGTATTACACGAAGGCCGAGGAATGCCTGTCGCGCCTACA
GGV904_42... ------------------------------------------------------------------------------

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            4265                                                                       4346
pTN6        GGCGACGGCGATGGGCTTCACGTCCGACCGCGTTGGGCACCTGGAATCGGTGTCGCTGCTGCACCGCTTCCGCGTCCTGGAC
GGV904_42... ------------------------------------------------------------------------------

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            4347                                                                       4428
pTN6        CGTGGCAAGAAAACGTCCCGTTGCCAGGTCCTGATCGACGAGGAAATCGTCGTGCTGTTTGCTGGCGACCACTACACGAAAT
GGV904_42... ------------------------------------------------------------------------------

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            4429                                                                       4510
pTN6        TCACATGGGAGAAGTACCGCAAGCTGTCGCCGACGGCCCGACGGATGTTCGACTATTTCAGCTCGCACCGGGAGCCGTACCC
GGV904_42... ------------------------------------------------------------------------------

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            4511                                                                                4592
pTN6        GCTCAAGCTGGAAACCTTCCGCCTCATGTGCGGATCGGATTCCACCCGCGTGAAGAAGTGGCGCGAGCAGGTCGGCGAAGCC
GGV904_42... --------------------------------------------------------------------------------

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            4593                                                                                4674
pTN6        TGCGAAGAGTTGCGAGGCAGCGGCCTGGTGGAACACGCCTGGGTCAATGATGACCTGGTGCATTGCAAACGCTAGGGCCTTG
GGV904_42... --------------------------------------------------------------------------------

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            4675                                                                                4756
pTN6        TGGGGTCAGTTCCGGCTGGGGGTTCAGCAGCCACCTGCATCGCAAGCTTGCTAGAGGGTCAGCTTTATGCTTGTAAACCGTT
GGV904_42... --------------------------------------------------------------------------------

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            4757                                                                                4838
pTN6        TTGTGAAAAAATTTTTAAAATAAAAAAGGGGACCTCTAGGGTCCCCAATTAATTAGTAATATAATCTATTAAAGGTCATTCA
GGV904_42... --------------------------------------------------------------------------------

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            4839                                                                                4920
pTN6        AAAGGTCATCCACCGGATCAATTCCCCTGCTCGCGCAGGCTGGGTGCCAAGCTCTCGGGTAACATCAAGGCCCGATCCTTGG
GGV904_42... --------------------------------------------------------------------------------

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            4921                                                                                5002
pTN6        TAGTCGGCAAATAATGTCTAACAATTCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCAAGCGTTAGATGCACTAA
GGV904_42... --------------------------------------------------------------------------------

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            5003                                                                                5084
pTN6        GCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTTTTATTATTTTTAAGCGTGCATAATAAGCCCTACACAAAT
GGV904_42... --------------------------------------------------------------------------------

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            5085                                                                                5166
pTN6        TGGGAGATATATCATGAAAGGCTGGCTTTTTCTTGTTATCGCAATAGTTGGCGAAGTAATCGCAACATCCGCATTAAAATCT
GGV904_42... --------------------------------------------------------------------------------

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            5167                                                                                5248
pTN6        AGCGAGGGCTTTACTAAGCTGATCCGGTGGATGACCTTTTGAATGACCTTTAATAGATTATATTACTAATTAATTGGGGACC
GGV904_42... --------------------------------------------------------------------------------

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            5249                                                                              5327
pTN6        CTAGAGGTCCCCTTTTTTATTTTAAAAATTTTTTCACAAAACGGTTTACAAGCATAAAGCTGACTCTAGCTAGAGGATC
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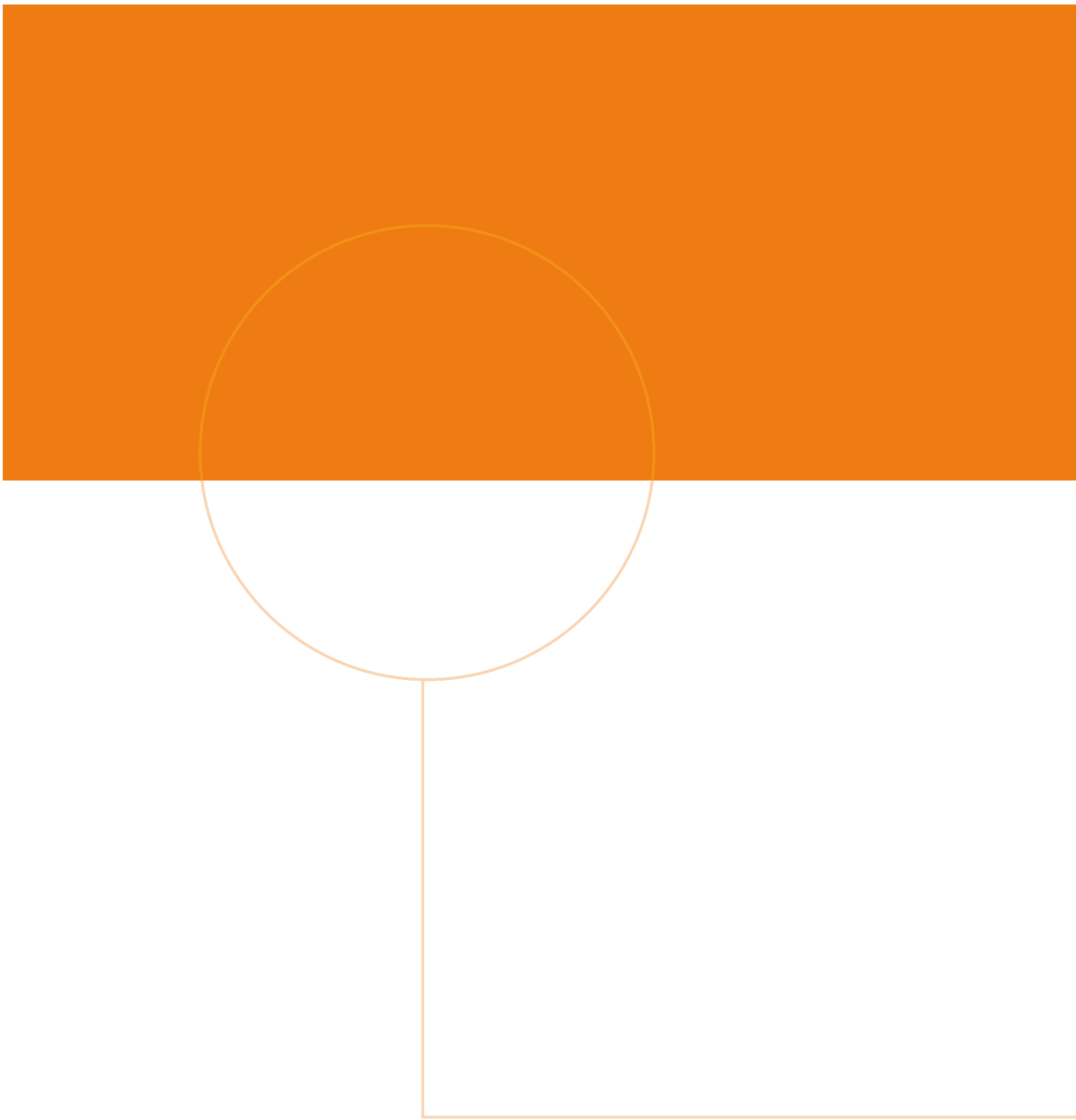

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