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Advancements in Construction of XylS/*Pm* Expression Vectors in Gram-positive Bacteria

Evaluated in *Bacillus subtilis* and *Lactobacillus plantarum*

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Biotechnology (5 year)
Submission date: May 2018
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Preface

This thesis concludes my Master of Science in Biotechnology at the Norwegian University of Science and Technology (NTNU) in Trondheim. The thesis was written at the Department of Biotechnology and Food Science (IBT) in collaboration with Vectron Biosolutions AS under the supervision of Professor Trygve Brautaset (IBT) and Dr. Morteza Moghadam (Vectron Biosolutions AS). The experimental work presented was conducted during the fall of 2017 and spring of 2018 at the laboratories of IBT.

Acknowledgements

First of all, I would like to thank my supervisor at NTNU, Professor Trygve Brautaset, and Vectron Biosolutions AS for giving me the opportunity to work on this project.

I would also like to thank Dr. Jostein Malmo and the employees at Vectron Biosolutions AS for all they have taught me, and for including me in the great social and working environment of the company. I am genuinely grateful for all your help, enthusiasm and answers to my many questions.

Dr. Morteza Moghadam deserves a special thank you for his patience, support, and valuable insight during this project.

In addition, I would like to thank all my fellow students. They have made long days in the lab rewarding and kept my enthusiasm up. A special thank you to Maiken Johnsgaard as well for all her help with LaTeX. My friends and family also deserve a thank you for all their help and support.

Abstract

Escherichia coli (*E. coli*) is the most commonly used host for expression of recombinant proteins. A large fraction of recombinant proteins produced in *E. coli* are stored in an insoluble form making them inaccessible for therapeutic use. Moving production into a Gram-positive host, could increase the availability. Many Gram-positive species can secrete proteins into the growth medium, and have a different intercellular environment potentially allowing for soluble protein expression in cases where *E. coli* falls short. The aim of this thesis was to make further advances in the adaption of the XylS/*Pm* expression cassette for production of recombinant proteins in Gram-positive bacteria, such as *Bacillus subtilis* (*B. subtilis*) and *Lactobacillus plantarum* (*L. plantarum*). Due to time constraints, little work was completed for *L. plantarum*.

In total 12 *B. subtilis*/*E. coli* shuttle vectors harboring the XylS/*Pm* cassette were constructed. Six plasmids contained a codon-optimized *xylS*, while the remaining six had *xylS* replaced by the codon-optimized *xylS-GFP*. The constructed vectors, pMSI58-69, were successfully transformed into both *E. coli* and *B. subtilis*. Following induction of *m*-toluate, only one construct was found functional in *E. coli* producing measurable mCherry levels. None of the vectors had mCherry production in *B. subtilis*. Fluorescence from the XylS-GFP fusion protein was not observed in either species.

Further investigations into the low mCherry production, led to attempts of identifying possible bottlenecks at the XylS or mCherry levels of expression. It was desirable to determine if transcription of these two proteins were obstructed. XylS levels were evaluated for all 12 constructs in *B. subtilis*. Only constructs with the medium promoter, P_{met} had considerable XylS production. The constructs with the weak and strong promoters, P_{liaG} and P_{43} respectively, showed little or no XylS production. XylS expression for the constructs with P_{met} in *E. coli* was evaluated and compared to *B. subtilis*. Both hosts appeared to have similar expression levels in both soluble and insoluble fractions.

Expression levels of XylS for the most promising pMSI-plasmid (pMSI66) was compared to the expression levels of a previously constructed shuttle vector, pSH3. pSH3 had mCherry expression in *E.coli*, although none was observed in *B. subtilis*. Comparison in *B. subtilis* was not possible due to problems with lysis of the pSH3 harboring cells. The comparison pMSI66 in *B. subtilis* and pSH3 in *E. coli* revealed similar levels of XylS expression.

The replacement of the original promoters controlling XylS production in the XylS/*Pm* cassette has lead to a solution for the reported bottleneck in XylS production. A new bottleneck has been identified in its stead, expression from the P_m promoter. This work is a contribution to the ongoing research in developing *B. subtilis* as an alternative bacterial host to the much used *E. coli*.

Sammendrag

Escherichia coli (*E. coli*) er en av de mest brukte artene for rekombinant proteinproduksjon. En stor del av de rekombinante proteinene produsert i *E. coli* lagres i en uløselig form, noe som gjør de utilgjengelige for terapeutisk bruk. Ved å flytte produksjonen til en gram-positiv art kan tilgjengeligheten økes. Mange gram-positive bakterier kan frigi proteiner i vekstmediet, og de har et annerledes intercellulært miljø som potensielt gjør det mulig å uttrykke løselige proteiner *E. coli* ikke klarer. Målet for denne masteroppgaven var å gjøre fremskritt i tilpasningen av ekspresjonskassetten XylS/*Pm* for produksjon av rekombinante proteiner i gram-positive bakterier, slik som *Bacillus subtilis* (*B. subtilis*) and *Lactobacillus plantarum* (*L. plantarum*). På grunn av tidsnød ble lite arbeid fullført for *L. plantarum*.

Totalt 12 *B. subtilis*/*E. coli* skyttelvektorer som inneholder ekspresjonskassetten XylS/*Pm* ble konstruert. Seks plasmider hadde en kodonoptimalisert variant av genet *xylS*, mens de resterende seks hadde *xylS* byttet ut med en kodonoptimalisert *xylS-GFP*. Vektorene pMSI58-69 ble suksessfullt transformert inn i både *E. coli* og *B. subtilis*. Etter å ha blitt indusert av *m*-toluat, var kun ett plasmid funksjonelt i *E. coli* etter at målbare nivåer av mCherry ble observert. Ingen av vektorene gav mCherry-produksjon i *B. subtilis*. Fluorescens fra fusjonsproteinet XylS-GFP ble ikke observert i noen av artene.

Videre undersøkelser av den lave mCherry-produksjonen førte til forsøk på å identifisere flaskehalser i ekspresjonen av XylS og mCherry. Det var ønskelig å finne ut om transkripsjonen av disse to proteinene var hindret på noen måte. XylS-nivåene ble undersøkt for alle 12 plasmidene i *B. subtilis*. Kun de med den medium sterke promoteren, P_{met}, hadde betydelig XylS produksjon. Plasmidene med den svake og sterke promoteren P_{liaG} og P_{43} henholdsvis, hadde liten eller ingen XylS produksjon. Videre ble XylS-uttrykket for vektorene med P_{met} sammenlignet i *E. coli* og *B. subtilis*. Begge artene synes å ha lignende uttrykksnivå både i den løselige og uløselige fraksjonen.

Nivået av XylS-uttrykk for det mest lovende pMSI-plasmidet (pMSI66) ble sammenlignet med nivået til en tidligere konstruert skyttelvektor, pSH3. pSH3 hadde mCherry produksjon i *E. coli*, men ikke målbare mengder i *B. subtilis*. Sammenligning av de to plasmidene i *B. subtilis* var ikke mulig da lyseringen av cellene med pSH3 feilet. Sammenligningen av pMSI66 i *B. subtilis* og pSH3 i *E. coli* viste lignende nivåer av XylS-uttrykk.

Erstatningen av de originale promoterene som kontrollerer XylS-produksjonen i ekspresjonskassetten XylS/*Pm* gav en løsning på flaskehalsen observert. En ny flaskehals har blitt oppdaget i dens sted, da uttrykk fra P_m promoteren. Dette arbeidet er et bidrag til den pågående forskningen rundt utviklingen av *B. subtilis* som en alternativ vert til den mye brukte *E. coli*.
norsk

Nomenclature

Amp	Ampicillin
bla	Ampicillin resistance gene
Bp	Base pair
Chl	Chloramphenicol
cat	Chloramphenicol resistance gene
MRS	De Man, Rosa and Sharpe Medium
DNA	Deoxyribonucleic acid
dH_2O	Distilled H_2O
GFP	Green fluorescent protein
IB	Inclusion bodies
IPTG	Isopropyl-β-D-1-thiogalactopyranosid
Kan	Kanamycin
kan	Kanamycin resistance gene
LA	Lysogeny Broth Agar Medium
LB	Lysogeny Broth Medium
mRNA	Messenger ribonucleic acid
NB	Nutrient Broth
OD_{600}	Optical density measured at wavelength 600 nm
ORF	Open reading frame
PCR	Polymerase chain reaction
RFU	Relative fluorescence unit
RPM	Rotations per minute
SD	Shine-Dalgarno sequence
tRNA	Transfer RNA
TOL	Toluene-degenerative
UTR	Untranslated region
wt	Wild type
α	Alpha
β	Beta
σ	Sigma

Contents

1 Introduction

1.1 Recombinant DNA Technology

United Nations Educational, Scientific and Cultural Organisation (UNESCO) defines biotechnology as the utlization of living organisms, such as plants, animals or microorganisms, to promote development to benefit mankind. This definition is, however, changing. Generations ago, processes such as baking, brewing or selective breeding would have been viewed as biotechnology. Today, most think of application of modern genetics or the involvement of molecular biology and technology belonging to this era [1]. Often, biotechnology today is divided into several branches representing their own use of biotechnology. Green biotechnology typically refers to agricultural use, blue to marine use, white to industrial use, and red to medical use of biotechnology. This thesis will cross into mostly red biotechnology, utilizing molecular biology and genetic engineering to further develop an already established expression cassette. Advancements made could be utilized in the remaining three realms.

Within the field of biotechnology, the technology behind recombinant DNA is vital. Recombinant DNA is created though the use of separating and recombining DNA fragments, often from different sources. This can be done to achieve new traits beneficial for the host, or it can be used in production of a desired substance which in turn can be harvested. The use of recombinant DNA technology in bacteria allows for production of recombinant proteins. In the past decades, this technology has seen major improvements, making it possible to design plasmid-based vectors carrying DNA elements combined from different sources allowing for production of recombinant proteins [1].

1.2 Platforms for Recombinant Protein Production

DNA is universal for all living organisms on Earth. This enables DNA fragments to be transferred between organisms, and allow for production of recombinant proteins. Model organisms are organisms which have been extensively studied, are easy to cultivate, manipulate genetically and often the entire genome has been sequenced. These organisms can be used to study the genes of more complex organisms or be used for other purposes within biotechnology [1].

Expression of recombinant proteins at high levels can often lead to formation of inclusion bodies (IB) in the cytoplasm. Formation of IBs may occur due to accumulation of partially unfolded recombinant proteins. Proteins encased in inclusion bodies are neither soluble nor active [2]. Contributing factors to formation of IBs are high protein concentration due to overexpression, reducing conditions in the cytoplasm, improper interactions with enzymes involved in folding of protein and the lack of post-translational modification [3]. Other factors affecting solubility are temperature, pH and nutrient supply [2]. In order to remain functional, the proteins will need to be soluble and biologically active. Recovery of proteins from IBs is often difficult and demanding. Obtaining functional protein requires denaturation, solubilization and finally renaturation [3]. These methods are, unfortunately, often ineffective [2][3].

The continuous desire for novel therapeutics drives the development of efficient production systems of pharmaceutical proteins forward. The choice of host and production conditions are essential for the downstream processing of the product. Some examples of the most frequently used prokaryotic hosts are *E.coli* and members of the *Bacillus* species. The two species are mostly favored due their easy and inexpensive cultivation for large-scale production [4].

Depending on the host used and recombinant protein in question, different levels of expression can be achieved. Some recombinant proteins may be toxic to the cell, and thus decrease cell survival. Other factors influencing protein production, in addition to the choice of host, include use of vector, culturing parameters, co-expression of other genes or optimizing the sequence of the recombinant gene. Even if protein production is satisfactory, it is not necessarily folded properly or available in its soluble and biologically active form [5].

1.2.1 *E. coli* as a Host

The gram-negative bacteria *E.coli* is one of the biggest workhorses in biotechnology and was first described by Theodor Escheric in 1885. It was the first bacteria to be used for expression of recombinant bacteria, and today it is one of the most commonly used species for this purpose [6]. The species is desired as a host for recombinant DNA because of its simple media requirement, fast growth rate and it is well characterized. Many expression systems exist in *E. coli*, enabling a more efficient protein production. Even though the bacteria have several advantages and a lot is known about its genetics and molecular biology, not all recombinant proteins are expressed efficiently in *E. coli*. This can be due to several factors. Some examples are difference in structural features of the sequence, use of codons, mRNA instability, toxicity of resulting gene products, inappropriate protein folding, lack of post-translational modifications and lack of proper secretion mechanisms [7].

For the most common host organisms, several different expression systems are available. Depending on what is desired, the characteristics of the expression system dictates which to choose. An optimal expression system has tight control of expression, preferably induced by a cheap agent not metabolized by the host, and does not require a transport system for uptake. It is also beneficial if the system is stable for scale-up to high-density cultivation and can be used across several species. For most cases, not all these criteria can be met. The optimal system in each case will therefore vary [8].

1.2.2 Gram-Positive Expression Hosts

E. coli as a host do have some imperfections, as previously mentioned. Not all recombinant proteins can be expressed well, and the outer membrane present in all gram-negative bacteria hinder extracellular excretion leading to accumulation of protein and increased formation of inclusion bodies [9]. The outer membrane contains lipopolysaccharides, which can contaminate the final protein product. If this occurs, lipopolysaccharides are referred to as endotoxins, which can be pyrogenic in humans and other mammals [4]. Another advantage of gram-positive species relates to their naturally high capacity to secrete proteins, often extracellularly into the medium, leading to a simpler downstream processing of the recombinant protein. *E. coli* folds proteins in a reducing environment in the cytoplasm, while extracellular secretion provide better folding conditions with its oxidizing properties. This favors formation of disulfide bonds, and increase the chances of correct protein folding [9].

Bacillus subtilis is a rod-shaped, abundant gram-positive soil bacteria [4][10]. The species was first discovered in 1835 by Christian Gottfried Ehrenberg, although he named it *Vibrio subtilis*. Ferdinand Cohn later renamed the organism *Bacillus subtilis* in 1872 [11]. If *B. subtilis* is exposed to nutrient limitation while growing, it has several responses increasing its chances for survival. These include production of antibiotics, extracellular enzymes, formation of biofilm, motility and natural competence. The cells are also capable of forming heat-resistant and dormant spores capable germinating, which is perhaps the most dramatic response. *B. subtilis* has been intensively studied partly because of its importance for the industry [10]. Roughly 60% of commercially available enzymes are produced by *Bacillus* species, such as amylase or alkaline proteases. *B. subtilis* shows great promise as an expression host, but is rarely used outside industrial production of enzymes. Some contributing

factors might be too few suitable expression vectors, presence of proteases, plasmid instability and misfolded proteins. Possible solutions have been presented in recent years to increase the amount of human pharmaceutical proteins expressed with *B. subtilis* as a host. Some of these include optimization of strains by reducing the genome size to only essential genes, reducing protease activity degrading recombinant proteins produced, or co-production of chaperones to ensure proper folding. [4].

Lactobacillus plantarum belongs to the rod-shaped gram-positive family *Lactobacillaceae*. *Lactobacillus* can grow in both aerobic and anaerobic conditions, and are generally non-motile [12]. *L. plantarum* is often grouped with other family members under the umbrella term lactic acid bacteria (LAB) [13], referring to their production of lactic acid during glucose metabolism. *L. plantarum* is classified as homofermentative, since most of its glucose metabolism results in lactic acid production. LABs are used all over the world in preparation of fermented foods, such as yogurt, wine brewing and sourdough bread [12]. Several *Lactobacillus* species are part of the normal flora in animals and humans, and some strains are used commercially as probiotics to restore and improve a healthy flora [12][14]. Several expression systems have been developed for *Lactococcus lactis*, whilst the interest for expression systems in other LAB-species, among them *L. plantarum*, is increasing [13][14].

1.3 Gene Expression in Bacteria

The process of gene expression occurs through first transcription of DNA to mRNA, then the translation of mRNA to produce a peptide. It is mainly these two processes which regulate the expression of a gene. By tightly controlling the components involved in them, an expression system with preferred characteristics can be achieved. Figure 1.1 illustrates the flow of information in gene expression [1].

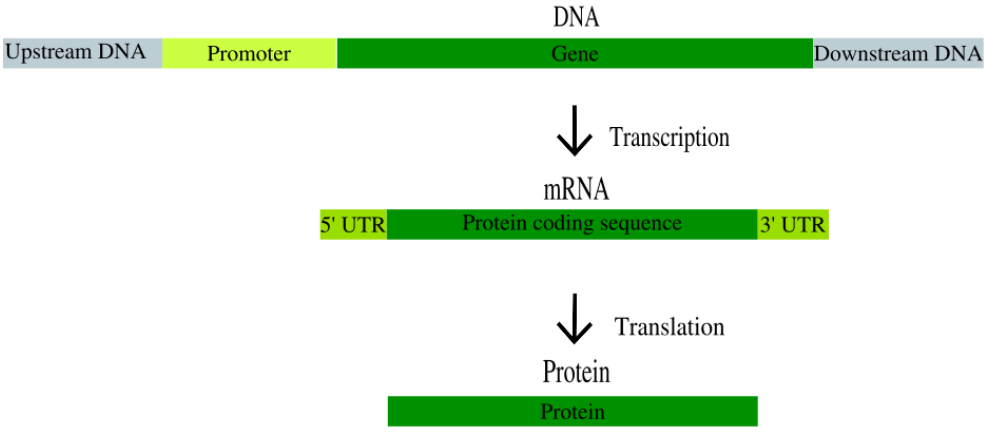

Figure 1.1: Overview of the process of gene expression i prokaryotes. DNA is transcribed into mRNA before mRNA is translated into protein. Figure adapted from [15].

1.3.1 Transcription

Transcription is the process where DNA is translated into mRNA. The process of transcription is typically divided into three major steps; initiation, elongation and termination. Initiation begins when the enzyme RNA polymerase recognizes the promoter sequence of the gene in question. The promoter sequence is the starting site for the transcriptional process. In some cases RNA polymerase is unable to recognize the promoter region without the help of transcription factors. Transcription factors are proteins capable of influencing the rate of transcription, since on occasion they are needed for regulating the initiation. Following initiation, RNA polymerase utilize the template strand of DNA to synthesize a complementary mRNA strand in the 5' to 3' direction. This is the elongation step. This step will last until RNA polymerase reaches a termination signal, thus triggering the final step; termination. The rate of transcription can be regulated by both activator and repressor proteins. They will bind to the promoter region, and can either stimulate (positive regulation) or hinder (negative regulation) binding of RNA polymerase, respectively [1].

1.3.2 Translation

The process of translation is where mRNA is translated into a peptide. Translation is also typically divided into three major steps; initiation, elongation and termination. As is illustrated in figure 1.1, a region called 5' untranslated region (5' UTR) is located downstream of the promoter sequence. This region is not translated into peptide, hence its name. Instead of being translated, it contains important regulatory binding sites for translation. One such example is the Shine-Dalgarno (SD) sequence. The SD sequence is where the ribosome binds to the mRNA, and is located approximately 9 bp upstream from the start codon [1].

mRNA is read by ribosomes, linking the appropriate amino acids together to produce a peptide. During initiation, the ribosome bind to the SD sequence, and required transcription factors will join to form the translational complex. The information stored in the mRNA molecule is deciphered in triplet bases, also known as codons. This allows for several combinations, enough to have one or more combinations representing the 20 different amino acids required to build a peptide. The combinations are mostly universal for all organisms, with some exceptions. Translation will start at the first AUG codon following the SD sequence. AUG is the most common start codon. During elongation, the mRNA is passed through the ribosome as new amino acids are added. The codons are "read" by by transfer RNA (tRNA), which contain an anticodon complementary to the codon present in the mRNA. If the codon and anticodon match, the amino acid carried by the tRNA will be added to the chain. The elongation will continue until the ribosome reaches a stop codon. This initiates binding of release factors, stimulating release of the polypeptide chain and dissociation of the ribosome [16].

1.3.3 Sigma-Factors

RNA polymerase is a holoenzyme essential for transcription of genes, as explained in section 1.3.1. Nature defines a holoenzyme as an apoenzyme together with its cofactor. An apoenzyme is defined as an active enzyme working in combination with a coenzyme determining its specificity [17]. The core or RNA polymerase consists of four subunits, β, β' and two α subunits. The cofactors needed by the enzyme are called sigma (σ) factors, and are required for RNA polymerase to be fully functional. After transcription is initiated, the σ factor is released from the holoenzyme, and only the apoenzyme continues to elongate the RNA chain [18].

In *E. coli* the predominant σ factor is σ^{70}, while in *B. subtilis* σ^{43} is the equivalent. In many species of bacteria there are alternative σ factors allowing RNA polymerase to recognize and bind to genes with different promoter sequences [18]. σ factors can be named with a number representing their weight in kDa [18][19]. Often, this terminology is abandoned and substituted with letters, allowing for incorporation of the identity of the σ factor into the name of its corresponding gene. For instance, σ^{55} has been renamed to σ^{A}, and the gene encoding it *sigA*. This further lessened the confusion introduced when the mass of the σ factors were overestimated, thus resulting in σ^{55} becoming σ^{43} which has been united under the name σ^{A} [19].

The two species *E. coli* and *B. subtilis*, have six and 17 different σ factors, respectively [20]. The choice of σ factor depends on what growth phase the cell is in. For instance in *B. subtilis*, σ^{A} is also called the housekeeping factor, while σ^{B} is involved in the stress response [19]. The remaining factors can be activated during cell growth, spore formation or extracytoplasmic stress [20].

1.3.4 Promoters

A promoter is a region located upstream of the coding sequence. This is the region recognized by RNA polymerase, and the initiation site for transcription. Promoters in bacteria have two major recognition sites called -10 and -35. The numbers they are named after refer to their estimated position upstream of the start site of transcription. The sequences of the two regions will vary, but an example of a consensus sequence for each are TATAA and TTGACA, respectively [16]. Promoters can be classified based on their estimated strength. Strength in this context refers to how frequent transcription is initiated. A strong promoter has an initiation sequence conforming to the consensus sequence, leading to frequent and tighter binding of the transcription machinery and as a consequence, initiation. A weaker promoter has substitutions in its initiation frequency, making it more demanding for the machinery to bind, thus decreasing rates of initiation and transcription [21].

1.4 Reporter Genes

Reporter genes are commonly used in genetic analyses due to their easily detectable protein products. Often they can be used to indicate the presence of a specific DNA segment in a cloning vector, but can also aid in detecting the location or amount of protein present. Two frequently used reporter genes are *GFP* and *mCherry*. Both genes encode fluorescent proteins which can be visualized *in vivo* [15][22], thus producing a gauge for their expression. Other selectable markers involve resistance genes to antibiotics. A vector can confer resistance to for instance ampicillin (*bla*), allowing only cells transformed with the plasmid to grow on plates containing ampicillin [21].

1.4.1 mCherry

mCherry is a monomeric derivative of red fluorescent protein (DsRed)[23] isolated from the coral *Discosoma*[24][25]. It was produced by directed evolution of an earlier monomeric derivative of DsRed, mRFP1 [26]. The wavelength of light required to yield maximum excitation is 485 nm, while the emission maximum of the reporter protein is 515 nm [23]. Compared to DsRed, it has decreased maturation time, high photostability and increased pH resistance [26]. A pink-colored colony or culture indicate successful uptake of the vector carrying *mCherry*, and the functional protein is produced, i.e. demonstrating a functional expression system. A visual representation is illustrated in figure 1.2.

1.4.2 Green Fluorescent Protein

GFP was originally isolated from the jellyfish *Aequorea victoria*, but has since been produced in genetically engineered and enhanced variants [15]. The protein has a size of 27 kDa, and excitation and emission maxima of 395 nm and 478 nm, respectively [27]. Fusion of *GFP* to a gene or regulatory sequence of interest, allow the expression of a desired gene to be assayed. This can produce a fusion protein if *GFP* is fused to the protein itself, or a gene fusion if *GFP* is fused to the regulatory region of the gene of interest. Fusion proteins are often used to investigate the spatial distribution and levels of the protein itself, while gene fusions rather provides insight into the regulatory region controlling expression of the gene of interest [15].

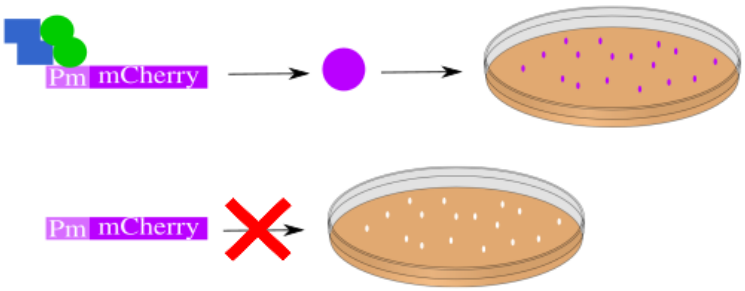

Figure 1.2: mCherry production from P_m. When functional, a XylS dimer (here XylS-GFP fusion proteins) bind to P_m to give transcription and the final protein product mCherry. This results in an observable color change in colonies cultivated on an agar plate. Without binding of a XylS dimer, there is no transcription from P_m, and thus no color change is observed.

1.5 RK2 Plasmid

The RK2 replicon is a member of a group of broad host-range plasmids, capable of regulated replication in most gram-negative bacteria [28][29]. This group is collectively referred to as the IncP plasmids, and were discovered in *Pseudomonas aeruginosa* in 1969. The ability to be transferred, replicated and maintained in several hosts makes it invaluable to genetic engineering. The most essential regions of the RK2 replicon are the origin of vegetative replication (*oriV*), and the gene trans-acting replication function (*trfA*)[29]. TrfA binds to iterons, repeating sequences, present in *oriV*. Mutated versions of TrfA have been found to affect the copy number of RK2 [30].

A new model of the initiation of replication for plasmid RK2 has been suggested, where a second protein, DnaA, is also required. DnaA is a universal initiator protein for replication from the bacterial chromosomal replication origin (*oriC*). It will recognize and bind to consensus sequences named DnaA boxes present in many *oriC* and replication origins of DnaA-dependent plasmids. Together with TrfA bound to iterons present, an open complex will form allowing helicase to bind and continue the process of replication. TrfA alone is only capable of forming a partially open complex. This protein is host specific, meaning DnaA from *E. coli* will not be able to fully function in for instance *B. subtilis*. Non-native DnaA proteins will be able to bind to the DnaA-boxes, but not engage in the DnaA-mediated unwinding of *oriC* required to allow helicase to bind. This was identified as a limiting step for plasmid host range in *B. subtilis*. DnaA from *B. subtilis* was able to interact with the DnaA box of RK2, although it failed to form a stable binding and initiate the open complex formation and as a consequence replication [31].

Minimal replicons based on RK2 have been engineered, containing only *oriV* and *trfA* [32]. One example of such a minimal replicon is pJB658. pJB658 also contains the XylS/*Pm* cassette [32][33]. *trfA* will affect the copy number of the plasmid, meaning the number of copies of said plasmid present in a cell. Regulation of a plasmid's copy number is done though a process called handcuffing. This process involves protein interactions between TrfA and the origin of replication [34].

1.6 XylS/*Pm* Expression Cassette

The XylS/*Pm* expression system originally came from the toluene-degrading (TOL) plasmid pWW0 isolated from *Pseudomonas putida* [35], This system has been found functional in a range of gram-negative bacterial species. The promoter present in the system, P_m, comes from the transcriptional regulation of the *meta*-cleavage pathway used for the breakdown of benzoate and alkylbenzoate. It is controlled by its activator XylS, which will dimerize in the presence of benzoate-derived effectors, where *m*-toluate is an example [36]. XylS belongs to the family of positive transcriptional regulators called AraC [37]. The protein consist of 321 amino acids, with a molecular mass of 36 kDa [38]. The XylS dimer binds to P_m, interacting with the transcription initiation machinery and initiating transcription [36][39]. A simplified overview of the cassette is shown in figure 1.3. Dimerization has been found to be essential for activation. It is not required for an inducer to be present for XylS to dimerize, although it happens increasingly in its presence [38]. The *xyls* gene is expressed by two tandem promoters, P_{s1} and P_{s2}, when on its native pWW0. P_{s1} is inducible and σ^{54}-dependent, while P_{s2} is constitutive and σ^{70}-dependent. When an effector is added, the XylS regulator is activated and stimulate transcription from the *meta*-operon promoter P_m [37].

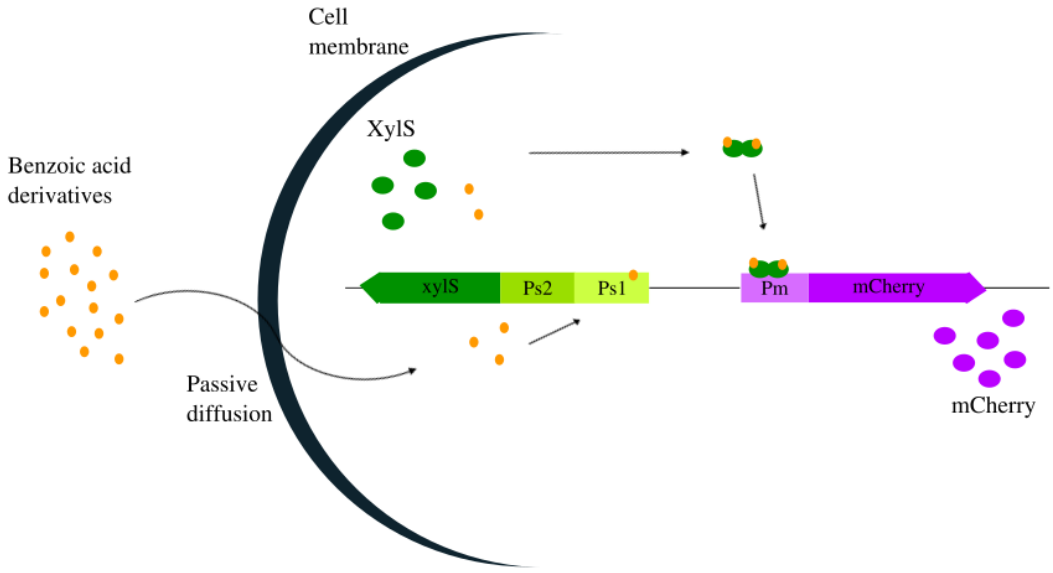

Figure 1.3: The XylS/*Pm* expression system. Inducer molecules (benzoic acid derivatives) enter the cells through passive diffusion. Inside the cell, the inducer bind to the inducible promoter P_{s1} responsible for XylS expression together with the constitutive promoter P_{s2}. XylS will dimerize in the presence of inducer, binding to P_m, activating expression of mCherry. Figure adapted from [8].

XylS has been proposed to exists in three states; monomeric, dimeric and as aggregates. When *m*-toluate is absent, XylS will be in its monomeric form, and most likely not able to activate P_m. In the presence of its effector *m*-toluate, an unknown fraction of the monomeric

XylS will form dimers and in turn activate transcription of the gene controlled by P_m. When levels of XylS are low the conversion to active dimers is seemingly dependent on inducer present. Above a certain threshold of XylS, the activity rate from P_m levels out. The explanation provided by the model is formation of XylS in a third state, as inactive aggregates. This indicates that the concentration of dimers will remain the same independent of added inducer when levels of XylS are high [40]. Overproduction of XylS typically occurs when its inducer, XylR, has been produced from the upper operon of the TOL-plasmid. XylR production is controlled by the promoter P_u, which is inducible by xylene or toluene, for instance [41].

1.7 Previous Work

A previous attempt to construct functional vectors containing the XylS/*Pm* cassette controlling expression of the reporter protein mCherry has been made. From the results achieved, it became clear mCherry expression in Gram-positive bacteria could prove difficult. Expression was observed for the vectors pSH2 and pSH3 in *E. coli* DH5α, while no expression was observed in the intended host *B. subtilis*. The plasmid copy number and mRNA levels of *mCherry* and *XylS* were evaluated to identify possible bottlenecks. Copy numbers and mRNA levels were found lower in *B. subtilis* than *E.coli*. pSH2 and pSH3 contained *xylS* placed under the native XylS/*Pm* promoters, P_{s1} and P_{s2}. Replacing of the two XylS/*Pm* promoters with native *B. subtilis* promoters was suggested as a continuation. This is believed to increase XylS production in *B. subtilis*, and in turn improve mCherry expression. An observable expression of mCherry will confirm the constructs are functional [42].

1.8 Aim of Study

As mentioned previously, *E. coli* is one of the biggest workhorses within production of recombinant proteins produced in bacteria. Often, a large fraction of protein produced is in an inactive form and trapped in inclusion bodies. Several potential advantages come with moving production to other species of bacteria. Two promising candidates are *B. subtilis* and *L. plantarum*. Gram-positive species make downstream processing less demanding by for instance extracellular secretion of produced protein. They also have a different intercellular environment, possibly resulting in appropriate folding and increased solubility in cases where *E. coli* falls short.

The work done in this thesis was intended to make advancements in the development of a toolbox for alternative expression hosts, in this case the Gram-positive species *B. subtilis* and *L. plantarum*. Some work on the two species had already been completed [42]. General growth studies on the two prospective hosts, *B. subtilis* and *L. plantarum*, are to be carried out to optimize growth conditions. An inducer diffusion study aiming to determine if the inducer of the XylS/*Pm* cassette, *m*-toluate, is able to permeate the thicker cell wall of Gram-positive bacteria is also to be completed. A potential toxicity level of *m*-toluate is to be identified. Due to time constraints, the work on *L. plantarum* was halted following growth and inducer diffusion studies.

To further investigate the bottleneck in mCherry production identified [42], the two promoters P_{s1} and P_{s2} controlling *xylS* expression are to be replaced with native *B. subtilis* promoters. Three promoters with varying strengths are to be used. Protein production from the resulting constructs are to be evaluated to determine what effect the change have on XylS and consequently mCherry production. Measuring protein expression of XylS can determine if sufficient amounts of the protein is produced to induce P_m and result in mCherry production. GFP is to be used as a reporter protein to gauge XylS levels *in vivo*.

2 Materials and Methods

2.1 Media and Solutions

The composition of media and other solutions used can be found in appendix A

2.2 Bacteria Strains, Plasmids and Growth Conditions

An overview and description of the different bacteria strains and plasmids used or engineered in this study can be found in tables 2.1 and 2.2, respectively.

E. coli DH5α was used as a general cloning host for plasmid construction, while *E. coli* BL21 was used as an expression host. *E. coli* was cultivated in Lysogeny Broth (LB) medium or LB Agar medium. Bacteria cultivated in LB were incubated at 37°C and 225 rotation per minute (rpm), while bacteria cultivated on LA were incubated at 37 °C without shaking. Different antibiotics were used as selective pressure, and was selected depending on the presence of antibiotic resistance genes contained by the plasmid harbored by the bacteria. Three different resistance genes were used, *bla*, *cat* and *kan*. These genes confer ampicillin, chloramphenicol and kanamycin resistance, respectively. Generally, *E. coli* cultures had a final concentration of 100 μg/mL of ampicillin or 50 μg/mL of kanamycin added. Chloramphenicol was only used for cultivation of *B. subtilis*, and had a final concentration of 5 μg/mL added. The antibiotic resistance conferred by each individual plasmid can be found in table 2.2.

Throughout this study several instruments were used repeatedly. For incubation of overnight cultures or shake flasks, the incubator used was HT Infors Multitron. The optical density at 600 nm (OD_{600}) of overnight or shake flask cultures were determined using the spectrophotometer Unicam Helios Epsilon, while for 96-well plates the spectrophotometer used was Molecular Devices SpectraMax Plus 384.

Table 2.1: Bacteria strains used in this study

Strains	Description	Reference
E. coli DH5α	General cloning host. Enables high-efficiency transformations.	Bethesda Research Laboratories (BSL)
E. coli BL21	Expression host for recombinant proteins. Does not express T7 RNA Polymerase.	New England Biolabs (NEB)
B. subtilis 168	Expression host for recombinant proteins.	Novozymes
L. plantarum NC8	Expression host for recombinant proteins.	Culture Collection, University of Gothenburg (CCUG)

2.2.1 Growth Study for *B. subtilis* and *L. plantarum*

This study had two prospective expression hosts, *B. subtilis* 168 and *L. plantarum* NC8. Due to time constraints, only work on *B. subtilis* 168 was continued following growth and inducer diffusion studies. For *B. subtilis*, the optimal medium and temperature of the bacteria was to be determined. Two different media were tested, LB medium and Nutrient Broth (NB), in two sets, 30°C and 37°C, both at 225 rpm. The second prospective host, *L. plantarum* NC8 was cultivated in De Man, Rosa and Sharpe (MRS) broth at 30°C and 40 rpm. The results of the growth studies conducted on the two hosts can be found in section 3.1.

Table 2.2: Plasmids used and constructed in this study

Plasmids		
pVB-1A0B1-mCherry	PJB658 based (RK2-based minimal replica) vector. Promoter: P_{s1}, P_{s2} and P_m. Regulator gene: *xylS*. Reporter gene: *mCherry*. Antibiotic resistance gene: *bla* and *neo*.	[43]
pVB-1B0A1-GFP	PJB658 based (RK2-based minimal replica) vector. Promoter: P_{s1}, P_{s2} and P_m. Regulator gene: *xylS*. Reporter gene: *GFP*. Antibiotic resistance gene: *kan*.	Vectron Biosolutions AS
pVB-1A0B10-mCherry	pVB-1A0B1-mCherry with PCR-fused *xylS-GFP* fragment inserted. *xylS* was amplified from pVB-1A0B1-mCherry, and *GFP* from pVB-1B0A1-GFP.	This study
pUC57mini-B.sub-PliaG-xylS-GFP-mCherry	pUC57 harboring the synthetic sequence *xylS-GFP* under P_{liaG}. Reporter gene *mCherry* is placed under wild type P_m. *xylS* and *mCherry* are codon optimized for *B. subtilis*. Reporter gene: *mCherry*. Resistance gene: *bla*.	Synthetic vector GenScript Promoter sequence [44]
pUC57-B.sub-PliaG-xylS-mCherry	pUC57 harboring the synthetic sequence *xylS* under P_{liaG}. Reporter gene *mCherry* is placed under wild type P_m. *xylS* and *mCherry* are codon optimized for *B. subtilis*. Reporter gene: *mCherry*. Resistance gene: *bla*.	Synthetic vector GenScript Promoter sequence [44]
pUC57-P43	pUC57 harboring the synthetic sequence of promoter P_{43}. Reporter gene: *mCherry*. Resistance gene: *bla*.	Synthetic vector GenScript Promoter sequence [45]
pUC57-Pmet	pUC57 harboring the synthetic sequence of promoter P_{met}. Reporter gene: *mCherry*. Resistance gene: *bla*.	Synthetic vector GenScript Promoter sequence [46]
pSH2	MTLBS72 based shuffle vector for *E. coli* and *B. subtilis*. Expression cassette: wild type XylS/*Pm*. Reporter gene: *mCherry* Antibiotic resistance gene: *bla* and *cat*.	[42]
pSH3	MTLBS72 based shuffle vector for *E. coli* and *B. subtilis*. Expression cassette: wild type XylS/*Pm*. Reporter gene: *mCherry* Antibiotic resistance gene: *bla* and *cat*.	[42]
pMSI58	pSH2 with insertion of P_{liaG}-*xylS-GFP* from pUC57mini-B.sub-PliaG-xylS-GFP-mCherry.	This study
pMSI59	pMSI58 with insertion of P_{43} promoter from pUC57-P43.	This study
pMSI60	pMSI58 with insertion of P_{met} promoter from pUC57-Pmet.	This study
pMSI61	pSH3 with insertion of P_{liaG}-*xylS-GFP* from pUC57mini-B.sub-PliaG-xylS-GFP-mCherry.	This study
pMSI62	pMSI61 with insertion of P_{43} promoter from pUC57-P43.	This study
pMSI63	pMSI61 with insertion of P_{met} promoter from pUC57-Pmet.	This study
pMSI64	pSH2 with insertion of P_{liaG}-*xylS* from pUC57-B.sub-PliaG-xylS-mCherry.	This study
pMSI65	pMSI64 with insertion of P_{43} promoter from pUC57-P43.	This study
pMSI66	pMSI64 with insertion of P_{met} promoter from pUC57-Pmet.	This study
pMSI67	pSH3 with insertion of P_{liaG}-*xylS* from pUC57-B.sub-PliaG-xylS-mCherry.	This study
pMSI68	pMSI67 with insertion of P_{43} promoter from pUC57-P43.	This study
pMSI69	pMSI67 with insertion of P_{met} promoter from pUC57-Pmet.	This study

2.3 Growth Study of *B. subtilis*

In order to learn more about the optimal growth conditions for *B. subtilis*, a growth study was conducted.

B. subtilis 168 was inoculated from a freezer stock, and transferred to 5 mL of NB or LB medium. The cultures were cultivated in an incubator overnight at 37°C and 225 rpm. The following day, 50 mL of their respective media was inoculated with 1% of overnight culture in baffled flasks. Three biological replicates were prepared in two sets for each media. One set was grown at 30°C and 225 rpm, while the other was grown at 37°C and 225 rpm. All cultures were cultivated in an incubator. OD_{600} was measured every hour using a spectrophotometer for 8 hours, then at longer intervals until the cultures had reached the stationary phase.

2.4 Growth Study of *L. plantarum*

In order to learn more about the optimal growth conditions for *L. plantarum*, a growth study was conducted.

L. plantarum NC8 was inoculated from a freezer stock, and transferred to 5 mL MRS medium. The culture was cultivated in an incubator at 30°C at 225 rpm overnight. The following day, 50 mL MRS medium was inoculated with 1% of overnight culture in a non-baffled flask. Four biological replicates were prepared. The new cultures were cultivated in an incubator at 30°C and 40 rpm. *L. plantarum* is a facultative anaerobe [12]. Aeration was limited by not using baffles or high rpm during cultivation. The low rpm used was intended to avoid settling of cells. OD_{600} was measured every hour using a spectrophotometer for 8 hours, then at longer intervals until the cultures had reached the stationary phase.

2.5 Inducer Diffusion Study

In this study, the constructed plasmids to be used for protein expression will contain the expression cassette XylS/*Pm*. For this expression cassette to be functional, it is fundamental for the inducer, *m*-toluate, to be able to permeate the cell in order to carry out its intended function. Without inducer stimulation, gene expression from the cassette will be minimal and the vector non-functional. It was therefore important to investigate if the inducer was able to pass the thicker cell wall of the two gram-positive species *B. subtilis* and *L. plantarum*. The effects of increasing concentrations of *m*-toluate on the overall condition and growth of cells was also to be investigated. A study exploring the diffusion of the inducer *m*-toluate was therefore conducted in the two gram-positive species. The protocol was carried out according to the work done by [36].

Stock solutions with varying concentrations of the inducer were prepared from a pre-made stock solution of 1.8 M *m*-toluate. The final concentrations prepared were 0, 1, 2, 4, 6, 8, 10, 15, 20 and 30 mM. The compositions of the stock solutions can be found in appendix A.5. The different concentrations were prepared to allow the same volume to be added to each culture. This is done to avoid possible sources of error regarding differences in volumes added. One set of triplicate cultures had dH_2O added to investigate the possible effects of ethanol on growth, the solvent used for the *m*-toluate solutions. These triplicates are denoted "No EtOH" in table 2.3.

Overnight cultures of *B. subtilis* 168 and *L. plantarum* NC8 were made by inoculating cells from a freezer stock and grown in 5 mL of MRS or LB, respectively. The cultures were cultivated in an incubator overnight at 37°C and 225 rpm. The following day, the overnight culture was diluted to give a final OD_{600} of 0.1 in 50 mL LB or MRS. OD_{600} measurements were conducted in a 96-well plate using a spectrophotometer. In a deep-well plate, 1.2 mL

of the fresh culture was added to each well. In addition to this, 20 μL of inducer of varying concentrations was added according to the schematic in figure 2.3. *B. subtilis* was grown in LB at 37°C and 800 rpm, while *L. plantarum* was grown in MRS at 30°C and 200 rpm. Both plates were cultivated in incubators. The OD_{600} was monitored using a spectrophotometer every 2-4 hours by transferring 100 μL culture to a 96-well plate.

Table 2.3: Distribution of *m*-toluate concentrations in deep-well plate used for inducer diffusion study of *B. subtilis* and *L. plantarum*. The numbers (1-5) and letters (A-H) refers to the position on the plate. The cultures had equal volume of inducer added, although with varying *m*-toluate concentrations. The concentrations used were 0, 1, 2, 4, 6, 8, 10, 15, 20 and 30 mM *m*-toluate. Cultures labelled with "no EtOH" had the equivalent volume of dH_2O added.

	1	2	3	4	5
A	no EtOH	1 mM	6 mM	15 mM	30 mM
B	no EtOH	2 mM	6 mM	15 mM	
C	no EtOH	2 mM	8 mM	15 mM	
D	0 mM	2 mM	8 mM	20 mM	
E	0 mM	4 mM	8 mM	20 mM	
F	0 mM	4 mM	10 mM	20 mM	
G	1 mM	4 mM	10 mM	30 mM	
H	1 mM	6 mM	10 mM	30 mM	

2.6 Standard Molecular Techniques

2.6.1 Preparation of Super Competent Cells

B. subtilis can be cultivated to a natural competency, while *E. coli* require high concentrations of $CaCl_2$ [47]. Treatment with calcium chloride ($CaCl_2$) is an established protocol for obtaining competent cells [48]. Treatment with rubidium chloride (RbCl) together with $CaCl_2$ has further improved the method of achieving competency [49]. For this study, the treatment solutions containing $CaCl_2$ and RbCl are TFB1 and TFB2 (see section A.5). The method used to produce competent cells in this study has been adapted from a previously described method [50].

E. coli cells were inoculated from a freezer stock and grown in 5 mL of LB medium. The culture was incubated at 37°C at 225 rpm overnight in an incubator. The following day, 1% of the overnight culture was used to inoculate 100 mL PSI medium. The new culture was incubated at 37°C and 225 rpm in an incubator until an OD_{600} of 0.4 was obtained. The OD_{600} measurements were done using a spectrophotometer. The culture was then incubated on ice for 15 minutes to halt cell growth, before being transferred to a 250 mL centrifugation bottle. It was then spun down for 5 minutes at 3000 rpm and 4°C using a centrifuge (Sorvall Lynx 6000 Centrifuge). The supernatant was discarded, and the pellet resuspended in 40 mL of cold TFB1 before incubation on ice for 15 minutes. The centrifugation step was repeated, and the cells resuspended in 3 mL cold TFB2. The culture was then aliquoted in Eppendorf tubes prepared on ice (100 μL each) before being snap-frozen in liquid nitrogen and stored at -80°C.

2.6.2 Transformation of *E.coli*

Transformation is the process where cells take up naked DNA from their environment. To be capable of doing this, the cells must first be made competent (see section 2.6.1). During transformation, DNA binds to the surface of the cell before crossing the membrane into the cytoplasm [48]. The heat-shock transformation protocol used has previously been described [50].

One previously prepared tube of 100 μL competent *E. coli* cells were thawed on ice. One tube is required for each ligation or circular plasmid mix. Up to 10 μL of ligation mix or 1 μL of plasmid was added before gently mixing by flicking the tube. The cells were incubated on ice for 30 minutes, heat-shocked in a water bath at 42°C for 45 seconds before an additional incubation period on ice of two minutes. To allow cell recovery, 900 μL of pre-warmed Super Optimal Catabolite-repression (SOC) medium (37°C) was added to the tube. Cells were left for a 2-hour incubation period at 37°C and 225 rpm in an incubator, before being plated out on LB agar (LA) plates containing the appropriate antibiotic. For each transformation tube, two plates were made. One plate had 100 μL of culture added directly. The remaining culture was spun down at 13 000 rpm for 5 minutes using a centrifuge (Eppendorf MiniSpin), the supernatant discarded and the pellet resuspended in the remaining volume. The entire volume was then plated out, making the second plate. The two plates were incubated at 37°C overnight.

2.6.3 Plasmid Isolation

Isolation of plasmids was done using the kit ZR Plasmid Miniprep - Classic from Zymo Research according to instructions from the manufacturer. Plasmids were isolated from both *E. coli* DH5α and *B. subtilis*.

The kit from Zymo Research uses a traditional 3-buffer procedure for isolation of plasmid DNA from *E. coli* or other species of bacteria. The protocol used is a modified alkaline lysis protocol, and allow for isolation of plasmid DNA for further use in cloning within minutes. Alkaline lysis is made up of four steps: resuspension, lysis, neutralization and clearing of lysate [51]. Cultures of bacteria harboring the desired plasmid are cultivated overnight in appropriate media and antibiotics. In this study, 5 mL of culture was spun down in a 13 mL tube at 10 000 rpm for 3 minutes in a centrifuge (Eppendorf 5804 R). Supernatant is poured off, and the pellet resuspended in buffer P1. Buffer P2 is added to lyse the cells, before buffer P3 is used to neutralize the solution after a 2-minute incubation period. The incubation period could be extended up to 10 minutes following addition of buffer P3. The samples were spun down in a centrifuge (Eppendorf 5424 R) at 10 000 rpm for 10 minutes to allow the cell debris to form a firm pellet. Supernatant was transferred to a Zymo-Spin Column in a collection tube provided by the kit, before being centrifuged at 10 000 rpm for 30 seconds using a centrifuge (Eppendorf 5424 R). This allows the DNA to bind to the column, preventing it from being washed away during the washing steps. The spin-column is transferred to a sterile 1.5 mL eppendorf tube, and the DNA eluted in the tube. For elution of DNA, 50 μL of DNA elution buffer was added. If the plasmid was >8 kb, the elution buffer was heated to 80 °C to increase amount of eluded DNA. Purified plasmid DNA was stored at -20°C.

For isolation from *B. subtilis*, some further adaptations had to be made. The kit ZR Plasmid Miniprep - Classic from Zymo Research was primarily used, although with some adjustements taken from the protocol "Isolation of Plasmid DNA from Bacillus subtilis using the QIAprep® Spin Miniprep Kit" (Qiagen). According to the Qiagen-protocol, the bacteria culture is to be grown until an OD_{600} of 0.8-1.2 was reached. This is done to minimize incomplete lysis, secretion of nucleases into medium and formation of lysis-resistant spores. A 10 mL culture of *B. subtilis* in LB was cultivated at 37 °and 225 rpm in an incubator. The

cultures were then spun down in a centrifuge (Eppendorf 5804 R) at 4°C and 10 000 rpm for 5 minutes. To lyse the thick peptidoglycan layer of Gram-positive bacteria, lysozyme was added to buffer P1 in a concentration of 1 mg/mL. The samples were incubated at 37°C for 10 minutes in a water bath. The rest of the protocol used was identical to the one described previously.

2.6.4 Measuring DNA Concentration

The concentration of plasmid present in a sample volume was determined using NanoDrop (NanoDrop One Thermo Scientific). For each measurement, 1.5 μL of sample was added to the pedestal. The device measures absorbance of the sample at 260 nm. To estimate protein contamination, absorbance at 230 and 285 nm is also measured.

2.6.5 Digestion of Plasmids Using Restriction Enzymes

Restriction enzymes are frequently used in making recombinant DNA. This type of enzymes are endonucleases, making them capable of introducing internal cuts in DNA. Many restriction enzymes make random cuts, while some have specific DNA sequence(s) they recognize resulting in cleavage of both DNA strands at that site and only that site. In biotechnology, restriction enzymes can be utilized to isolate DNA fragments, for instance a desired gene. The fragment can then be ligated into a vector digested with the same restriction enzyme(s), making the ends compatible [15][52]. In this study several restriction enzymes were used. They are listed alphabetically in figure 2.1 along with a diagram showing their restriction sites. Some of the enzymes were used for construction of the plasmids, others for confirmation of the constructs after transformation.

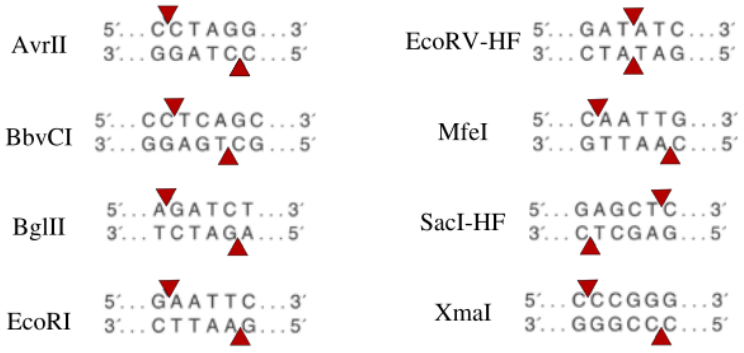

Figure 2.1: Overview of the restriction enzymes used in this study, along with their restriction sites. The introduced cuts are marked by red arrows. Digestion with all enzymes except EcoRV-HF results in sticky ends. EcoRV-HF will give blunt ends. Figures adapted from NEB.

A general set up for a digestion mix used in this study can be found in table 2.4. For each reaction the appropriate buffer was used according to the online tool "Restriction Enzyme Single/Double Digestion" from NEB (*https://nebcloner.neb.com/#!/redigest*). All reagents were transferred in appropriate volumes to Eppendorf tubes, briefly vortexed and spun down before being placed for incubation for 1-18 hours at 37°C in a water bath.

Table 2.4: Generalized digestion mix for a double digest. The amount of DNA added depended on the concentration of the sample. A DNA content of 300-500 ng was desirable.

Component	Volume
10x Buffer (NEB)	1 uL
Restriction enzyme 1 (NEB)	0.5 uL
Restriction enzyme 2 (NEB)	0.5 uL
DNA	2-8 uL
dH_2O	Up to 10 uL

2.6.6 Gel Electrophoresis

Gel electrophoresis is a method commonly used to separate DNA molecules according to size. The gel used often consists of the polysaccharide agarose dissolved in heated buffer. When the solution cools down, the gel solidifies. Wells are created by inserting a comb into the gel, and is removed after solidification. The gel is placed in a tank with a negative and positive electrode at opposite ends, which is then filled with buffer. Samples containing DNA is loaded separately into the wells produced by the comb, one sample in each. DNA is negatively charged due to its phosphate-rich backbone, and will move from the negative pole towards the positive when a current is applied. Larger DNA fragments will meet more resistance from the agarose and move slower, thus travel a shorter distance compared to smaller fragments within a given time frame. A molecular weight standard with fragments of known sizes, allowing for direct comparison of size, are included in a separate well [1].

DNA is not visual in the gel on its own. Dyes are therefore added either to the agarose solution before setting the gel, or after the gel has been run [1]. Previously, ethidium bromide have been used for this purpose. It will intercalate with the DNA of the sample allowing it to fluoresce under UV-light producing bright bands. However, this compound is known to be mutagenic, and has in most cases been replaced by safer dyes such as GelGreen (Biotium) [53].

The gel used in this study was made by dissolving 0.8 % agarose in 1x Tris-acetate-EDTA (TAE) buffer with GelGreen (Biotium) added (8 μL 10,000x per mL). Figures of the ladders used can be found in appendix D. Samples and ladder were loaded into separate wells in the gel prior to application of a current. Each sample to be run on gel had 6x loading dye (NEB) added to give a final concentration of 1x. The dye stops any digestion reactions, and gives sharper bands on agarose gels [54]. The gel was run at 90 V for 45-90 minutes, until the visual band of loading dye was about 1.5 cm from the bottom of the gel. The current was applied by a power supply (Bio-Rad PowerPac Basic Power Supply). The gel was imaged using ChemiDoc XRS+ (Bio-Rad) after completion.

2.6.7 DNA Extraction From Agarose Gel

The desired fragments separated by gel electrophoresis was excised from the gel using a clean scalpel and placed in sterile 1.5 mL Eppendorf tubes before purification. Purification was achieved using Zymoclean Gel DNA Recovery Kit from Zymo Research. This was carried out according to instructions from the manufacturer.

Agarose dissolving buffer is added to the fragments in a ratio according to their weight. To remove the gel, the fragments were incubated at 55°C until the gel was completely dissolved. The solution was then transferred to a Zymo-Spin Column in a collection tube, before being washed with washing buffers. The DNA was eluted from the column into a sterile 1.5 mL eppendorf tube using 12 μL DNA elution buffer. The DNA was stored at -20°C.

2.6.8 Ligation

Ligation is the final step in the construction of a complete recombinant plasmid. The enzyme T4 DNA ligase can be used to catalyze the reaction of joining the fragment vector and insert DNA. This enzyme catalyze the linkage reaction of the 3'-OH on one strand and the 5'-PO_4 on its neighboring strand [55]. The ends can be either blunt or sticky, depending on the presence of an overlapping fragment [56]. The following method is based on "Ligation Protocol with T4 DNA ligase" (NEB).

An online ligation calculator (NEB) was used to calculate the molar ratios and amount of DNA needed in order to reach a 1:5 vector to insert ratio. This calculator takes in the size of the two fragments to be ligated and the amount of vector DNA available, and gives the amount of insert DNA to be added (*https://nebiocalculator.neb.com/#!/ligation*). A general set up of a ligation mix used can be found in table 2.5. The components of the ligation mix were mixed in a sterile 1.5 mL Eppendorf tube, with T4 DNA ligase added last. A negative control was prepared where the insert DNA had been replaced by an equal volume of dH_2O. The mixture was briefly vortexed and micro-centrifuged before being left at 16°C for 3 hours to overnight in an incubator (Eppendorf ThermoMixer C). Following incubation, up to 10 μL of ligation mix was added to 100 μL previously prepared competent cells. The cells were then transformed according to the method described in section 2.6.2.

Table 2.5: Generalized ligation mix for ligation and re-ligation control. *x* refers to volume of insert DNA calculated by the online calculator (*https://nebiocalculator.neb.com/#!/ligation*) to give a 1:5 vector to insert ratio. *y* refers to the volume of vector DNA available. The volumes added depended on the concentration of the sample. A total DNA concentration of 50-100 ng was desirable.

Component	Ligation	Ligation control
10x T4 Ligase Buffer (NEB)	1 μL	1 μL
T4 Ligase (NEB)	0.5 μL	0.5 μL
Digested insert	x μL	No insert
Digested vector	y μL	y μL
dH_2O	Up to 10 μL	Up to 10 μL

2.7 Plasmid Construction

In this study, 12 expression vectors were constructed, and the functionality in their intended host was to be evaluated. mCherry was the reporter protein to be expressed, indicating a functional construct. Since it has previously been proven difficult to express mCherry in *B. subtilis* and *L. plantarum* [42], GFP was to be used as a second reporter protein to evaluate XylS production *in vivo*. XylS is the inducer of the P_m promoter controlling expression of mCherry. The DNA sequence of the engineered fusion protein consisted of *GFP* fused to the C-terminus of *xylS* via a linker. The sequence of the linker was GGATCCGCTGGCTC-CGCTGCTGGTTCTGGCGAATTC, encoding the amino acid linker GSAGSAAGSGEF [57]. *xylS-GFP* was to be placed under the control of three different native *B. subtilis* promoters, as can be seen in figure 2.2. The three promoters used were further described in section 2.8. Identical constructs without *GFP* fused to *xylS* were also to be engineered. Due to time constraints, only the constructs for *B. subtilis* were attempted.

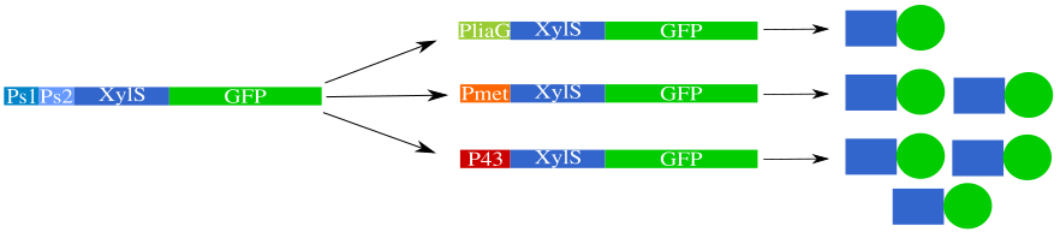

Figure 2.2: Changes made for promoters controlling XylS expression. The native XylS/*Pm* promoters P_{s1} and P_{s2} control expression of *xyls*. Xyls is shown as the fusion protein XylS-GFP. For each backbone, three promoters are used to exchange P_{s1} and P_{s2}. The relative strength of the promoters (P_{liaG}, P_{43} and P_{met}) are indicated by number of XylS-GFP fusion proteins produced in addition to their coloring.

The parental plasmids used during construction of the expression vectors are pSH2 and pSH3. pVB-1A0B1-mCherry and pVB-1B0A1-GFP are the parental vectors used in the construction of pVB-1A0B10-mCherry. pVB-1A0B1-mCherry and pVB-1B0A1-GFP are based on pJB658, a RK2 based minimal replicon containing the XylS/*Pm* cassette along with the reporter genes mCherry and GFP, respectively (see table 2.2). pSH2 is based on the *B. subtilis* expression vector pHCMC05 [58], and has had the XylS/*Pm* cassette and mCherry added from pVB-1A0B1-mCherry [42]. pSH3 is based on pHT10, a derivative of pHT01 with an added c-Myc tag [59]. pSH3 has also XylS/*Pm* cassette and mCherry added from pVB-1A0B1-mCherry [42]. Plasmid maps of the parental plasmids and the constructed expression plasmids pMSI58-69 can be found in appendix H.

The construction of the 12 expression vectors were engineered using the same principle. Identical constructs were made with or without *GFP* fused to the C-terminus of *xylS*. Out of the 12 constructs, 6 contained *GFP* (pMSI58-63), while the remaining six (pMSI64-69) were identical, although contained *xylS* without *GFP* fused. A fragment consisting of P_{liaG}, the weak promoter, along with *xylS-GFP* or *xylS* were taken from either pUC57mini-B.sub-PliaG-xylS-GFP-mCherry or pUC57mini-B.sub-PliaG-xylS-mCherry, depending on whether the final construct contained *GFP* or not, respectively. The two parental plasmids harboring the inserts were digested with SacI-HF and XmaI. Two different backbones were used, pSH2 or pSH3, both digested with SacI-HF and XmaI to ensure compatible ends to the insert. The inserts were ligated into either pSH2 (pMSI58 or pMSI64), or pSH3 (pMSI61 or pMSI67). These four constructs were then used further to construct the remaining 8 plasmids by replacing the P_{liaG} promoter using AvrII and BbvCI with P_{43} and P_{met}. pMSI59, pMSI62, pMSI65 and pMSI68 for P_{43}, while pMSI60, pMSI63, pMSI66 and pMSI69 for P_{met}. An overview of the construction of the plasmids can be found in tables 2.6 and 2.7 for constructs with and without GFP, respectively.

Table 2.6: Construction of pMSI expression vectors with the DNA sequence of *xylS-GFP*. Fragments where the size is indicated in bold font was used further.

Insert				Backbone					
Plasmid	Enzyme	Fragments		Plasmid	Enzyme	Fragments		Vector name	Promoter strength
pUC57mini-B.sub-PliaG-xylS-GFP-mCherry	SacI-HF+XmaI	**2840**	1845	pSH2	SacI-HF+XmaI	**6632**	2691	pMSI58	Weak
pUC57-P43	AvrII+BbvCI	2728	**98**	pMSI58	AvrII+BbvCI	**9335**	137	pMSI59	Strong
pUC57-Pmet	AvrII+BbvCI	2728	**1081**	pMSI58	AvrII+BbvCI	**9335**	137	pMSI60	Medium
pUC57mini-B.sub-PliaG-xylS-GFP-mCherry	SacI-HF+XmaI	**2840**	1845	pSH3	SacI-HF+XmaI	**6396**	2691	pMSI61	Weak
pUC57-P43	AvrII+BbvCI	2728	**98**	pMSI61	AvrII+BbvCI	**9099**	137	pMSI62	Strong
pUC57-Pmet	AvrII+BbvCI	2728	**1081**	pMSI61	AvrII+BbvCI	**9099**	137	pMSI63	Medium

Table 2.7: Construction of pMSI expression vectors with the *xylS* gene without fused *GFP*. Fragments where the size is indicated in bold font was used further.

Insert				Backbone					
Plasmid	Enzyme	Fragments		Plasmid	Enzyme	Fragments		Vector name	Promoter strength
pUC57-B.sub-PliaG-xylS-mCherry	SacI-HF+XmaI	2677	**2087**	pSH2	SacI-HF+XmaI	**6632**	2691	pMSI64	Weak
pUC57-P43	AvrII+BbvCI	2728	**98**	pMSI64	AvrII+BbvCI	**8582**	137	pMSI65	Strong
pUC57-Pmet	AvrII+BbvCI	2728	**1081**	pMSI64	AvrII+BbvCI	**8582**	137	pMSI66	Medium
pUC57-B.sub-PliaG-xylS-mCherry	SacI-HF+XmaI	2677	**2087**	pSH3	SacI-HF+XmaI	**6396**	2691	pMSI67	Weak
pUC57-P43	AvrII+BbvCI	2728	**98**	pMSI67	AvrII+BbvCI	**8346**	137	pMSI68	Strong
pUC57-Pmet	AvrII+BbvCI	2728	**1081**	pMSI67	AvrII+BbvCI	**8346**	137	pMSI69	Medium

2.8 Native *B. subtilis* Promoters

In this study, three different native promoters from *B. subtilis* were used to investigate XylS expression using mCherry as a reporter protein. It was desired to find one weak, one strong and one medium promoter to investigate the production of recombinant protein in the host.

2.8.1 P_{liaG}

P_{liaG} is a native, weak and constitutive promoter in *B. subtilis* dependent on the σ^A for transcription. Under normal stress-free conditions, P_{liaG} controls the expression of the last four genes in the liaIHGFSR locus, *liaGFSR*, allowing it to control the production of the LiaRS system. This locus, and its components, are involved in maintaining cell membrane integrity and detecting cell wall antibiotics. The cell envelope is the most important line of defence for a cell, in addition to providing its shape, transport of nutrients and withstanding the inner osmotic pressure. Maintaining the integrity of the cell membrane is therefore imperative for survival [44].

2.8.2 P_{43}

P_{43} is a native, strong, constitutive promoter in *B. subtilis*. Under normal conditions, it controls the expression of the P43 protein. The promoter is active both during exponential and lag phases of growth [45]. This is believed to be due to a characteristic of the promoter to contain overlapping sigma factor recognition sites, and therefore be recognized by both the major σ^{55} (σ^A in *B. subtilis*) and the minor σ^{37} (σ^B in *B. subtilis*) [45][60].

2.8.3 P_{met}

P_{met}, also referred to as P_{mdh}, is a promoter found in the thermotolerant and methylotrophic *Bacillus methanolicus*. Under normal conditions, the promoter controls the expression of methanol dehydrogenase (MDH). This enzyme allows the cell to oxidize methanol into formaldehyde. The promoter sequence identified upstream of MDH was found to be σ^{43}-dependent [61]. As explained previously (see section 1.3.3), σ^{43} has been unified with σ^{55} in the common term σ^A [19]. This is the predominant σ factor in *B. subtilis* [18].

2.9 Construction of Pilot-Vector pVB-1A0B10-mCherry

Polymerase chain reaction (PCR) is a technique used to amplify DNA *in vitro*. Since its invention in the 1980s, the technique has revolutionized DNA cloning by eliminating the need for utilizing bacteria to amplify DNA. In order for a DNA molecule to be amplified, two synthetic oligonucleotides, often referred to as primers, are needed. Primers are complementary to the target DNA, also referred to as template, making them capable of hybridizing to it. The primers will bind at opposite ends of the template, and thus defining the ends of the segment to be amplified [62]. The polymerase used in a PCR reaction must be thermostable to handle the high temperatures during the reaction. Typically, *Taq* DNA plymerase originating from the bacteria *Thermus aquaticus* is used for DNA synthesis [56].

A PCR reaction is commonly divided into three major steps, usually repeated 20-30 times [63].

- **Denaturation:** Heating of reaction mixture, usually up to 94-98°C.
- **Annealing:** Lowering of temperature to around 50-60°C, allowing primer to anneal to now single stranded DNA template.
- **Elongation:** Increase of temperature to about 75°C, allowing *Taq* DNA Polymerase to synthesize DNA from 5' to 3'.

An attempt was made to construct the synthetic sequence where *xylS* had *GFP* fused to its C-terminus. This was to be accomplished by PCR-amplifying the two genes from two existing vectors, before fusing the two fragments together also using PCR. *mCherry* was amplified from the vector pVB-1A0B1-mCherry, while *GFP* came from the vector pVB-1B0A1-GFP.

Two different PCR reactions were prepared, PCR1 and PCR2. PCR1 was divided into two separate reaction tubes, PCR1-1 and PCR1-2. Both reaction tubes had all components listed in table 2.8 added. For PCR1-1, the primers used were 1f-frag1 and 1r-frag1-o(frag2), while the template used was pVB-1A0B1-mCherry. For PCR1-2, the primers used were 2f-frag2-o(frag1) and 2r-frag2, while the template used was pVB-1B0A1-GFP. PCR2 also had the components listed in table 2.8 added. The primers used were 1f-frag1 and 2r-frag2, while the templates were the two reaction products from PCR1-1 and PCR1-2, PCR-XylS and PCR-GFP, respectively The primer sequences and their templates are listed in appendix G. A general PCR mix is listed in table 2.8, while the program used is listed in table 2.9.

The resulting DNA fragments were purified using gel electrophoresis and excised following both PCR1 and PCR2 (see sections 2.6.6 and 2.6.7). The final XylS-GFP fragment resulting from PCR2 was digested with BglII and EcoRI and purifed using gel. The backbone for the construct was prepared from pVB-1A0B1-mCherry digested sequentially with BglII and MfeI, before purification using gel. A ligation of the two was set up in a 5:1 ratio between insert and backbone (see section 2.6.8). The ligation mix was used to transform *E. coli* DH5α (see section 2.6.2). Resulting colonies from transformation was screened to see if they contained the correct construct. This was done using restriction enzymes and gel electrophoresis. The resulting band pattern was compared to the expected pattern produced by an *in silico* cutting done in Clone Manager 9 (Sci-Ed). Following verification of the plasmid, it was transformed into its expression host, *E. coli* BL21 (see section 2.6.2). The final plasmid was named pVB-1A0B10-mCherry.

Table 2.8: PCR reaction mix used for construction of XylS-GFP expression vector pVB1A0B10.

Component	Volume
5x Q5 buffer (NEB)	10 μL
Q5 polymerase (NEB)	0.5 μL
5x GC enhancer (NEB)	10 μL
dNTP (10 mM)	1 μL
dH_2O	24.5 μL
Primer 1 (10 mM)	1.5 μL
Primer 2 (10 mM)	1.5 μL
Template	1 μL
Total	50 μL

Table 2.9: PCR program used for denaturation of DNA template, annealing of primers and DNA synthesis by DNA polymerase. Denaturation, annealing and elongation was repeated 30 times. PCR1 had an elongation step for 30 seconds, while for PCR2 it was increased to 60 seconds.

Step	Temperature	Time	
Initial denaturation	98	30 sec	
Denaturation	98	10 sec	
Annealing	70	30 sec	x30
Elongation	72	30/60 sec	
Final elongation	72	2 min	
Hold	4	∞	

2.10 Transformation of *B. subtilis*

2.10.1 Preparation of Electrocompetent *B. subtilis*

The method used to make electrocompetent *B. subtilis* has been adapted from the work done by [64].

B. subtilis 168 was inoculated from freezer stock stored at -80 °C, and grown in 5 mL of LB medium. The culture was cultivated overnight at 37 °C at 225 rpm in an incubator. The following day, 48 mL of Growth medium was inoculated with 0.6% of the overnight culture. The new culture was grown at 37 °C and 225 rpm in an incubator, until an OD_{600} of 0.85-0.95 was reached. OD_{600} measurements were conducted using a spectrophotometer. The culture was then incubated on ice for 10 minutes before harvestation. The cells were transferred to a 50 mL Falcon tube and spun down at 4 °C and 10 000 rpm for 5 minutes using a centrifuge (Eppendorf 5804 R). After this step, the cells were always kept on ice. The cells were washed in ice-cold Electroporation medium before repeating the centrifugation step. This washing step was repeated a total of 4 times. Following washing, the cells were resuspended in 1.2 mL of Electroporation solution. The culture was then aliquoted in Eppendorf tubes, 60 μL in each, snap-frozen in liquid nitrogen and stored at -80 °C.

2.10.2 Electroporation of *B. subtilis*

Electroporation was first performed in 1982 [65]. Since then, the technique has been further developed and can now be used for most cell types. Electroporation utilizes the ability of the cell membrane to act as a capacitator and is unable to pass a current. When a membrane is subjected to a high-voltage electric field, it will temporarily break down leading to formation of pores. These pores are large enough to allow macromolecules such as DNA to enter or leave [66]. Electroporation of *B. subtilis* was used to transform the 12 expression vectors pMSI58-pMSI69. The method used for electroporation has been adapted from the work done by [64].

Electrocompetent cells *B. subtilis* were thawed on ice, and 500 ng of plasmid DNA was added to each aliquot of 60 μL. The mixture was homogenized by careful pipetting, before incubation on ice for 5 minutes. Following incubation, the mixture was transferred to a pre-chilled 2 mm cuvette. Moisture was wiped from the exterior of the cuvette, bubbles eliminated and the culture evenly distributed before it was placed in the electroporator (Bio-Rad Gene Pulser Xcell), equipped with a PC module. The settings used are displayed in table 2.10. The time constants were recorded, and remained within the interval of 4.7-5.0. Immediately following electroporation, 1 mL of Outgrowth medium was added and the cells transferred to a 13 mL tube for better aeration, before incubation for 4 hours at 37°C and

225 rpm in an incubator. The culture was spun down at 10 000 rpm for 2 minutes using a centrifuge (Eppendorf 5804 R). The supernatant was discarded, and the cells resuspended in remaining volume before being plated out on LA plates with appropriate antibiotic and incubated over night at 37°C.

Table 2.10: Settings used for electroporation of *B. subtilis* 168 using a Gene Pulser Xcell (Bio-Rad).

Setting	
Voltage	2500 V
Capacitance	25 μF
Resistance	200 Ω
Cuvette	2 mm

2.11 mCherry Fluorometry

mCherry intensity was determined by the fluorescence detector TECAN Infinite 200 Pro Multifunctional Microplate Reader. TECAN measures the amount of fluorescence in relative fluorescence units (RFU). To measure the intensity of mCherry, 100 μL of culture was transferred to a black bottomed 96-well plate. The excitation and emission maxima for the protein is 485 nm and 515 nm, respectively [23]. RFU was normalized against OD_{600}. Settings for the program used can be found in appendix F.

2.12 GFP Fluorometry

GFP intensity was determined by the fluorescence detector TECAN Infinite 200 Pro Multifunctional Microplate Reader. As with mCherry activity (see section 2.11), the result of the assay is given in RFU. To measure the intensity of GFP, 1 mL of cells from culture was washed twice in Phosphate Buffered Saline (PBS) before being resuspended in PBS with the equivalent volume. Following washing and resuspension, 100 μL of the culture was transferred to a black-bottomed 96-well plate. The excitation and emission maxima for the protein is 395 nm and 478 nm, respectively. RFU was normalized against OD_{600}. Settings for the program used can be found in appendix F.

2.13 Evaluation of Protein Expression From Native *B. subtilis* Promoters

2.13.1 mCherry Expression in *E. coli* DH5α

The performance of the 12 constructed plasmids, pMSI58-69 was to be evaluated. It was desired to investigate the full functionality of the plasmids by first measuring mCherry production in *E. coli* DH5α. Production of the reporter protein mCherry indicate an operational construct. Measurements of mCherry intensity will reveal which of the plasmids have the highest production, if any is observed.

Overnight cultures were prepared of *E. coli* DH5α harboring pMSI58-pMSI69 in LB medium. Three controls were included, all in *E. coli* BL21. The positive controls included were pVB-1A0B1-mCherry and pVB-1A0B10-mCherry, and the negative was pVB-1B0A1-GFP. The cultures were prepared in a 96-well plate, and left at 37°C and 800 rpm overnight. The next day, 1.2 mL of fresh medium was inoculated with 1% overnight culture. Four biological replicates were prepared for each construct. The cultures were incubated at 37°C and 800 rpm until an OD_{600} of 1 was reached. Only presence of inducer was investigated, and the

cultures were induced with a final concentration of 1 mM *m*-toluate. Following induction, the cultures were incubated at 16°C and 800 rpm until harvest 18 hours later. mCherry measurements were conducted at harvest using a fluorometer (TECAN Infinite 200 Pro) (see section 2.11).

2.13.2 mCherry and GFP Expression in *B. subtilis*

It was desired to see how the expression vectors behaved in its intended host *B. subtilis*. Both mCherry and GFP expression was investigated. Based on the observations made for the expression done in *E. coli* DH5α, only three expression vectors were tested. They had the same backbone, pSH3, although they represented the three different promoters.

Overnight cultures of *B. subtilis* harboring pMSI68-pMSI69 in LB medium were prepared. Several positive controls were included. They were pVB-1A0B1-mCherry and pVB-1A0B10-mCherry for mCherry production, and pVB-1B0A1-GFP for GFP production. All controls were in *E. coli* BL21. Wild type *B. subtilis* was used as a negative control. pHT10-mCherry in *B. subtilis* was borrowed from [42]. The cultures were prepared in a 96-well plate, and left at 37°C and 800 rpm overnight. The next day, 1.2 mL of fresh medium was inoculated with 1% overnight culture. Four different inducer concentrations were investigated, 0 mM, 1 mM, 2 mM and 4 mM *m*-toluate. One biological replicate was prepared for each plasmid and inducer concentration. The cultures were incubated at 37°C and 800 rpm until an OD_{600} of 1 was reached. The cultures were then induced and further incubated at 16°C and 800 rpm for 18 hours. mCherry and GFP measurements were conducted at harvest using a fluorometer (TECAN Infinite 200 Pro) (see section 2.11 and 2.12)

2.13.3 XylS Production in *B. subtilis*

Based on the observations made of mCherry and GFP production from the constructs in their intended host *B. subtilis* and mCherry production in *E. coli* DH5α, it was decided to further investigate the XylS production.

Overnight cultures were prepared of *B. subtilis* harboring pMSI58-pMSI69 in LB medium. In addition, overnight cultures of *E. coli* DH5α harboring pMSI67 and wild type *B. subtilis* were prepared and used as positive and negative control, respectively. pMSI67 was chosen as a negative control due to previously observed mCherry production (see section 3.3.1). The cultures were left at 37°C and 225 rpm overnight. 30 mL of fresh LB medium was inoculated with sufficient overnight culture to get a starting OD_{600} of 0.05. All cultures were grown in baffled flasks at 37°C and 225 rpm, until an OD_{600} of 2-3 was reached. None of the cultures were induced since the constitutive expression of XylS was to be investigated. The cultures were moved for further incubation at 16°C for 18 hours. At harvest, OD_{600} was determined, and GFP intensity was measured for the cultures harboring plasmids containing *XylS-GFP* fusion (pMSI58-63) (see section 2.12). The cultures were transferred to 50 mL Falcon tubes and centrifuged for 5 minutes at 10 000 rpm and 4°C using a centrifuge (Eppendorf 5804 R). Following this step, the supernatant was discarded and the pellets were weighed before being frozen at -20°C. The pellets were further processed using the protocols for SDS-PAGE and western blots, as described in sections 2.14.1 and 2.14.2, respectively.

2.13.4 XylS Production in *E. coli* DH5α

The observations made following protein isolation, SDS-PAGE and western blot of the *B. subtilis* expression experiment investigating XylS production prompted an similar expression experiment to be conducted. For this expression study, only the constructs containing the medium promoter, P_{met} (pMSI60, pMSI63, pMSI66 and pMSI69) were included, and the host used was *E. coli* DH5α. The same protocol as described in section 2.13.3 was followed.

The positive control used was still *E. coli* DH5α harboring pMSI67, while the negative control was changed to wild type *E. coli* DH5α.

2.14 Protein Isolation

Protein isolation is a method used to investigate the protein content of a cell. This involves breaking the cell open using either chemical methods such as detergents, or mechanical methods such as beads or a blender. Proteins will differ in for instance size, charge and solubility. making it difficult to assay protein content with a single procedure. Isolation of proteins typically involve denaturation and a 2D gel, such as SDS-PAGE [15].

2.14.1 SDS-PAGE

SDS polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to separate proteins using a similar principle as gel electrophoresis used for DNA separation. Proteins are somewhat more challenging to separate on gel. They come in different shapes, sizes and charges. The pore size of the gel can be adjusted to suit the size of the protein to be studied. Not all proteins carry a uniform charge, nor are all negatively charged as is the case for DNA. Proteins are therefore treated with a negatively charged detergent, sodium dodecyl sulfate (SDS). This detergent binds to hydrophobic regions of the protein, causing them to unfold and coats them in a uniform negative charge. Protein-protein or protein-lipid interactions will also be broken, rendering all proteins in the form of freely soluble, extended polypeptide chains. To break any disulfide bonds present, a reducing agent such as β-mercaptoethanol is also added. Similar to gel electrophoresis, the samples are loaded into wells, and an electric current is applied to make the proteins migrate through the gel. Bigger proteins will meet higher resistance, and will thus travel a shorter distance compared to smaller proteins. The migration results in distinctive bands, which can be visualized by a dye such as Coomassie blue. The now visual protein bands can be compared to a molecular weight standard to determine their molecular weight [62]. The following protocol was used to prepare samples for SDS-PAGE.

The pellets were thawed on ice. Based on the weight of the pellet, 1 mL per 100 mg pellet 0.9% NaCl solution was added. All pellets were resuspended by vortexing and placed back on ice. 1 mL of the resuspended solution was transferred to a 1.5 mL tube and spun down for 5 minutes at 10.000 rpm and 4°C in a centrifuge (Eppendorf 5424 R). The supernatant was discarded with vacuum (BioChem-VacuuCenter BVC Control). 500 μL of CelLytic B Cell Lysis Reagent (Sigma) with benzonase nuclease added (1 μL per 5 mL) was used to resuspend the pellets. The samples were incubated for 30 minutes at room temperature and 100 rpm on a rocking table (Unimax 1010 Heidolph Instruments). Tubes were inverted a couple of times during the incubation period. The samples were spun down for 5 minutes at 10.000 rpm and 4°C in a centrifuge (Eppendorf 5424 R) aiming to separate soluble and insoluble protein fractions. The supernatant (soluble fraction) was transferred to a 1 mL deep-well plate. The pellet (insoluble fraction) was resuspended in 500 μL freshly made SDS running buffer (Bio-Rad Tris/Glycine Buffer). For both the soluble and insoluble fractions 100 μL was transferred to a new well and diluted by adding 400 μL SDS running buffer (Bio-Rad Tris/Glycine Buffer). The diluted samples are used for SDS-PAGE. The samples were mixed with 2x Laemmli Sample buffer (Bio-Rad) with 50 mM DTT in a 96-well PCR plate. The plate was incubated for 5 minutes at 95°C in a PCR machine (Bio-Rad C1000 Touch Thermal Cycler).

A precast gel (Bio-Rad 12% Criterion TGX) was loaded with 10-40 μL of diluted and boiled culture. In preparation for western blotting, a replicate gel was prepared. Both gels were run for 40-50 minutes at 200 V, until the band of lading dye reached the bottom of the gel. Following SDS-PAGE, one gel was stained in InstantBlue (Expedeon), a Coomassie blue

based staining solution, for 1 hour before being destained in dH_2O. The replicate gel was used for western blotting as described in section 2.14.2.

2.14.2 Western Blotting

Western blotting is a technique used to detect a desired protein in a mixture of proteins by using antibodies capable of binding the target protein. After being separated according to size using SDS-PAGE, the protein mixture is transferred to a membrane, typically nitrocellulose, using electrophoresis. The membrane is positively charged, and the negatively charged proteins will adhere to it. In order to detect the target protein, the membrane must be exposed to a primary antibody. A primary antibody is an antibody which binds the target protein directly, as opposed to a secondary antibody which will bind to the primary antibody. The secondary antibody typically carries a tag making it easily detectable, amplifies the signal, and will indirectly label the desired protein [15]. In this study, the primary antibody used was XylS antibody raised in rabbit (Genscript, cat# SC1676), and the secondary was Goat Anti-Rabbit IgG F(c) Polyclonal Antibody (ImmunoReagents Inc, cat# IMMRIR2226).

SDS-PAGE and its preparations was carried out as described in section 2.14.1. The transfer sandwich was prepared using Trans-Blot® Turbo™ Midi PVDF Transfer Packs (Bio-Rad, cat# 1704157) containing a pre-wetted PVDF membrane and filter papers. Proteins were transferred to the membrane using Trans-Blot Transfer System (Bio-Rad, cat# 1704150). The cassette was then placed in the Trans-Blot Transfer System (Bio-Rad). The program used was the pre-set "Mixed molecular weight". The running conditions were 2.5 A, 25 V and the running time was 7 minutes.

Following transfer of proteins, the membrane was placed in an iBind Flex device (Invitrogen iBind Flex Western System) for detection using primary and secondary antibodies. The protocol supplied with the device was followed [67]. The composition of the solutions needed for operating the iBind Flex device can be found in tables 2.11, 2.12 and 2.13. The optimal amount of added antibodies was not known. Different quantities were therefore tested and are listed as a range. The antibody stock solutions used had a concentration of 0.408 mg/mL for XylS antibody raised in rabbit, and 0.5 mg/mL for goat anti-rabbit.

Table 2.11: Components needed to make 100 mL of 1x iBind Flex solution. Taken from the protocol supplied with the iBind Flex system [67].

Component	Volume
iBind Flex 5x Buffer	20 mL
iBind Flex 100x additive	1000 μL
Distilled water	79 mL

Table 2.12: Components needed to make a diluted solution of primary antibody, XylS antibody raised in rabbit (Genscript). The optimal amount of primary antibody was not known. Several quantities were tested, and the volume is given as a range. The concentration of the antibody stock solution was 0.408 mG/mL. Adapted from the protocol supplied with the iBind Flex system [67].

Component	Volume
1x iBind Flex solution	5 mL
XylS-rabbit antibody (Genscript)	5-25 μL

Table 2.13: Components needed to make a diluted solution of secondary antibody, Goat Anti-Rabbit IgG F(c) Polyclonal Antibody (ImmunoReagents Inc). The optimal amount of secondary antibody was not known. Several quantities were tested, and the volume is given as a range. The concentration of the antibody stock was 0.5 mg/mL. Adapted from the protocol supplied with the iBind Flex system [67].

Component	Volume
1x iBind Flex solution	5 mL
Goat Anti-Rabbit antibody (ImmunoReagents Inc.)	25-50 μL

The blot was left in the device for 2.5 hours to overnight. After the western blot had gone to completion, the membrane was washed in 20 mL dH_2O for 2 minutes. Alternatively, if the blot did not completely finish after being left overnight, it was first washed in 20 mL 1x iBind Flex solution then dH_2O. For developing the blot, 2 mL of Tetramethylbenzidine (TMB, SIGMA) was applied and re-applied before incubation at room temperature for 1-10 minutes. The reaction was stopped by rinsing the membrane with dH_2O. Both the gel and the membrane was imaged using ChemiDoc XRS+ (Bio-Rad).

3 Results

3.1 Growth Studies

A typical growth curve consist of four phases; lag phase, exponential phase, stationary phase and death phase. It is during the exponential phase the cells are the most healthy and dividing rapidly. This is also the stage where the generation time of a bacteria can be calculated. The generation time is the time needed for the cell mass to double. Based on the slope of a linear regression line fitted to the exponential phase, the generation time can be calculated [21].

3.1.1 Growth Study of *B. subtilis*

A growth study of *B. subtilis* 168 was conducted to determine the optimal conditions for cultivation. Two sets of three biological replicates were prepared in NB or LB medium. One set of replicates for each medium was cultivated at 30°C, the other at 37°C. The averages of the three biological replicates are displayed in figures 3.1 and 3.2 for NB and LB medium, respectively, along with the calculated standard deviation. A linear regression was calculated for the exponential phases. The regression lines and their slopes, μ [h^{-1}], are also displayed in figures 3.1 and 3.2. The generation times were calculated to be 65 minutes and 36 minutes for 30°C and 37°C in NB, respectively. While in LB the generation times were 57 minutes and 32 minutes for 30°C and 37°C. The calculated generation times reveal that *B. subtilis* 168 grows faster in LB compared to NB, and at 37°C compared to 30°C.

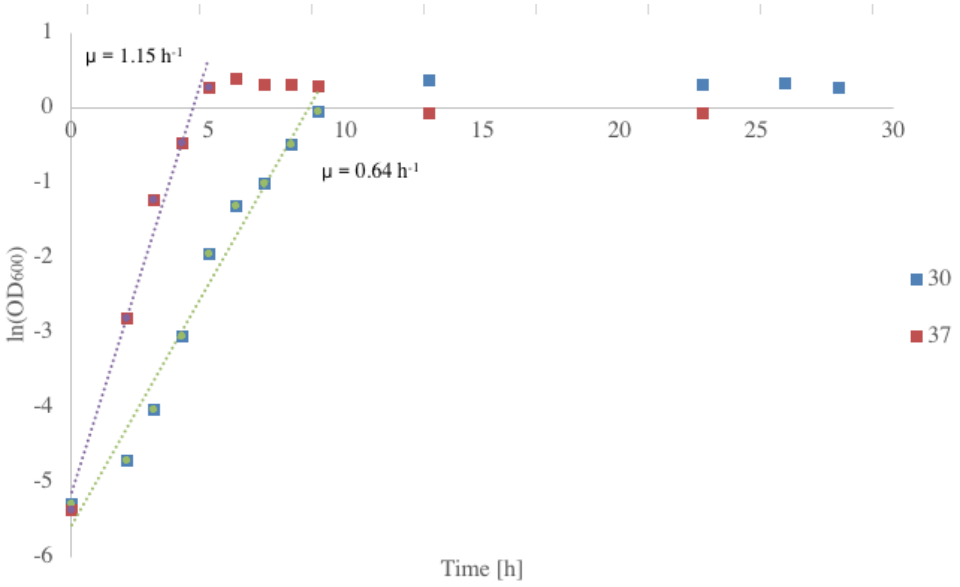

Figure 3.1: Semi-logarithmic plot of OD_{600} for *B. subtilis* 168 growth in NB medium at 30 and 37°C and 225 rpm. Error bars represent standard deviation of measurements for three biological replicates. The linear regression of the exponential phase and its slope, μ [h^{-1}], are also included.

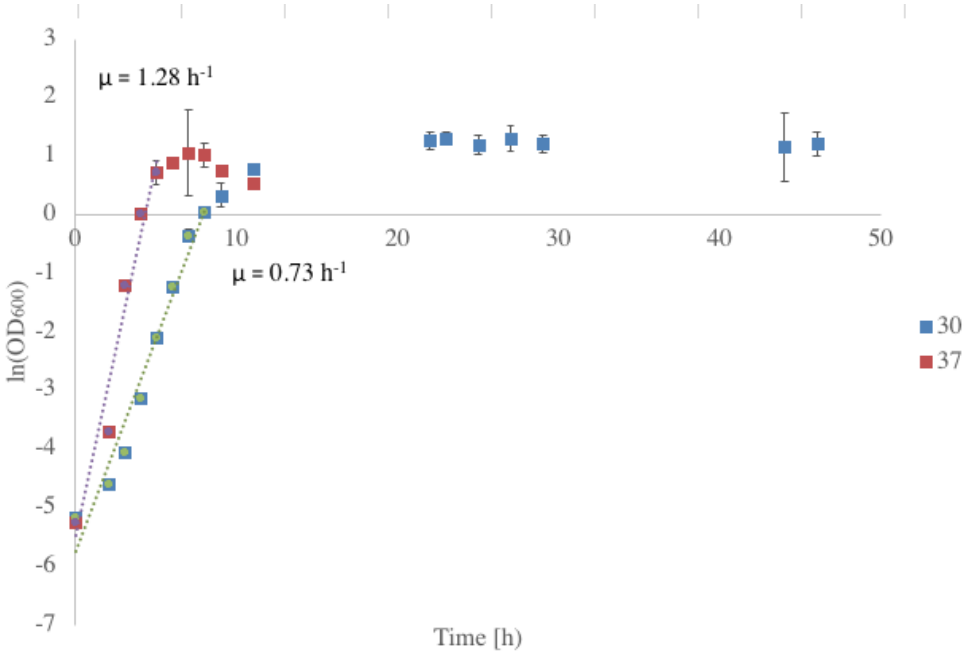

Figure 3.2: Semi-logarithmic plot of OD_{600} for *B. subtilis* 168 growth in LB medium at 30 and 37°C and 225 rpm. Error bars represent standard deviation of measurements for three biological replicates. The linear regression of the exponential phase and its slope, μ [h^{-1}], are also included.

3.1.2 Growth Study of *L. plantarum*

A growth study of *L. plantarum* NC8 grown in MRS was conducted to determine the optimal conditions for cultivation. MRS is a medium specifically designed for *Lactobacilli* [68]. A comparison of growth in LB and MRS had previously been carried out. MRS was found to give the shortest generation time [42], thus only MRS was used in this study. In the growth study previously conducted, *L. plantarum* was cultivated at 37°C and 225 rpm [42]. *L. plantarum* is a facultative anaerobe [12], and a decrease from 225 to 50-40 rpm have been observed in literature to be optimal for growth [69][70]. Three biological replicates were prepared, and the average OD_{600} is displayed in figure 3.3 along with the calculated standard deviation. A linear regression was calculated for the exponential phase. The regression line and its slope, μ [h^{-1}], is also displayed in figure 3.3. The generation time was calculated to be 42 minutes.

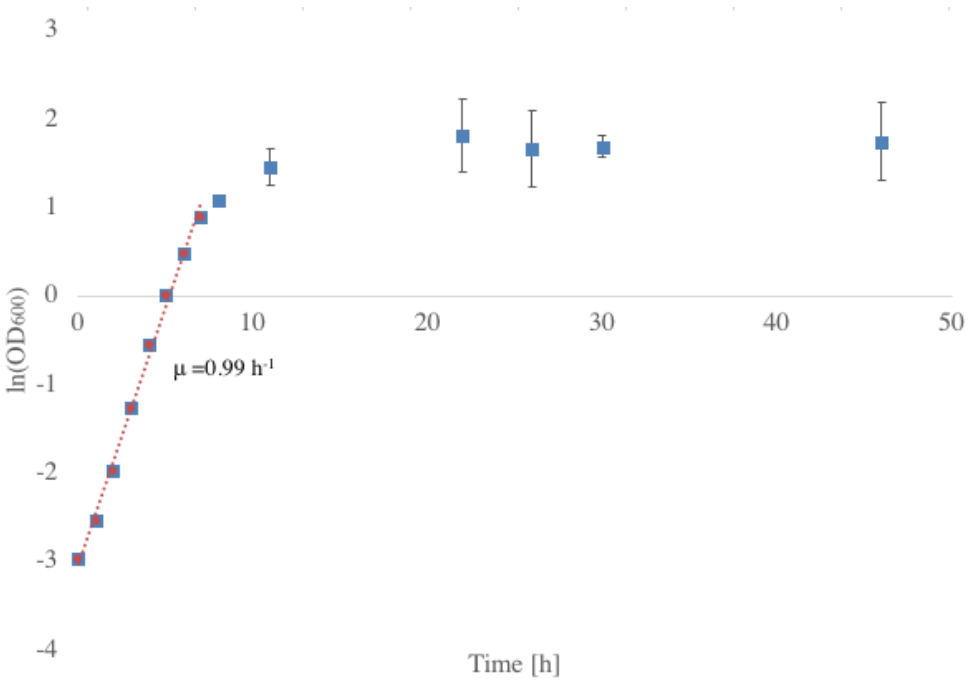

Figure 3.3: Semi-logarithmic plot of OD_{600} for *L. plantarum* NC8 growth in MRS medium at 30°C and 40 rpm. Error bars represent standard deviation for measurements of three biological replicates. The linear regression of the exponential phase and its slope, μ [h^{-1}], are also included.

3.1.3 Generation Time

The slope of each regression line displayed in figures 3.3, 3.1 and 3.2 was calculated using Microsoft Excel. Based on the slope, the generation time of the bacteria cultivated in different conditions was estimated. The complete method and a calculated example can be found in appendix C. The slopes and generation times are summarized in table 3.1.

Table 3.1: A summary of the calculated slopes and generation times of *B. subtilis* 168 and *L. plantarum* NC8. *B. subtilis* was grown in NB or LB medium, at 30°C or 37°C and 225 rpm. *L. plantarum* was grown in MRS medium at 30°C and 40 rpm. The slopes were estimated using linear regression in Microsoft Excel, and the generation time was derived based on the slope.

Species	Medium	Temperature [°C]	μ [h^{-1}]	Generation time [min]
B. subtilis	NB	30	0.64	65
B. subtilis	NB	37	1.15	36
B. subtilis	LB	30	0.73	57
B. subtilis	LB	37	1.28	32
L. plantarum	MRS	30	0.99	42

3.2 Inducer Diffusion Study

An inducer diffusion test was carried out to investigate the effects of increasing concentrations of inducer on growth. The expression cassette present in the engineered pMSI-plasmids is XylS/*Pm*. Expression from this cassette can be indirectly induced by *m*-toluate through XylS production. As of yet, *m*-toluate has not been shown capable of diffusing though the thicker cell wall of the Gram-positive species. Without the ability of permeating the cell, the inducer is not able to fulfill its intended role. It was expected to see a decrease in growth at higher *m*-toluate concentrations, indicating the inducer is able to enter the cells [36]. A potential toxicity level was also explored.

Cultures of *B. subtilis* 168 and *L. plantarum* NC8 were grown in a deep-well plate with inducer added at the time of inoculation. The inducers used gave a final concentration of 0, 1, 2, 4, 6, 8, 10, 15, 20 and 30 mM *m*-toluate. The volume of the solvent (ethanol) was constant in all samples. Controls investigating the effects of ethanol was prepared by replacing the volume of ethanol added for 0 mM *m*-toluate with dH_2O. For each concentration and species, three biological replicates were prepared. Figures 3.4 and 3.5 show the average OD_{600} as a function of time for *B. subtilis* and *L. plantarum*, respectively. Standard deviation was also calculated, and are included.

The effect of *m*-toluate on growth is most apparent at 15-30 mM in *B. subtilis* and 30 mM in *L. plantarum*. A considerable reduction in growth is observed partly for 8-10 mM in *B. subtilis* and 15-20 mM in *L. plantarum* when compared to the highest growth observed during the study. Concentrations of inducer in the range of 0-6 mM for *B. subtilis* and 0-10 mM for *L. plantarum* appear to have a limited effect on growth. The levels of inducer added which negligibly affect growth is about 2 mM for *B. subtilis*, and up to 4 mM for *L. plantarum*. In *B. subtilis*, a jump in OD_{600} can be seen for inducer concentrations of 8 and 10 mM from time point 13 to 23 hours. A similar jump for 6 mM from 5 to 13 hours.

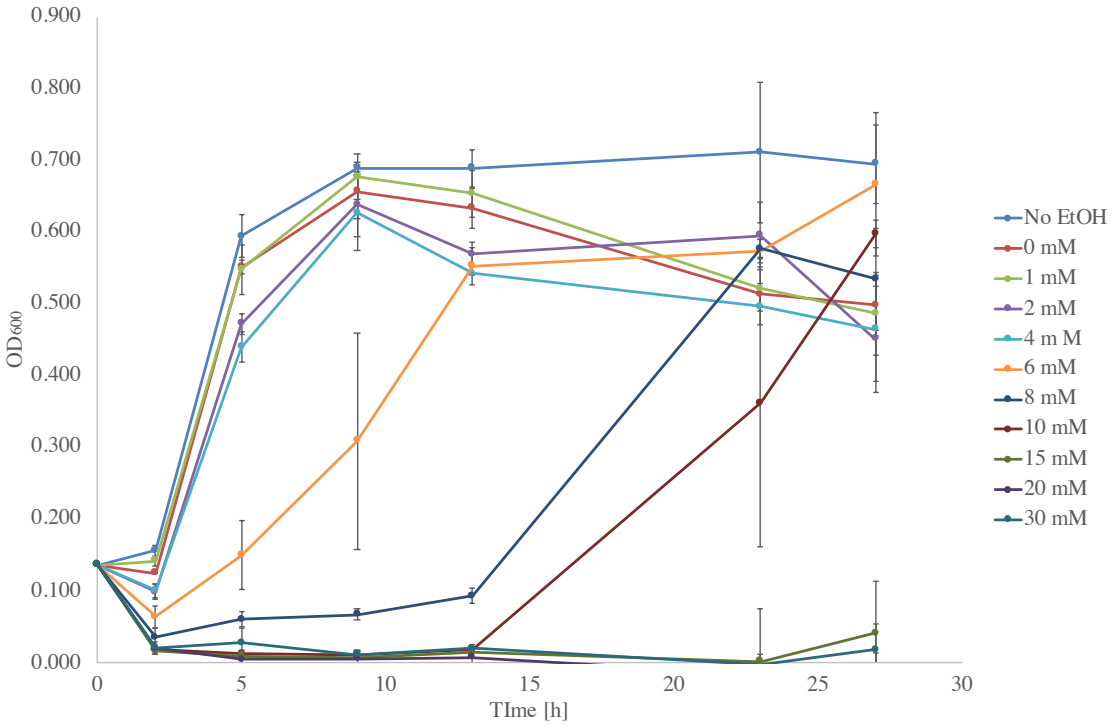

Figure 3.4: Average OD_{600} values for *B. subtilis* 168 grown in LB medium at 37°C and 800 rpm. Different concentrations of the inducer *m*-toluate was added (0-30 mM), including a control with dH_2O. Error bars represent standard deviation of measurements for three biological replicates.

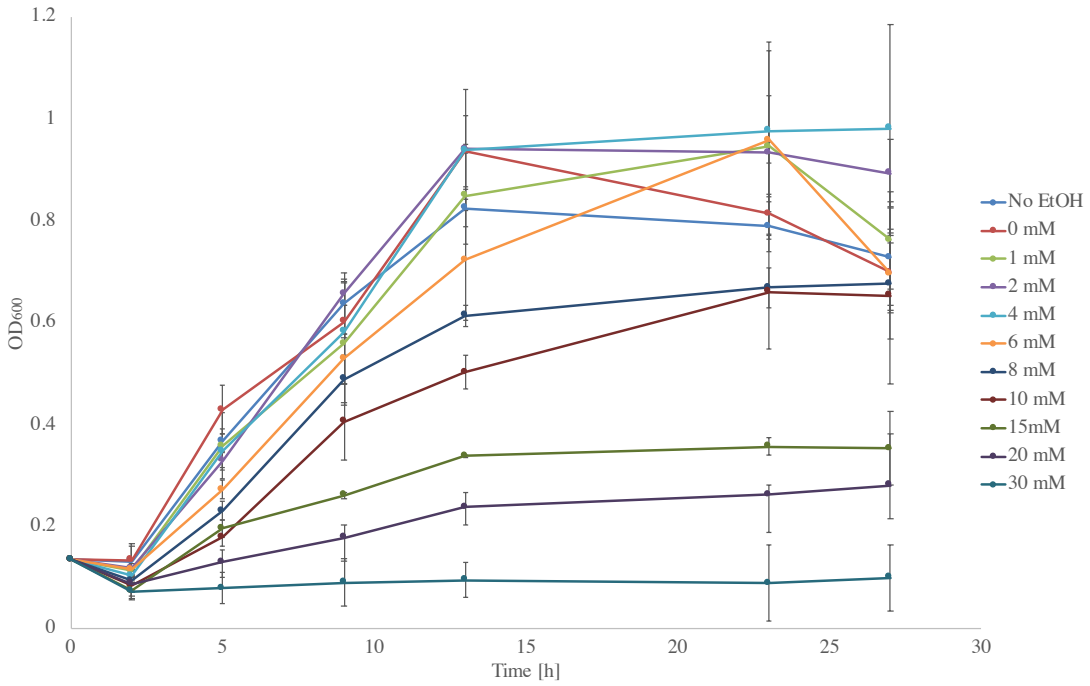

Figure 3.5: Average OD_{600} values for *L. plantarum* NC8 grown in MRS medium at 30°C and 800 rpm. Different concentrations of the inducer *m*-toluate was added (0-30 mM), including a control with dH_2O. Error bars represent standard deviation of measurements for three biological replicates.

3.2.1 pH-test of Media

A considerable decrease in growth was observed in cultures with a high *m*-toluate concentration. *m*-toluate is a carboxylic acid with a pK_a of 4.27 [71]. A change in pH was suggested as a contributing factor to the observed decrease. The possible acidifying effects of increasing concentrations of *m*-toluate on the medium used for cultivation of the two Gram-positive species *B. subtilis* and *L. plantarum* were investigated. pH-measurements of the media was conducted to see to what extent the media was acidified by *m*-toluate. The two different media used, LB and MRS, had an appropriate amount of the inducer m-toluate added and were mixed well. The pH was measured using a pH-meter. The results of the pH-test is summarized in table 3.2. A relative minor decline was observed for both media going from 1 to 10 mM of inducer. A more considerable decline in pH was observed for MRS and especially LB going from 10 to 30 mM. MRS had a small decrease of 0.19, while LB decreased 1.57 points. The most dramatic decrease of the two media was observed in LB.

Table 3.2: LB and MRS medium with inducer *m*-toluate added to give final concentrations of 1, 10 and 30 mM. The pH of the resulting media was measured using a pH-meter.

	1 mM	10 mM	30 mM
LB	6.74	6.37	4.80
MRS	5.73	5.58	5.39

3.3 Evaluation of Protein Expression From Native *B. subtilis* Promoters

Protein production from the 12 constructed plasmids, pMSI58-69 was evaluated. For six of the 12 pMSI-constructs engineered in this study, the sequence of the activator for P_m, *xylS*, was fused to *GFP*. The intention behind constructing plasmids containing this fusion was to use the fluorescent properties of the resulting GFP fusion protein to evaluate the levels of XylS present *in vivo*. This could serve as non-invasive indicator helpful in identifying bottlenecks in the production of the reporter protein mCherry. If mCherry is produced, it can be assumed the rest of the expression system is functional.

3.3.1 mCherry Expression in *E. coli* DH5α

The functionality of the plasmids in the construction host *E. coli* DH5α was investigated by measuring mCherry expression from the P_m promoter following induction with 1 mM *m*-toluate. Cultures were prepared of *E. coli* DH5α harboring pMSI58-pMSI69 in LB medium. The positive controls pVB-1A0B1-mCherry and pVB-1A0B10-mCherry, and the negative control pVB-1B0A1-GFP in *E. coli* BL21 was also included. Four biological replicates were prepared for each construct. A plot showing the average RFU normalized to OD_{600} for each construct is displayed in figure 3.6. The calculated standard deviations are included. The highest mCherry activity observed was in pMSI67 (J figure 3.6). It was even higher than the two positive controls included, pVB-1A0B1-mCherry and pVB-1A0B10-mCherry (M and N figure 3.6). The other pMSI-plasmids had low to no mCherry intensity measured. pMSI67 is identical to the construct pMSI61 (D figure 3.6), with the exception of *xylS-GFP* being present in pMSI61. pMSI61 display the second highest mCherry intensity of the pMSI-plasmids. A similar pattern where two constructs differ mostly in containing *xylS-GFP* is seen in the two positive controls pVB-1A0B1-mCherry and pVB-1A0B10-mCherry. pVB-1A0B10-mCherry contain *xylS-GFP*, and display a lower mCherry intensity when compared to pVB-1A0B1-mCherry which does not contain GFP.

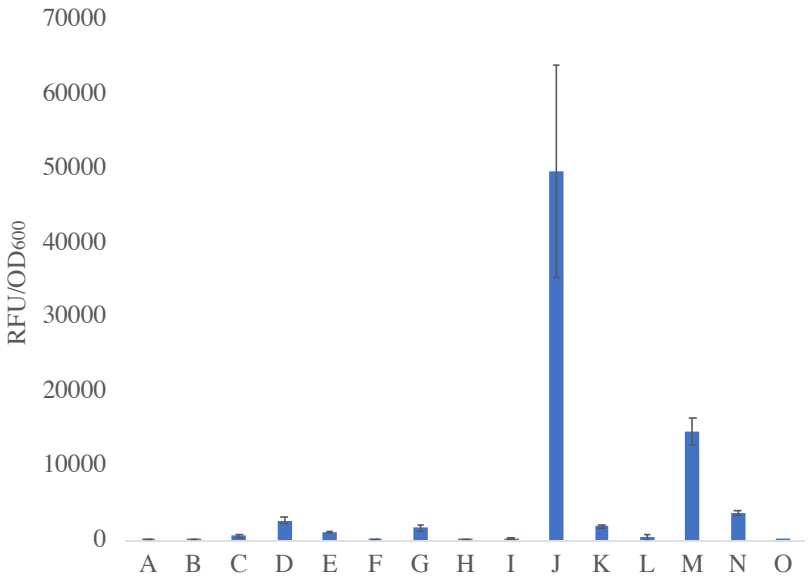

Figure 3.6: Average mCherry expression of pMSI-vectors in *E. coli* DH5α and *E. coli* BL21 harboring pVB-1A0B1-mCherry, pVB-1A0B10-mCherry and pVB-1B0A1-GFP. All cultures were cultivated in LB at 37°C and 800 rpm. Relative fluorescence units (RFU) was normalized to OD_{600}. Error bars represent standard deviation of measurements for four biological replicates. A: pMSI58; B: pMSI59; C: pMSI60; D:pMSI61; E: pMSI62; F: pMSI63; G: pMSI64; H: pMSI65; I: pMSI66; J: pMSI67; K: pMSI68; L: pMSI69; M: pVB-1A0B1-mCherry; N: pVB-1A0B10-mCherry; O: pVB-1B0A1-GFP.

3.3.2 mCherry and GFP Expression in *B. subtilis*

Since so few of the constructs were shown to express mCherry in *E. coli* DH5α, only some were further investigated in *B. subtilis*. The choice fell on pMSI67 containing P_{liaG} displaying the highest mCherry expression, and pMSI68 and pMSI69. pMSI68 and pMSI69 are the corresponding constructs containing the two other *B. subtilis* promoters, P_{43} and P_{met}, respectively.

Cultures of *B. subtilis* harboring pMSI67-pMSI69 in LB medium were prepared, including the controls pVB-1A0B1-mCherry, pVB-1A0B10-mCherry, and pVB-1B0A1-GFP in *E. coli* BL21, wild type *B. subtilis* and harboring pHT10-mCherry. pVB-1B0A1-GFP served as a positive control for GFP expression, while pVB-1A0B1-mCherry and pVB-1A0B10-mCherry served as positive controls for mCherry expression. Wild type *B. subtilis* served as a negative control. pHT10-mCherry was intended to be used to compare mCherry production from the most promising pMSI-vector to a confirmed mCherry producing construct borrowed from [42]. Four cultures for each construct were prepared, each induced with a final concentration of either 0, 1, 2, or 4 mM *m*-toluate. A plot showing the average RFU normalized to OD_{600} is displayed in figures 3.7 and 3.8 for GFP and mCherry, respectively. Neither of the *B. subtilis* constructs (A-C figures 3.7 and 3.8) resulted in higher intensity of GFP or mCherry than the negative control (G figures 3.7 and 3.8). The positive controls (F figure 3.7 and D and figure 3.8) displayed a far higher fluorescence intensity measurement compared to pMSI67-69. pHT10-mCherry from [42] displayed some mCherry production, although at far lower levels compared to the positive controls.

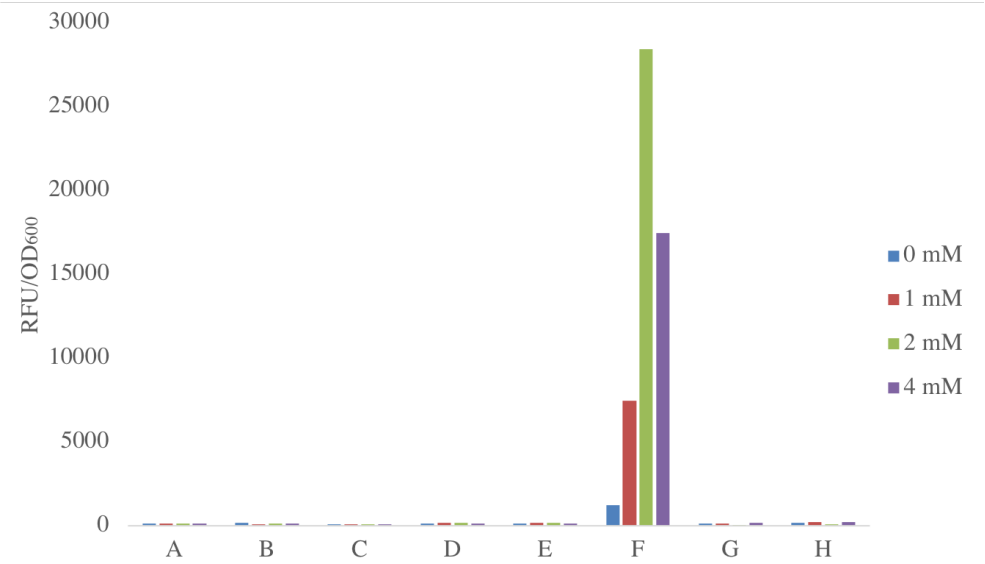

Figure 3.7: GFP expression of wild type, pMSI67-69 and pHT10 in *B. subtilis* 168 and pVB-1A0B1-mCherry, pVB-1A0B10-mCherry and pVB-1B0A1-GFP in *E. coli* BL21. All cultures were cultivated in LB medium at 37°C and 800 rpm. Relative fluorescence units (RFU) was normalized to OD_{600}. Four cultures induced with a final concentration of either 0 mM, 1 mM, 3 mM or 4 mM *m*-touluate were included for each construct. Relative fluorescence units (RFU) was normalized to OD_{600}. A: pMSI67; B: pMSI68; C: pMSI69; D:pVB-1A0B1-mCherry; E: pVB-1A0B10-mCherry; F: pVB-1B0A1-GFP; G: Wild type *B. subtilis*; H: pHT10-mCherry.

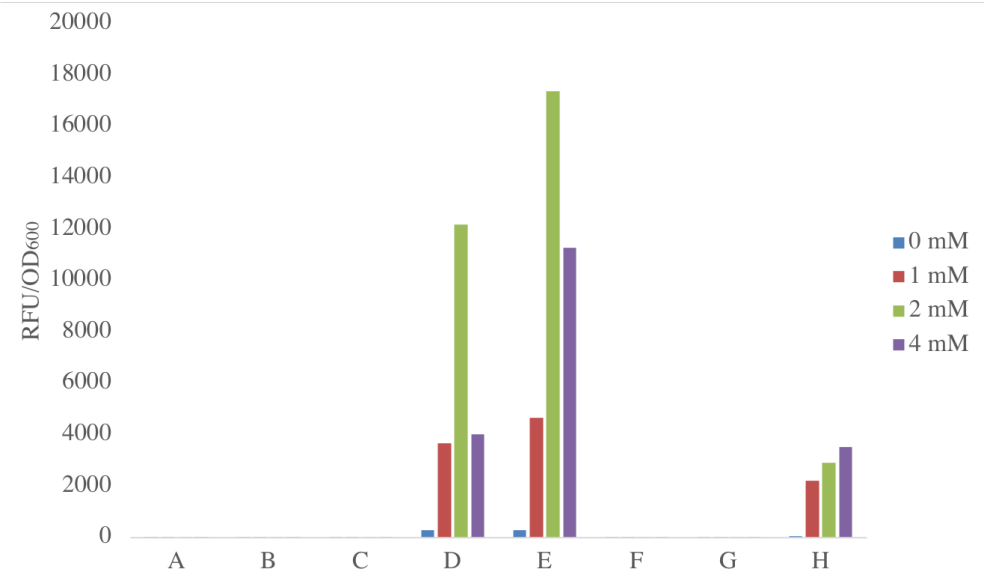

Figure 3.8: mCherry expression of wild type, pMSI67-69 and pHT10 in *B. subtilis* 168 and pVB-1A0B1-mCherry, pVB-1A0B10-mCherry and pVB-1B0A1-GFP in *E. coli* BL21. All cultures were cultivated in LB medium at 37°C and 800 rpm. Relative fluorescence units (RFU) was normalized to OD_{600}. Four cultures induced with a final concentration of either 0 mM, 1 mM, 3 mM or 4 mM *m*-touluate were included for each construct. Relative fluorescence units (RFU) was normalized to OD_{600}. A: pMSI67; B: pMSI68; C: pMSI69; D:pVB-1A0B1-mCherry; E: pVB-1A0B10-mCherry; F: pVB-1B0A1-GFP; G: Wild type *B. subtilis*; H: pHT10-mCherry.

3.3.3 XylS Production in *B. subtilis*

Due to the low mCherry production observed in *B. subtilis* 168, it was speculated if the lack of sufficient XylS production might be a bottleneck. XylS is the activator of P_m, controlling mCherry expression. GFP expression was investigated using pVB-1A0B10-mCherry (data not shown). The data obtained followed the same trend as figure 3.7, indicating GFP had not been able to fulfill its intended role as reporter protein for XylS levels. It was therefore decided to use antibodies for the assay instead.

Cultures of *B. subtilis* harboring pMSI58-pMSI69 were prepared, together with *E. coli* DH5α harboring pMSI67 and wild type *B. subtilis*. The cultures were not induced since the constitutive XylS production was to be assayed. GFP levels for PBS-washed cells were determined, and the measurements are displayed in figure 3.9. The cells were lysed and separated into soluble and insoluble fractions. The two fractions were further analyzed using SDS-PAGE and western blot. Figures 3.10 and 3.11 display the resulting gel (a) and blot (b).

None of the six pMSI-constructs investigated showed a considerable increase in GFP expression when compared to wild type *B. subtilis*. Plasmids pMSI60 and pMSI63 show a minor increase compared to wild type. A similar expression study using the constructs containing the medium promoter, P_{met}, was also carried out using *E. coli* DH5α as the expression host. This expression study achieved similar results regarding measured RFU from expressed GFP (data not shown).

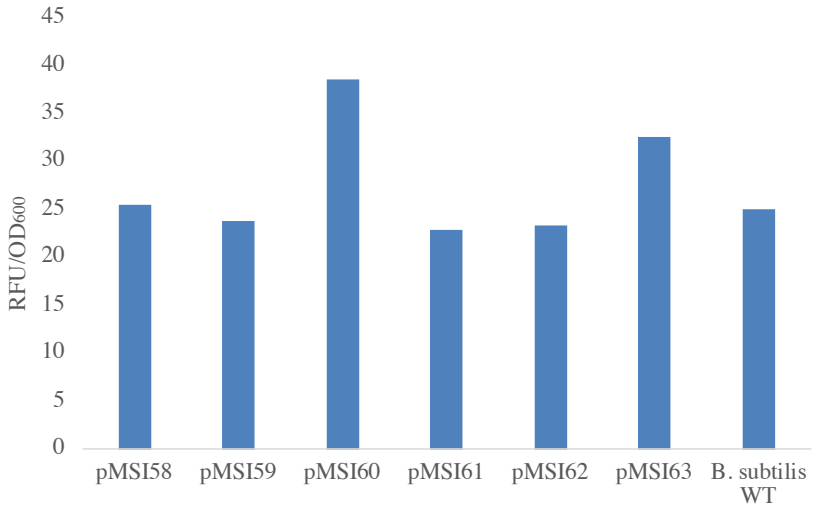

Figure 3.9: GFP expression of wild type and pMSI58-63 in *B. subtilis* 168. All cultures were cultivated in LB medium at 16°C and 225 rpm. Relative fluorescence units (RFU) was normalized to OD_{600}.

The gel and blot revealed both XylS-GFP and XylS production in the pMSI-constructs (figures 3.10 and 3.11). The highest amount of protein expressed was found in the constructs with the medium promoter, P_{met}. These constructs are pMSI60, pMSI63, pMSI66 and pMSI69. pMSI60 and pMSI63 contain *xyls-GFP* (lanes C and F figure 3.10), while pMSI66 and pMSI69 contain unfused *xylS* (lanes I and J figure 3.10). For some of the constructs with the weak and strong promoters, P_{liaG} and P_{43} respectively, faint bands of the correct size can be observed on the blot. Since the antibody concentration was not optimized, the bands appear very saturated and a lot of background staining can be observed. The bands for lanes C and D appear to have been oversaturated and possibly some of the protein has come loose from the membrane, revealing a white spot underneath.

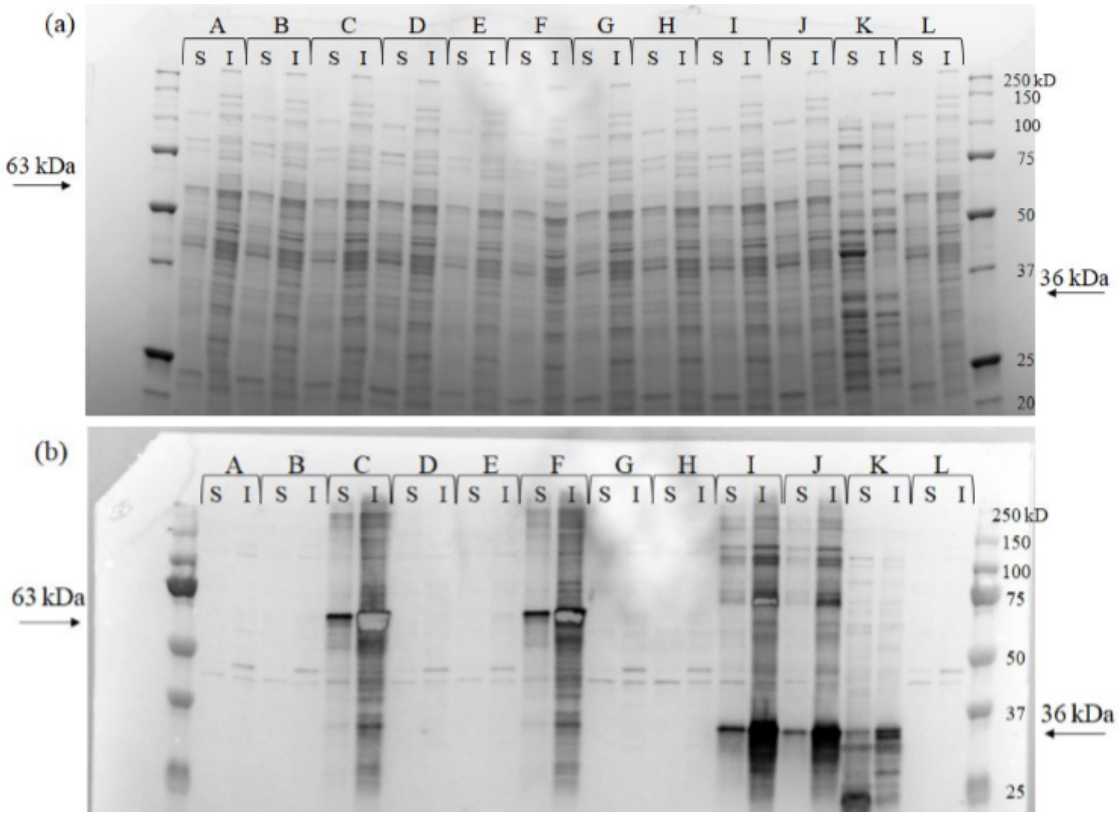

Figure 3.10: SDS-PAGE (a) and western blot (b) of XylS expression in wild type and pMSI58-69 in *B. subtilis* 168. All cultures were cultivated in LB medium at 16°C and 225 rpm. At harvest, the cells were lysed and separated into soluble (S) and insoluble (I) fractions. Unless otherwise stated, 10 μL of sample was loaded in each well and the expression host is *B. subtilis*. Lane A: pMSI58; lane B: pMSI59; lane C: pMSI60; lane D: pMSI61; lane E: pMSI62; lane F: pMSI63; lane G: pMSI64; lane H: pMSI65; lane I: pMSI66; lane J: pMSI69; lane K: pMSI67 in *E.coli* DH5α; lane L: wild type *B. subtilis*. The ladders used were a) Precision Plus Protein Unstained Standards (Bio-Rad) b) Precision Plus Protein Standards Dual Color (Bio-Rad).

3.3.4 XylS Production in *E. coli* DH5α

The results of XylS expression for all 12 pMSI-constructs in *B. subtilis* prompted a similar expression experiment in *E. coli* DH5α. It was mostly the constructs containing the medium promoter, P_{met}, which displayed XylS expression (pMSI60, pMSI63, pMSI66 and pMSI69). Only those constructs were therefore included. It was desirable to compare the XylS production in *B. subtilis* to expression from a host known to produce mCherry, and thus XylS. This was accomplished by running the soluble and insoluble fractions of the same construct from the two hosts next to each other.

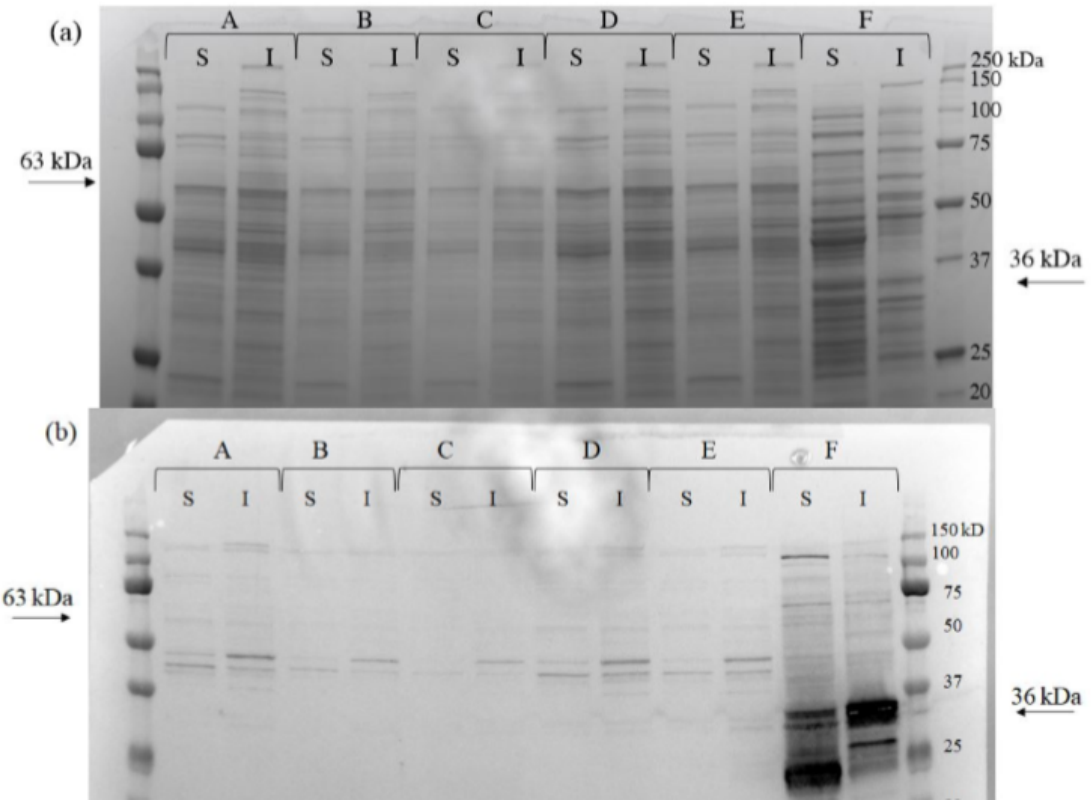

Figure 3.11: SDS-PAGE (a) and western blot (b) of XylS expression in wild type and pMSI58-69 in *B. subtilis* 168. All cultures were cultivated in LB medium at 16°C and 225 rpm. At harvest, the cells were lysed and separated into soluble (S) and insoluble (I) fractions. Unless otherwise stated, 10 μL of sample was loaded in each well and the expression host is *B. subtilis*. Lane A: pMSI61 20 μL; lane B: pMSI67; lane C: pMSI68; lane D: pMSI67 20 μL; lane E: pMSI68 20 μL; lane F: pMSI67 20 μL in *E.coli* DH5α. The ladders used were a) Precision Plus Protein Unstained Standards (Bio-Rad) b) Precision Plus Protein Standards Dual Color (Bio-Rad).

Cultures of *E. coli* harboring constructs with the medium promoter were prepared (pMSI60, pMSI63, pMSI66 and pMSI69), together with *E. coli* DH5α harboring pMSI67 and wild type *E. coli*. pMSI67 was used as a positive control following the high expression of mCherry observed previously in *E. coli* DH5α (see figure 3.6). Wild type *E. coli* DH5α served as a negative control, not harboring *xylS*. The cultures were not induced since the constitutive expression of XylS is to be assayed. GFP levels for PBS-washed cells containing the *xylS-GFP* fusion (pMSI60 and pMSI63) were determined. The measurements are not shown, but briefly discussed in section 3.3.3. The cells were lysed and separated into soluble and insoluble fractions. The two fractions were further analyzed using SDS-PAGE and western blot. Only the constructs containing the medium promoter P_{met} were included from both hosts. The same construct in the two expression hosts were run adjacent to each other. Figure 3.12 shows the resulting blot (gel not shown).

The comparison revealed almost identical XylS expression levels of the same construct in the two hosts. For pMSI60 and pMSI63 (A and B, C and D figure 3.12), the soluble fraction appear to contain greater amounts of XylS-GFP protein when expressed in *E. coli* when compared to the expression observed in *B. subtilis*. For the two other constructs, pMSI66 and pMSI69 (E and F, G and D figure 3.12), the expression appear to be similar in both hosts.

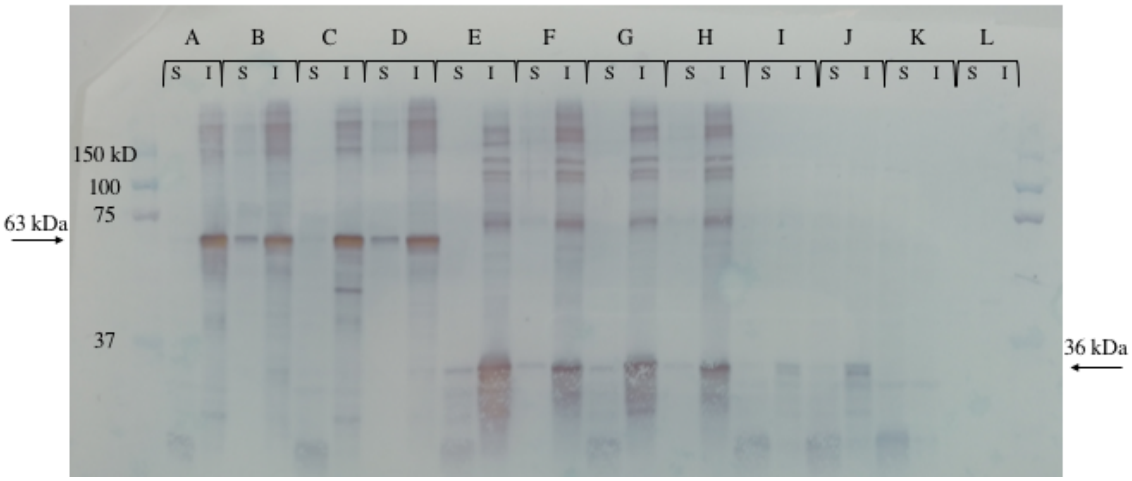

Figure 3.12: Western blot of XylS expression in wild type *B. subtilis* 168 and cells harboring the plasmids pMSI60, pMSI63, pMSI66 and pMSI69. The identical constructs in *E. coli* DH5α were also prepared, along with wild type *E. coli* DH5α. All cultures were cultivated in LB medium at 16°C and 225 rpm. At harvest, the cells were lysed and separated into soluble (S) and insoluble (I) fractions. Lane A: pMSI60 in *E. coli* DH5α; lane B: pMSI60 in *B. subtilis*; lane C: pMSI63 in *E. coli* DH5α; lane D: pMSI63 in *B. subtilis*; lane E: pMSI66 in *E. coli* DH5α; lane F: pMSI66 in *B. subtilis*; lane G: pMSI69 in *E. coli* DH5α; lane H: pMSI69 in *B. subtilis*; lane I: pMSI67 in *E. coli* DH5α (positive control); lane J: pMSI67 in *E. coli* DH5α (positive control from previous expression study); lane K: wild type in *E.coli* DH5α; lane L: wild type *B. subtilis*. The ladder used was Precision Plus Protein Standards Dual Color (Bio-Rad).

3.3.5 Comparison of Expression: pSH3 and pMSI66

It was desirable to see how the most promising pMSI-vector competed with the best performing vector from [42]. A suggestion was to see how native *B. subtilis* promoters would perform compared to the wild type pWW0 promoters, P_{s1} and P_{s2}, controlling XylS expression. pSH3, had XylS expression placed under the inducible P_{s1} and constitutive P_{s2} [42], while the pMSI-constructs have XylS expression placed under native *B. subtilis* promoters (see plasmid maps appendix H). The comparison included pSH3 in two hosts, *E. coli* DH5α and *B. subtilis*, while pMSI66 was in *B. subtilis*. As a reference, two protein standards of XylS with different concentrations were included.

B. subtilis 168 was used as an expression host for pSH3 borrowed from [42] and pMSI66. Cultures with *E. coli* DH5α harboring pSH3 was also included. pSH3 expression was investigated both in the presence (1 mM *m*-toluate) and absence of inducer, while pMSI66 was only investigated in the absence of inducer. Samples for pMSI66 was taken from a similar, previously conducted expression study (see section 2.13.3). The mCherry measurements obtained for pSH3 are displayed in figure 3.15. An illustration of the pink color obtained in harvested cultures upon mCherry expression is displayed in figure 3.14. The soluble and insoluble fractions for both pSH3 and pMSI66 were further analyzed using SDS-PAGE and western blot. The purified XylS standard was included in two different amounts, 100 and 200 ng applied. Figures 3.15 display the resulting gel (a) and blot (b).

No mCherry production was reported for non-lysed cells, neither induced nor uninduced *B. subtilis* harboring pSH3 (see figure 3.13). As for *E. coli* DH5α harboring pSH3, the induced culture had considerable mCherry expression. As expected, the uninduced culture had some production, albeit lower than the induced one. The mCherry production was also visually compared using harvested pellets (see figure 3.14). As expected from the fluorometry measurements, the pellet of the induced *E. coli* DH5α culture exhibited a bright pink color, and its uninduced control a slight pink color change. Neither pellets of *B. subtilis* showed any color change.

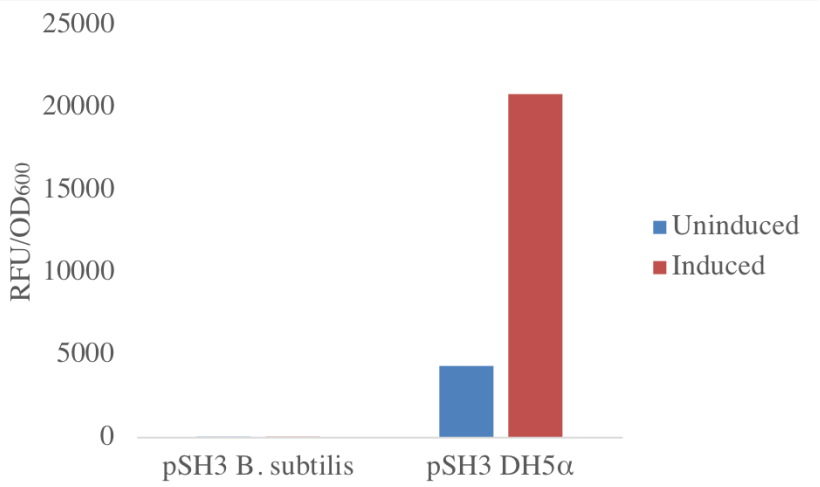

Figure 3.13: mCherry expression of pSH3 in *B. subtilis* 168 and *E. coli* DH5α. All cultures were cultivated in LB medium at 37°C and 225 rpm. One induced and uninduced culture was included for each host. Relative fluorescence units (RFU) was normalized to OD_{600}.

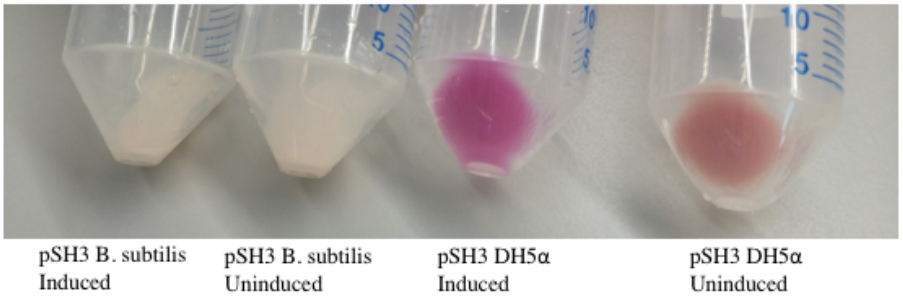

Figure 3.14: Color change observed in pellets of pSH3 in *B. subtilis* 168 and *E. coli* DH5α. All cultures were cultivated in LB medium at 37°C and 225 rpm. One induced and uninduced parallel was included for each culture. Plasmid and expression host are included below each tube.

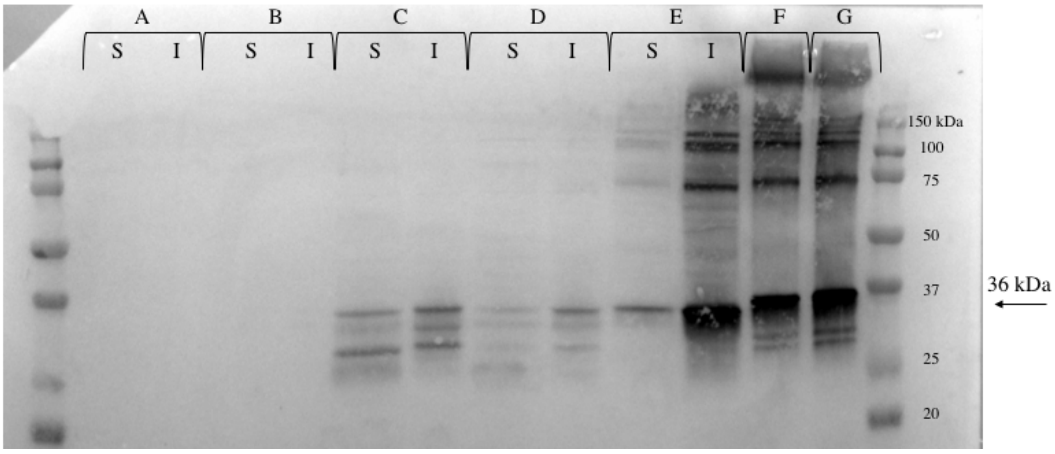

Figure 3.15: Western blot of XylS expression in *B. subtilis* 168 and *E. coli* DH5α harboring the plasmid pSH3. Also included is pMSI66 in *B. subtilis*. All cultures were cultivated in LB medium at 16°C and 225 rpm. One induced and uninduced culture was included for pSH3, pMSI66 was uninduced. At harvest, the cells were lysed and separated into soluble (S) and insoluble (I) fractions. A protein standard of purified XylS was included in two different amounts, 100 and 200 ng applied. Lane A: induced pSH3 in *B. subtilis*; lane B: uninduced pSH3 in *B. subtilis*; lane C: induced pSH3 in *E. coli* DH5α; lane D: uninduced pSH3 in *E. coli* DH5α; lane E: uninduced pMSI66 in *B. subtilis*; lane F: XylS protein standard 100 ng; lane G: XylS protein standard 200 ng.

Unfortunately, the *B. subtilis* cells harboring pSH3 did not lyse, thus no direct comparison was possible to make between the two constructs in the intended host. A second attempt was made, increasing the incubation period in lysis buffer from 30 to 60 minutes. However, the same results were obtained (data not shown). Further attempts were not made. *B. subtilis* cells harboring pMSI66 was lysed in parallel to pSH3 in the second lysis attempt. The cells lysed as expected both after the incubation period in lysis buffer of both 30 and 60 minutes (data not shown). The *E. coli* DH5α cells lysed as expected, and allow for some degree of comparison of XylS levels for the two plasmids. *B. subtilis* cells harboring pMSI66 used in figure 3.15 was lysed in a previous expression study.

As expected, the uninduced sample of *E. coli* DH5α has the lowest expression, albeit some XylS can be observed for both fractions. The level of XylS expressed from the induced *E. coli* DH5α harboring pSH3 in the soluble fraction appear to be similar or slightly lower compared to the amount observed in pMSI66 (C vs E figure 3.15). In the insoluble fraction, pMSI66 in *B. subtilis* have a far higher XylS production than any fraction from *E. coli* DH5α harboring pSH3. The band pattern for both soluble (faintly) and insoluble fractions in pMSI66 greatly resembles the pattern observed in the purified XylS protein standard (E, F and G figure 3.15).

The fluorometric mCherry measurements done on *B. subtilis* and *E. coli* DH5α harboring pSH3 indicated the induced sample of *E. coli* DH5α had the highest expression. This was also supported by the visual observation of the pellets. Some color change was observed in the uninduced sample, indicating XylS is able to activate P_m without the presence of *m*-toluate.

4 Discussion

4.1 Effects of *m*-Toluate

The main aim of this study was to construct functional expression vectors for *B. subtilis* containing recombinant proteins under control of the inducible XylS/*Pm* cassette. To allow for induction of the expression cassette, its inducer, *m*-toluate, must be capable of permeating the cell wall and enter the cell. In *E. coli*, *m*-toluate is known to enter the cells by passive diffusion [39]. In this thesis, it was presumed a similar model could be used for induction of gram-positive bacteria. However, the thicker cell wall was believed to hinder the passive diffusion required for induction. Increasing inducer concentrations have been proposed to lead to a decrease in observed growth. This effect is believed to come from an increase in cytoplasmic concentration, suggesting successful permeation of the cell wall by *m*-toluate through diffusion [36].

In this thesis, an inducer diffusion study was carried for *B. subtilis* and *L. plantarum* to look for effects of increasing *m*-toluate concentrations on growth. Because of the proposed increasing toxicity of the inducer, a decrease in growth was expected at higher concentrations as evidence of *m*-toluate permeating the cell wall. For both *B. subtilis* and *L. plantarum*, the resulting growth curves suggest this is the case. Increasing concentrations appear to affect growth to a greater extent. Results showed considerable decrease in growth for concentrations higher than 10 mM for *B. subtilis* and 20 mM in *L. plantarum*. This is believed to be the level where the inducer becomes toxic under the conditions tested. For later experiments where *m*-toluate is to be used as an inducer for *B. subtilis* and *L. plantarum*, the level of inducer added should remain lower than this level. It is not desirable to add levels of inducer negatively affecting growth of cells during protein expression experiments. This could potentially lead to lowered protein yields. The reduction in growth observed was used as an indicator to verify the ability of *m*-toluate to pass the cell wall of Gram-positive bacteria.

The extensive cell death observed on the higher concentrations of *m*-toluate was hypothesized to be caused by acidification of the medium used for cultivation in addition to the reported toxicity. *m*-toluate is a carboxylic acid with a pK_a of 4.27 [71]. The change in pH of the the media used for cultivation of the two Gram-positive species were therefore investigated. Little change in pH was observed moving from 1 to 10 mM *m*-toluate, while from 10 to 30 mM a dramatic decrease was observed in LB medium. For inducer concentrations lower or equal to 10 mM, it is unlikely the change in pH can be held accountable for the decrease in growth observed. The variations observed are more likely due to the toxic effects of *m*-toluate reported [36]. For higher inducer concentrations, above 10 mM *m*-toluate, pH appear to have played a bigger role. *B. subtilis* seem more affected by the assumed decrease in pH observed in the medium at higher inducer concentrations compared to *L. plantarum*. The most likely explanation for this is the smaller change in pH observed in MRS compared to LB medium. Another possible explanation could be related to *L. plantarum* being classified as a lactic acid bacteria. This group of bacteria produce lactic acid as a by-product of glucose metabolism [12]. Several defense systems are found in *Lactrobacilli* to increase their tolerance to acidification of the environment. One example is the H^+-ATPase proton pump which forces H^+ out of the cytoplasm allowing for an increase of pH intracellularly compared to the external pH [72]. It is possible this ability to adapt to acidic environments partly explains the higher growth observed in *L. plantarum* compared to *B. subtilis* at equal *m*-toluate concentrations. *L. plantarum* has been found to be capable of growing in medium containing ethanol and with a pH as low as 3.2 [73]. This is more than 2 points lower than the pH measured for MRS with 30 mM *m*-toluate added. For *B. subtilis* grown in LB, the combination of toxicity and acidification is suspected to account for the greater growth impairments observed. An unexpected increase in growth at inducer concentrations 8 mM and 10 mM, and somewhat 6 mM, was observed. It is difficult to provide an explanation for

this other than the cell must have adapted in some way, or possibly *m*-toluate was degraded by an unknown mechanism.

The pH test of the media was carried out after the inducer diffusion study was completed. It would be interesting to see how another medium with a buffering capacity would behave, or a medium with adjusted pH. This could eliminate the uncertainty whether the cell death observed was due to the decrease in pH observed, or the potential toxicity posed by *m*-toluate. From the results presented, it is difficult to draw a line separating the two possible causes behind the growth impairment observed. A distinction would also be easier to make if the effects of pH on growth was tested separately. Based on the results observed, it is likely *m*-toluate is capable of permeating the thicker cell wall of gram-positive bacteria. If this was not the case, a different expression cassette with a promoter inducible by a different compound capable of diffusing into the cells should have been considered.

4.2 Evaluation of Expression in Buffered Medium

Since the pH of LB medium is affected by the addition of the inducer *m*-toluate for the concentration range used, a second expression study including all 12 pMSI-constructs in *B. subtilis* was carried out. The choice of medium for this study fell on Hi+Ye medium, believed to have a better buffering capacity than LB medium.

The results obtained were, unfortunately, inconclusive. During the 18-hour incubation period following induction, an unknown red chromophore had formed in all *B. subtilis* cultures, including wild type. The two constructs from [42], pHCMC05-mCherry and pHT10-mCherry in *B. subtilis*, were exempt from this occurrence for unknown reasons. Cultures of *E. coli* BL21 appeared unaffected. *B. subtilis* has been reported to produce an extracellular, insoluble red pigment [74]. The red pigment is believed to be a response to iron present in the medium. It consists of Fe^{3+} and pulcherriminic acid forming pulcherrimin [74][75]. LB medium, which was used for cultivation of *B. subtilis* for previous experiments in this study, do not contain considerable amounts of iron. Hi+Ye medium used for this expression study has Fe(III) citrate added (see appendix A). It is therefore believed the change of medium is to blame for the discoloration observed. For cultivation of *B. subtilis* in the future where the pH has to be adjustable, a different medium with limited iron-content should be considered.

It was believed the production of chromophore and the presence of the chromophore itself could affect mCherry and GFP measurements. These results are therefore not included. The red chromophore formed has been reported to have two absorption maxima, at 385 and 490 nm [74]. The maximum at 490 nm is close to the range of UV excitation used to excite GFP (excitation 485 nm), and could potentially have affected the measurements (see appendix F). mCherry would most likely not have been affected since the excitation and emission maxima used for measurements were 584 nm and 620 nm, respectively (see appendix F).

The optimal inducer concentration, mCherry and GFP expression levels could not be determined due to lack of reliable results. For proper determination of optimal inducer concentration and protein expression, the experiment should to be repeated in a suitable medium. The medium should be capable of counteracting the change in pH observed by addition of *m*-toluate. In addition, the levels of iron present need to be considered. Replicates for each *m*-toluate concentration should be included to be able to make a more assertive conclusion.

4.3 Evaluation of GFP Expression

For six of the 12 pMSI-constructs, *GFP* was fused to *xylS* with the intention of providing a method to measure XylS production *in vivo*. Plasmids pMSI60 and pMSI63 show some increase compared to to controls not harboring *GFP*, but this is not enough to confidently say the system is working as it was intended. The fluorescence levels measured are therefore assumed to be background fluorescence, and not a real detection of fluorescence emitted by GFP. Since the expression was tested in three different hosts, the problem is probably not host-specific. *GFP* was codon-optimized for *B. subtilis*, which could provide some insight into the low expression observed in the two *E. coli* strains. Another possible explanation is that the gene is not present in the vector used. This is found to be highly unlikely since the sizes of fragments used during construction was investigated using gel electrophoresis, and found to be as expected. The presence of a protein of similar size to GFP fused to XylS was also confirmed by western blot. A band with an estimated size of 63 kDa can be observed, especially strong in pMSI60 and pMSI63. This size corresponds to the estimated size of the fusion protein, where XylS of 36 kDa [39] and GFP of 27 kDa [27] is joined. The blots therefore confirm expression of what is assumed to be the desired XylS-GFP fusion protein present in both *B. subtilis* and *E. coli* DH5α.

A more likely explanation for the low fluorescence observed involves steric effects of the fusion on the two proteins fused together. GFP is a smaller protein than XylS (27 kDa vs 36 kDa), and the fusion could hinder or alter how the protein folds. Improper folding can affect its ability to fluoresce, and thus pose as an explanation for the low fluorescence observed. Unfortunately, this means GFP is not a good candidate to use as a reporter protein for these constructs. It would be interesting to try with a different reporter, for instance luciferase. Luciferase catalyzes the reaction of its substrate luciferin to produce light among other products[76]. The light produced can be measured, and used to indicate protein levels, similar to the intended function of GFP in this study.

4.4 Evaluation of mCherry Expression

The fluorescence emitted by mCherry was investigated for all pMSI-construct in *E. coli* DH5α first since it was in this host the greatest success was reported previously [42]. Little or no fluorescence was observed for most pMSI-constructs, with the exception of pMSI67. Some mCherry production was expected, similar to the previous observations reported [42]. The use of native *B. subtilis* promoters, together with mCherry and XylS codon-optimized for *B. subtilis*, could provide some explanation for the little expression observed in the remaining 11 pMSI-constructs in *E. coli* DH5α. However, the expression from pMSI67 was higher than even the included positive controls, indicating it is unlikely the exchange of promoters and codon-optimization should impede expression to a great extent.

For constructs with the *xylS-GFP* fusion, some indications of lowered mCherry expression was observed as a consequence of the GFP fusion. pMSI67, containing unfused *xylS*, had considerable mCherry expression, while the corresponding construct with the *xylS-GFP* fusion, pMSI61, displayed an expression barely higher than the negative control. The only difference between the two constructs is the fusion, and it is therefore reasonable to assume the addition of GFP is causing the effect observed. The added load of GFP fused to XylS appear to curb the intended function of the protein as an activator for P_m, and yields greatly reduced mCherry production. Even though the standard deviation of pMSI67 is considerable, the difference between the two corresponding constructs is still substantial. This pattern is supported by a similar observation in the two positive controls, pVB-1A0B1-mCherry and pVB-1A0B10-mCherry. pVB-1A0B10-mCherry contains the fusion *xylS-GFP*, and display a lower mCherry intensity compared to pVB-1A0B1-mCherry containing only *xylS*. These observations could indicate the fusion either lowers mCherry production because transcription is affected, or the fusion proteins produced have reduced functionality

and are not able to induce P_m to give transcription from *mCherry* as well as wild type. To give further weight to this argument, the levels of XylS-GFP and XylS produced were evaluated. The difference for the constructs containing the medium promoter P_{met} reveal a similar pattern between pMSI60 containing *XylS-GFP* and the corresponding construct pMSI66 (see lanes A and E figure 3.12). For both soluble and insoluble fractions, pMSI66 show higher expression. This observation indicate it is rather the functionality of XylS, not transcription that is affected by fusion to GFP.

RNA polymerase is required to bind to the promoter of a gene in order to initiate transcription [1]. This enzyme is a holoenzyme, dependent on native σ factors for optimal function [18]. When expressed in *E. coli* DH5α, the required σ factors for expression from a native *B. subtilis* promoter are not necessarily present. Without activation of the *B. subtilis* promoters, no XylS is produced, and consequently no mCherry to produce an observable color change. The high expression observed for pMSI67 indicates the σ factor required for expression from P_{liaG} is present in *E. coli* DH5α. Expression was therefore expected to be similar to pMSI67 for the other three constructs containing this promoter: pMSI58, pMSI61 and pMSI64. The low mCherry levels observed for the other two *B. subtilis* promoters are probably due to the change from the XylS/*Pm* promoters P_{s1} and P_{s2} controlling XylS expression to native *B. subtilis* promoters. *E. coli* was not the intended expression host, and no further problem solving was carried out.

The expression of the highest performing pMSI-construct, pMSI67, and the corresponding plasmids containing the two other promoters were investigated in *B. subtilis*. No mCherry expression was observed for either of the three constructs. Why the expression level was so low is unknown, and should be subject to further testing. By using the native host, the lack of σ factor would not pose a sufficient explanation. The versions of *mCherry* and *xylS* used in the constructs were optimized for *B. subtilis*, thus yielding no further explanation. The fusion of GFP could not be to blame either, since neither of the three constructs tested contained *GFP*. Expression studies conducted in this host was expected to yield higher expression rates rather than lower compared to *E. coli* DH5α since transcription and translation should be optimized. To further investigate possible bottlenecks of the system, it was decided to assay XylS production.

4.5 Evaluation of XylS Expression

Further investigations were carried out to determine at what stage the expression system is dysfunctional. Due to the problems experienced with GFP as a reporter protein (see section 4.3), it was decided to use antibodies for detection of XylS levels instead. The constitutive production of XylS from native *B. subtilis* promoters was assumed to be the next plausible bottleneck. Without presence of XylS, the P_m promoter is not activated, and consequently little or no mCherry is produced.

Two expression studies, one for *B. subtilis* harboring all 12 pMSI-constructs, the other for *E. coli* DH5α harboring the pMSI-constructs containing the medium promoter, P_{met}, were carried out. Expression of XylS in the 12 pMSI-vectors was mostly limited to the constructs containing the medium promoter. Little to no expression was observed for the constructs with the weak or strong promoter, P_{liaG} and P_{43}, respectively. This was quite unexpected since the highest performing plasmid at this point, pMSI67, contain P_{liaG}, not P_{met}. Why the expression of XylS was so low for pMSI67 is hard to explain when taking into consideration the plasmid performed so well in *E. coli* DH5α. A possible explanation for the change in performance could be related to the altered functionality of the three native *B. subtilis* promoters when moved to a different host. The promoter showing the highest expression in one host, will not necessarily be the best performing promoter in a different species. Expression of mCherry indirectly indicates expression of XylS, since this it is the activator for

the promoter controlling mCherry expression. Another possible explanation is the amount of XylS needed to activate P_m could be lower in *E. coli* than the levels observed in and required for *B. subtilis*. The high XylS expression observed in the plasmids containing P_{met} makes it reasonable to say XylS expression has been strongly improved in pMSI60, pMSI63, pMSI66 and pMSI69.

Much of the produced XylS in both *B. subtilis* and *E. coli* can be found in the insoluble fraction, and is believed to be inactive. Before the antibody concentrations were optimized, the bands of bigger size than expected (64 and 37 kDa for XylS-GFP and Xyls, respectively) were believed to be unspecific binding. When comparing the banding pattern of XylS expressed from pMSI66 and the purified XylS protein standard, it becomes apparent this is probably not the case. The band observed slightly below 75 kDa for constructs expression XylS can be explained by dimer formation. The size could be argued to be close to 72 kDa, which is twice the size of a XylS monomer of 36 kDa. The bands observed higher up could be aggregates, which XylS has been reported to form [40].

4.5.1 Comparison to pSH3

It was not previously known what levels of XylS is required to yield detectable amounts of mCherry either by the naked eye or using a fluorometer in *B. subtilis*. It was therefore decided to compare the XylS expression from the most promising pMSI-construct containing P_{met} to a construct known to produce mCherry. pSH3 hosted by *B. subtilis* and *E. coli* DH5α, both taken from [42], was compared to pMSI66 displaying the highest XylS levels of the pMSI-plasmids in both soluble and insoluble fractions.

An expression study similar to the one conducted for the XylS expression in *B. subtilis* and *E. coli* DH5α was carried out. In accordance with the findings reported [42], mCherry was expressed only for pSH3 in *E. coli* DH5α and not *B. subtilis*. It was therefore desirable to look into the XylS levels in the two hosts, and possibly compare the levels produced. Unfortunately, *B. subtilis* harboring pSH3 did not lyse even after two attempts. A direct comparison of pSH3 and pMSI66 in *B. subtilis* could therefore not be made. However, it can be argued the levels observed in *E. coli* DH5α might be more representative since pSH3 contain the XylS/*Pm* native promoters P_{s1} and P_{s2} [42]. The levels of available and biologically active XylS (soluble fraction) appear to be similar or even higher for pMSI66. It can therefore be argued pMSI66 should have been able to produce enough mCherry to turn the culture visibly pink, as was demonstrated for pSH3 in *E. coli* DH5α. However, the amount of mCherry required to change the color of a *E. coli* DH5α culture might not be the same as the amount required by *B. subtilis*. This comparison, nonetheless, indicate the problem causing low mCherry expression might be with the P_m promoter controlling mCherry expression, rather than with the newly introduced native *B. subtilis* promoters. mCherry is codon-optimized for *B. subtilis*, and should pose little problems for expression in the species. This should be fully established, and could be subject for further testing by placing the codon-optimized *mCherry* under the control of a promoter known to be functional in *B. subtilis*.

Further investigations should be done on the functionality of the P_m promoter in *B. subtilis*. The lack of mCherry expression, despite the observed similar levels of XylS production compared to a culture with proven mCherry expression, indicates a possible bottleneck located at P_m. The native pWW0-promoter has been reported to be dependent on the two *E. coli* σ factors σ^{32} and σ^{38} [77], also known by their alternative names σ^{S} (RpoS) or σ^{H} (RpoH), respectively. σ^{S} is needed for expression of general stress response genes, while σ^{H} is used during heat-shock [78]. In *B. subtilis*, σ^{H} is involved in sporulation, and is utilized by a minor species of RNA polymerase mostly active in the stationary phase [79]. σ^{S} from *E. coli* and σ^{B} from *B. subtilis* are functionally similar, although they are not homologous

proteins [80]. No entry for σ^{S} in *B. subtilis* could be found in the databases of Uniprot (*www.uniprot.org*). It is possible no such σ factor is exists or is needed in *B. subtilis* since σ^{B} carries out the same function.

The two σ factors required for expression from P_m appear more difficult to come by than their counterparts in *E. coli*. This could provide some explanation for why mCherry expression in *E. coli* was observed in pMSI67 and pSH3, while the identical construct gave none in *B. subtilis*. A possible way to counteract this obstacle is to include the sequences of *E. coli* σ^{32} and σ^{38} on the pMSI-plasmid, or co-transform with a second plasmid harboring the sequence. The expression of the σ factors should be controlled by constitutive promoters, or possibly promoters inducible by either *m*-toluate or another inducer, to ensure expression. This is believed to resolve the hypothesized bottleneck located at expression from P_m.

5 Conclusion

E. coli is the most commonly used expression host for production of recombiant proteins. Gram-positive bacteria can give several possible advantages. Two examples are extracellular secretion, making downstream processing less demanding, and different intercellular environment possibly resulting in more appropriate folding and soluble expression in cases *E. coli* would fail. The aim of this study was to make advancements in the adaptation of the XylS/*Pm* cassette for recombinant protein production in the two Gram-positive species *B. subtilis* and *L. plantarum*. Due to time constraints, little work was completed for *L. plantarum*.

In total 12 *B. subtilis*/*E. coli* shuttle vectors were constructed. All were successfully transformed into both *B. subtilis* and *E. coli*. *m*-toluate was found able to permeate the cell wall of Gram-positive bacteria, and deemed a suitable inducer. mCherry production was observed only from pMSI67 in *E. coli* after induction with *m*-toluate. No production was observed for any of the 12 constructs in *B. subtilis*. Six of the 12 constructs contained the fusion protein XylS-GFP. Fluorescence from the fusion protein was not observed in either species. A protein of the appropriate size was observed in both *B. subtilis* and *E. coli* using western blot.

XylS production in *B. subtilis* was evaluated. Only constructs with the medium promoter, P_{met} had considerable XylS production. The constructs with the weak and strong promoters, P_{liaG} and P_{43} respectively, showed little or no XylS production. XylS production for the constructs with P_{met} in *E. coli* was evaluated and compared to the expression observed in *B. subtilis*. The two hosts appeared to have similar expression levels in both soluble and insoluble fractions.

Expression levels of XylS for the most promising pMSI-plasmid, pMSI66, was compared to the expression levels of pSH3, the most promising construct from the previous work done [42]. pSH3 had mCherry expression in *E.coli*, albeit none in *B. subtilis*. Comparison in *B. subtilis* was not possible due to problems with lysis of the pSH3 harboring cells. The comparison pMSI66 in *B. subtilis* and pSH3 in *E. coli* revealed similar levels of XylS expression.

Replacing the original promoters controlling XylS production in the XylS/*Pm* cassette has lead to a solution for the reported bottleneck in XylS production. A new bottleneck has been identified in its stead, expression from the P_m promoter. Hopefully, the work presented in this thesis provide valuable knowledge for further advancements in adapting the XylS/*Pm* cassette for Gram-positive bacteria.

6 Further Work

To improve the ability for *in vivo* measurement of XylS levels, adaptations to GFP should be considered. One possibility is to introduce a longer linker between XylS and GFP in an attempt to reduce steric hindrance to their folding. This could improve the low fluorescence levels observed. It is also possible a different reporter gene should be considered. A suggestion is the enzyme luciferase, which has previously been proven functional in *E. coli* DH5α in conjunction with XylS/*Pm* [40]. Another study has used luciferase as a reporter gene in *B. subtilis* [81], thus demonstrating the functionality of the protein in the expression host used in this study.

It would be interesting to investigate the levels of *xylS* and *mCherry* mRNA after introducing the changes proposed to give higher yields of recombinant protein [42]. XylS levels were estimated to have increased from the levels reported, and the assumed increase would be interesting to quantify and compare. The further effects on mCherry expression would be beneficial to test at mRNA level, in addition to the protein level investigated in this thesis. This could aid in explaining why the measurable mCherry levels are low, even though XylS levels appear to be at a sufficient level for induction of P_m. It is possible the problem lies with induction of P_m. The σ factors required seem to be present at low concentrations under normal conditions in *B. subtilis*, thus hindering expression of mCherry. By supplying the required σ factors artificially, one can attempt to ensure expression from P_m. The sequences encoding σ^{32} and σ^{38} could be added to the expression vector itself, or be co-transformed with a second vector. It is recommended to place the sequences under control of promoters shown to be functional in *B. subtilis* to avoid problems with expression. Hopefully, this results in transcription of sufficient amounts of the two σ factors to give expression from P_m.

To continue the work on *L. plantarum*, a functional and suitable vector should be identified. This allows for insertion of the XylS/*Pm* cassette, along with the reporter gene *mCherry* to produce an expression vector. Detection of the product from the reporter gene will indicate a functional expression vector and cassette. It is possible the native XylS/*Pm* promoters, P_{s1} and P_{s2}, must be replaced with native *L. plantarum* promoters, similar to the work done on *B. subtilis* in this thesis. The addition of the sequences of σ^{32} and σ^{38} to the constructed vectors should also be considered. It is possible the observed bottleneck at the P_m promoter is also present in *L. plantarum*, and this modification could circumvent the assumed bottleneck.

References

1. Clark, D. P. & Pazdernik, N. J. *Biotechnology Applying the Genetic Revolution* (Academic Cell/Elsevier Inc., 2009).
2. Rao Dasari, V. K., Are, D., Rao Joginapally, V., Mangamoori, L. N. & Rao Adibhatla, K. S. B. Optimization of the downstream process for high recovery of rhG-CSF from inclusion bodies expressed in Escherichia coli. *Process Biochemistry* **43,** 566–575 (May 2008).
3. Fink, A. L. Protein aggregation: Folding aggregates, inclusion bodies and amyloid. *Folding and Design* **3,** R9–R23 (Feb. 1998).
4. Westers, L., Westers, H. & Quax, W. J. Bacillus subtilis as cell factory for pharmaceutical proteins: A biotechnological approach to optimize the host organism. *Biochimica et Biophysica Acta - Molecular Cell Research* **1694,** 299–310 (Nov. 2004).
5. Gopal, G. J. & Kumar, A. Strategies for the Production of Recombinant Protein in Escherichia coli. *The Protein Journal* **32,** 419–425 (Aug. 2013).
6. Gellissen, G. *Production of Recombinant Proteins: Novel Microbial and Eukaryotic Expression Systems* (Wiley-VCH, 2005).
7. Makrides, S. C. Strategies for achieving high-level expression of genes in Escherichia coli. *Microbiological reviews* **60,** 512–538 (1996).
8. Brautaset, T., Lale, R. & Valla, S. Positively regulated bacterial expression systems. *Microbial Biotechnology* **2,** 15–30 (Jan. 2009).
9. Anné, J., Vrancken, K., Van Mellaert, L., Van Impe, J. & Bernaerts, K. Protein secretion biotechnology in Gram-positive bacteria with special emphasis on Streptomyces lividans. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1843,** 1750–1761. ISSN: 0167-4889 (Aug. 2014).
10. Piggot, P. Bacillus Subtilis. *Encyclopedia of Microbiology,* 45–56 (2009).
11. Pokhrel, B., Bashyal, B. & Magar, R. T. Production, Purification and Characterization of Cellulase from Bacillus subtilis Isolated from Soil. *European Journal of Biotechnology and Bioscience EJBB* **2,** 31–37. ISSN: 2321-9122 (2014).
12. The Editors of Encyclopaedia Britannica. *Lactobacillus* Accessed on 04.03.2018. 2017. `https://www.britannica.com/science/Lactobacillus`.
13. Pavan, S. *et al.* Adaptation of the nisin-controlled expression system in Lactobacillus plantarum: a tool to study in vivo biological effects. *Applied and environmental microbiology* **66,** 4427–32. ISSN: 0099-2240 (Oct. 2000).
14. Halbmayr, E. *et al.* High-level expression of recombinant B-galactosidases in Lactobacillus plantarum and Lactobacillus sakei using a sakacin P-based expression system. *Journal of Agricultural and Food Chemistry* **56,** 4710–4719 (2008).
15. Clark, D. P. & Pazdernik, N. J. *Molecular Biology* 2nd ed. (Academic Cell/Elsevier Inc., 2013).
16. Clark, D. P. & Pazdernik, N. J. *Biotechnology* (Academic Cell/Elsevier Inc., 2012).
17. Nature. *Holoenzymes* Accessed on 26.04.2018. `https://www.nature.com/subjects/holoenzymes`.
18. Helmann, J. D. Structure and Function of Bacterial Sigma Factors. *Ann. Rev. Biochem* **57,** 839–72 (1988).
19. Haldenwang, W. G. The sigma factors of Bacillus subtilis. *Microbiological reviews* **59,** 1–30. ISSN: 0146-0749 (Mar. 1995).
20. Li, W., Zhou, X. & Lu, P. Bottlenecks in the expression and secretion of heterologous proteins in Bacillus subtilis. *Research in Microbiology* **155,** 605–610. ISSN: 0923-2508 (Oct. 2004).

21. Madigan, M. T., Martinko, J. M., Bender, K. S., Buckley, D. H. & Stahl, D. A. *Brock Biology of Microorganisms* 14th (Pearson, Harlow, 2015).

22. Lagendijk, E. L., Validov, S., Lamers, G. E., De Weert, S. & Bloemberg, G. V. Genetic tools for tagging Gram-negative bacteria with mCherry for visualization in vitro and in natural habitats, biofilm and pathogenicity studies. *FEMS Microbiology Letters* **305,** 81–90. ISSN: 03781097 (Mar. 2010).

23. Lansford, R., Bearman, G. & Fraser, S. E. Resolution of multiple green fluorescent protein color variants and dyes using two-photon microscopy and imaging spectroscopy. *Journal of Biomedical Optics* **6,** 311. ISSN: 10833668 (2001).

24. Bevis, B. J. & Glick, B. S. Rapidly maturing variants of the Discosoma red fluorescent protein (DsRed). *Nature Biotechnology* **20,** 83–87. ISSN: 10870156 (Jan. 2002).

25. Matz, M. V. *et al.* Erratum: Fluorescent proteins from nonbioluminescent Anthozoa species. *Nature Biotechnology* **17,** 969–973. ISSN: 1087-0156 (Oct. 1999).

26. Shaner, N. C. *et al.* Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nature Biotechnology* **22,** 1567–1572. ISSN: 1087-0156 (Dec. 2004).

27. Inouye, S. & Tsuji, F. I. Aequorea green fluorescent protein. Expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS letters* **341,** 277–80. ISSN: 0014-5793 (Mar. 1994).

28. Thomas, C., Cross, M., Hussain, A. & Smith, C. Analysis of copy number control elements in the region of the vegetative replication origin of the broad host range plasmid RK2. *The EMBO Journal* **3,** 57–63. ISSN: 1460-2075 (Jan. 1984).

29. Thomas, C. M. Molecular genetics of broad host range plasmid RK2. *Plasmid* **5,** 10–19 (1981).

30. Durland, R. H., Toukdarian, A., Fang, F. & Helinski, D. R. Mutations in the trfA Replication Gene of the Broad-Host-Range Plasmid RK2 Result in Elevated Plasmid Copy Numbers. *JOURNAL OF BACTERIOLOGY* **172,** 3859–3867 (1990).

31. Caspi, R., Helinski, D. R., Pacek, M. & Konieczny, I. Interactions of DnaA proteins from distantly related bacteria with the replication origin of the broad host range plasmid RK2. *The Journal of biological chemistry* **275,** 18454–61. ISSN: 0021-9258 (June 2000).

32. Blatny, J. M., Brautaset, T., Winther-Larsen, H. C., Karunakaran, P. & Valla, S. Improved Broad-Host-Range RK2 Vectors Useful for High and Low Regulated Gene Expression Levels in Gram-Negative Bacteria. *PLASMID* **38,** 35–51 (1997).

33. Sletta, H. *et al.* Broad-host-range plasmid pJB658 can be used for industrial-level production of a secreted host-toxic single-chain antibody fragment in Escherichia coli. *Applied and environmental microbiology* **70,** 7033–9. ISSN: 0099-2240 (Dec. 2004).

34. Toukdarian, A. E. & Helinski, D. R. TrfA dimers play a role in copy-number control of RK2 replication. *Gene* **223,** 205–211. ISSN: 0378-1119 (Nov. 1998).

35. Worsey, M. J. & Williams, P. A. Metabolism of toluene and xylenes by Pseudomonas (putida (arvilla) mt-2: evidence for a new function of the TOL plasmid. *Journal of bacteriology* **124,** 7–13. ISSN: 0021-9193 (Oct. 1975).

36. Dragset, M. S. *et al.* Benzoic acid-Inducible gene expression in mycobacteria. *PLoS ONE* **10.** ISSN: 19326203. doi:10.1371/journal.pone.0134544 (2015).

37. Gallegos, M. T., Marqués, S. & Ramos, J. L. Expression of the TOL plasmid xylS gene in Pseudomonas putida occurs from a σ70-dependent promoter or from σ70- and σ54-dependent tandem promoters according to the compound used for growth. *Journal of Bacteriology* **178,** 2356–2361. ISSN: 00219193 (1996).

38. Domínguez-Cuevas, P., Marín, P., Busby, S., Ramos, J. L. & Marqués, S. Roles of effectors in XylS-dependent transcription activation: Intramolecular domain derepression and DNA binding. *Journal of Bacteriology* **190,** 3118–3128 (2008).

39. Gawin, A., Valla, S. & Brautaset, T. The XylS/Pm regulator/promoter system and its use in fundamental studies of bacterial gene expression, recombinant protein production and metabolic engineering. *Microbial Biotechnology* **10,** 702–718. ISSN: 17517915 (July 2017).

40. Zwick, F., Lale, R. & Valla, S. Regulation of the expression level of transcription factor XylS reveals new functional insight into its induction mechanism at the Pm promoter. *BMC Microbiology* **13,** 262. ISSN: 1471-2180 (Nov. 2013).

41. Abril, M.-A., Michan, C., Timmis, K. N. & Ramos, J. L. Regulator and Enzyme Specificities of the TOL Plasmid-Encoded Upper Pathway for Degradation of Aromatic Hydrocarbons and Expansion of the Substrate Range of the Pathway. *JOURNAL OF BACTERIOLOGY* **171,** 6782–6790 (1989).

42. Hverven, S. K. *Construction of XylS/Pm Expression Vectors for Bacillus subtilis* MA thesis (Norwegian University of Science and Technology, 2017).

43. Nygård, T. S. *Evaluation of Pseudomonas putida and Pseudoalteromonas haloplanktis as Alternative Hosts for Recombinant Gene Expression - by Evaluating the xylS/Pm Expression System with mCherry as Reporter Gene* MA thesis (Norwegian University of Science and Technology, 2017).

44. Jordan, S., Junker, A., Helmann, J. D. & Mascher, T. Regulation of LiaRS-dependent gene expression in bacillus subtilis: identification of inhibitor proteins, regulator binding sites, and target genes of a conserved cell envelope stress-sensing two-component system. *Journal of bacteriology* **188,** 5153–66. ISSN: 0021-9193 (July 2006).

45. Wang, P. Z. & Doi, R. H. Overlapping promoters transcribed by bacillus subtilis sigma 55 and sigma 37 RNA polymerase holoenzymes during growth and stationary phases. *The Journal of biological chemistry* **259,** 8619–25. ISSN: 0021-9258 (July 1984).

46. Jakobsen, O. M. *et al.* Overexpression of wild-type aspartokinase increases L-lysine production in the thermotolerant methylotrophic bacterium Bacillus methanolicus. *Applied and environmental microbiology* **75,** 652–61. ISSN: 1098-5336 (Feb. 2009).

47. Weston, A., Brown, M. G. M., Perkins, H. R., Saunders, J. R. & Humphreys, G. O. Transformation of Escherichia coli with plasmid deoxyribonucleic acid: calcium-induced binding of deoxyribonucleic acid to whole cells and to isolated membrane fractions. *Journal of Bacteriology* **145,** 780–787. ISSN: 00219193 (1981).

48. Bergmans, H. E. N., Van Die, I. M. & Hoekstra, W. P. M. Transformation in Escherichia coli: stages in the process. *Journal of Bacteriology* **146,** 564–570 (1981).

49. Hanahan, D. Studies on transformation of Escherichia coli with plasmids. *Journal of Molecular Biology* **166,** 557–580. ISSN: 0022-2836 (June 1983).

50. Dagert, M. & Ehrlich, S. D. Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells. *Gene* **6,** 23–38. ISSN: 03781119 (1979).

51. Birnboim, H. C. & Doly, J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic acids research* **7,** 1513–23. ISSN: 0305-1048 (Nov. 1979).

52. Snustad, D. P. & Simmons, M. J. *Genetics* 6th ed. (John Wiley & Sons, 2012).

53. Haines, A. M., Tobe, S. S., Kobus, H. J. & Linacre, A. Properties of nucleic acid staining dyes used in gel electrophoresis. *Electrophoresis* **36,** 941–944 (Mar. 2015).

54. New England Biolabs Inc. *Gel Loading Dye, Blue (6X)* Accessed on 16.03.2018. `https://www.neb.com/products/b7021-gel-loading-dye-blue-6x#Product%5C%20Information`.

55. Lehman, I. R. DNA ligase: structure, mechanism, and function. *Science (New York, N.Y.)* **186,** 790–7. ISSN: 0036-8075 (Nov. 1974).

56. Nelson, D. L. & Cox, M. M. *Lehninger Principles of Biochemistry* 6th (W.H. Freeman and Company, New York, 2013).

57. Waldo, G. S., Standish, B. M., Berendzen, J. & Terwilliger, T. C. Rapid protein-folding assay using green fluorescent protein. *Nature Biotechnology* **17,** 691–695. ISSN: 1087-0156 (July 1999).

58. Lifescience Market. *pHCMC05 Plasmid* Accessed on 29.04.2018. `https://www.lifescience-market.com/p/phcmc05-plasmid-p-63798.html`.

59. MoBiTech. *Bacillus subtilis Expression System* Accessed on 24.04.2018. `https://www.mobitec.com/cms/products/bio/04_vector_sys/bacillus_subtilis_expression.html`.

60. Chappell, J. *Promoter 43: Constitutive Promoter for B. subtilis* Accessed on 08.04.2018. 2008. `http://parts.igem.org/Part:BBa_K143013`.

61. De Vries, G. E., Arfman, N., Terpstra, P., Dijkhuizenl & L. Cloning, Expression, and Sequence Analysis of the Bacillus methanolicus Cl Methanol Dehydrogenase Gene. *JOURNAL OF BACTERIOLOGY* **174,** 5346–5353 (1992).

62. Alberts, B. *et al. Molecular Biology of the Cell* 6th ed. (Garland Science, 2015).

63. Reece, R. J. *Analysis of Genes and Genomes* (John Wiley & Sons, Chichester, West Sussex, 2004).

64. Xue, G. P., Johnson, J. S. & Dalrymple, B. P. High osmolarity improves the electrotransformation efficiency of the gram-positive bacteria Bacillus subtilis and Bacillus licheniformis. *Journal of Microbiological Methods* **34,** 183–191 (1999).

65. Wong, T.-K. & Neumann, E. Electric field mediated gene transfer. *Biochemical and Biophysical Research Communications* **107,** 584–587. ISSN: 0006-291X (July 1982).

66. Potter, H. & Heller, R. Transfection by electroporation. *Current protocols in molecular biology* **Chapter 9,** Unit 9.3. ISSN: 1934-3647 (May 2003).

67. Invitrogen. *iBind Flex Western System* Accessed on 06.04.2018. 2015. `https://www.thermofisher.com/no/en/home/life-science/protein-biology/protein-assays-analysis/western-blotting/detect-proteins-western-blot/ibind-western-system/ibind-flex-system.html`.

68. De Man, J., Rogosa, d. & Sharpe, M. E. A medium for the cultivation of lactobacilli. *Journal of Applied Microbiology* **23,** 130–135 (1960).

69. Sedewitz, B., Schleifer, K. H. & Gotz, F. Physiological Role of Pyruvate Oxidase in the Aerobic Metabolism of Lactobacillus plantarum. *JOURNAL OF BACTERIOLOGY* **160,** 462–465 (1984).

70. Noori, F., Ebrahimi, M. T. & Jafari, P. Growth Optimization of Lactobacillus plantarum T5jq301796.1, an Iranian Indigenous Probiotic in Lab Scale Fermenter. *Applied Food Biotechnology* **3,** 188–193. ISSN: 2423-4214 (July 2016).

71. Wan, Q. H., Shaw, P. N., Davies, M. C. & Barrett, D. A. Chromatographic behaviour of positional isomers on porous graphitic carbon. *Journal of Chromatography A* **697,** 219–227. ISSN: 0021-9673 (Apr. 1995).

72. Cotter, P. D. & Hill, C. Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiology and molecular biology reviews : MMBR* **67,** 429–53. ISSN: 1092-2172 (Sept. 2003).

73. G-Alegría, E. *et al.* High tolerance of wild Lactobacillus plantarum and Oenococcus oeni strains to lyophilisation and stress environmental conditions of acid pH and ethanol. *FEMS Microbiology Letters* **230,** 53–61 (Jan. 2004).

74. Canale-Parola, E. A red pigment produced by aerobic sporeforming bacteria. *Archiv für Mikrobiologie* **46,** 414–427. ISSN: 03028933 (1963).

75. Feng, X. *et al.* Structural and functional analysis of Bacillus subtilis YisP reveals a role of its product in biofilm production. *Chemistry and Biology* **21,** 1557–1563. ISSN: 10745521 (2014).

76. Naylor, L. H. Reporter gene technology: the future looks bright. *Biochemical Pharmacology* **58,** 749–757. ISSN: 0006-2952 (Sept. 1999).

77. Marqués, S., Manzanera, M., González-Pérez, M. M., Gallegos, M. T. & Ramos, J. L. The XyIS-dependent Pm promoter is transcribed in vivo by RNA polymerase with σ32 or σ38 depending on the growth phase. *Molecular Microbiology* **31,** 1105–1113 (Feb. 1999).

78. Lovley, D. R. *et al.* Geobacter: The Microbe Electric's Physiology, Ecology, and Practical Applications. *Advances in Microbial Physiology* **59,** 1–100. ISSN: 0065-2911 (Jan. 2011).

79. Weir, J., Predich, M., Dubnau, E., Nair, G. & Smith, I. Regulation of spoOH, a Gene Coding for the Bacillus subtilis CH Factor. *JOURNAL OF BACTERIOLOGY,* 521–529 (1991).

80. Kazmierczak, M. J., Wiedmann, M. & Boor, K. J. Alternative sigma factors and their roles in bacterial virulence. *Microbiology and molecular biology reviews : MMBR* **69,** 527–43. ISSN: 1092-2172 (Dec. 2005).

81. Hutter, B., Fischer, C., Jacobi, A., Schaab, C. & Loferer, H. Panel of Bacillus subtilis reporter strains indicative of various modes of action. *Antimicrobial agents and chemotherapy* **48,** 2588–94. ISSN: 0066-4804 (July 2004).

82. New England BioLabs Inc. PCR Using Q5 High-Fidelity DNA Polymerase. Accessed on 27.04.2018. `https://www.neb.com/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491` (2015).

7 Appendices

A Media and Solutions

A.1 Growth Media

Lysogeny Broth (LB) medium
5.0 g Tryptone (OXOID)
2.5 g Yeast extract (OXOID)
2.5 g NaCl (VWR)
Up to 500 mL dH_2O
Autoclaved

LB Agar (LA) medium
5.0 g Tryptone (OXOID)
2.5 g Yeast extract (OXOID)
2.5 g NaCl (VWR)
7.5 g Agar (OXOID)
Up to 500 mL dH_2O
Autoclaved

Nutrient Broth
2.5 g Peptone (OXOID)
1.5 g Meat extract (OXOID)
Up to 500 mL dH_2O
pH adjusted to 7.0 before autoclaving

De Man, Rosa and Sharpe (MRS) Broth Medium
25.5 g MRS (Sigma-Aldrich)
0.5 mL Tween 80 (Sigma)
Up to 500 mL dH_2O
Autoclaved

MRS Agar medium
25.5 g MRS (Sigma-Aldrich)
0.5 mL Tween 80 (Sigma)
7.5 g Agar (OXOID)
Up to 500 mL dH_2O
Autoclaved

Basis medium 1+Yeast extract Hi (Hi+YE Basis medium)
Basis medium 1
8.6 g $Na_2HPO_4{\cdot}2H_2O$ (Merck)
3 g KH_2PO_4 (Acros Organics)
1 g NH_4Cl (Sigma-Aldrich)
0.5 NaCl (VWR)
10 mL Fe(III) citrate hydrate
0.1 mL H_3BO_3
1.5 mL $MnCl_2{\cdot}4H_2O$
0.1 mL $EDTA{\cdot}2H_2O$
0.1 mL $CuCl_2{\cdot}2H_2O$
0.1 mL $Na_2Mo_4O_4{\cdot}2H_2O$
0.1 mL $CoCl_2{\cdot}H_2O$
2 mL $Zn(CH_3COO)_2{\cdot}2H_2$
Up to 900 mL dH_2O
Autoclaved

Compositions of stock solutions are given in appendix B.

Yeast extract Hi
10 g Yeast extract (OXOID)
Up to 100 mL tap water
Autoclaved

1M $MgSO_4$
12. 3 g $MgSO_4$ (VWR)
Up to 50 mL dH_2O
Autoclaved
2.5 mL added to 1 L Hi+Ye medium.

Glucose solution
11.35 g Glucose (VWR)
Up to 50 mL tap water
Autoclaved
8 mL added to 1 L Hi+Ye medium.

Glycerol solution
50.15 g 100% Glycerol (VWR)
Up to 100 mL tap water
Autoclaved
20 mL added to 1 L Hi+Ye medium.

After separate autoclaving, mix Basis medium 1, Yeast extract Hi, 1M $MgSO_4$, glucose solution and glycerol solution to make 1 L Hi + Ye Basis medium.

A.2 Media for Preparation of Competent *E. coli*

PSI medium
10 g Tryptone (OXOID)
2.5 g Yeast extract (OXOID)
5.12 g $MgSO_4 \cdot 7H_2O$ (VWR)
Up to 500 mL dH_2O
pH adjusted to 7.6 using KOH
Autoclaved

TFB1, super competent cells
0.588 KAc (Merck)
2.42 g RbCl (Acros Organics)
0.389 g $CaCl_2 \cdot 2H_2O$ (Merck)
3.146 g $MnCl_2 \cdot 4H_2O$ (J.T. Baker)
30 mL 99.5 % Glycerol (VWR)
Up to 200 mL dH_2O
pH adjusted to 5.8 using diluted acetic acid.
Filter sterilized

TFB2, super competent cells
0.21 MOPS (Fisher Scientific)
1.1 g $CaCl_2 \cdot 2H_2O$ (Merck)
0.121 g RbCl (Acros Organics)
15 mL 99.5 % Glycerol (VWR)
Up to 100 mL dH_2O
pH adjusted to 6.5 using diluted NaOH.
Filter sterilized

A.3 Media for Transformation of Competent *E. coli*

Super Optimal Broth (SOB) medium
2 g Tryptone (OXOID)
0.5 g Yeast extract (OXOID)
0.058 g NaCl (VWR)
0.019 KCl (Merck)
0.5 g $MgSO_4 \cdot 7H_2O$ (VWR)
Up to 100 mL dH_2O
Autoclaved

Super Optimal Catabolite-repression (SOC) medium
100 mL SOB medium
2 mL 20% glucose solution (VWR)
Autoclaved

A.4 Media for Preparation of Competent *B. subtilis* and Electroporation

Growth Medium
18.2 g Sorbitol (Sigma)
2 g Trypotone (OXOID)
1 g Yeast exctract (OXOID)
1 g NaCl (VWR)
Up to 200 mL dH_2O
Autoclaved

Electroporation/Washing Solution
18.2 g Sorbitol (Sigma)
18.2 g Mannitol (Sigma)
20 g 99.5 % Glycerol (VWR)
Up to 200 mL dH_2O
Autoclaved

Outgrowth Medium
9.1 g Sorbitol (Sigma)
6.9 g Mannitol (Sigma)
1 g Trypotone (OXOID)
0.5 g Yeast exctract (OXOID)
0.5 g NaCl (VWR)
Up to 100 mL dH_2O
Autoclaved

A.5 Other Solutions

Phosphate Buffered Saline (PBS), GFP fluorescence
8 g NaCl (VWR)
0.2 g KCl (Merck)
1.44 g Na_2HPO_4 (Merck)
15 mL 99.5 % Glycerol (VWR)
Up to 800 mL dH_2O
pH adjusted to 7.4 using HCl or NaOH.
Volume adjusted to 1 L using dH_2O.
Autoclaved

0.8 % Agarose with GelGreen, gel electrophoresis
3.2 g Agarose (Lonza)
20 μL GelGreen 10,000x (Biotioum)
400 mL 1xTAE
Heated in a microwave for 2 minutes.
Cooled down to 50 °C

50xTris-acetate-EDTA(50xTAE) Buffer, gel electrophoresis
242 g Tris-base (Sigma)
57.1 mL Acetic acid (VWR)
100 mL 0.5 M EDTA, pH 8 (VWR)
Autoclaved

1 M Isopropyl-β-D-1-thiogalacto-pyranosid (IPTG)
2.38 g IPTG (VWR)
Up to 10 mL dH_2O
Sterile filtered

1.8 M *m*-Toluic acid, inducer
2.451 g *m*-Toluic acid (Aldrich)
10 mL 99.8 % Ethanol absolute (VWR)
Sterile filtered

From the 1.8 M stock solution, several stock solutions were prepared with varying *m*-toluate concentrations. The solutions prepared were to give final concentrations of 0, 1, 2, 4, 6, 8, 10, 15, 20 and 30 mM. Table 7.1 displays the compositions of the stock solutions.

Table 7.1: Composition of *m*-toluate stock solutions with varying concentrations used for induction study.

Final concentration (mM)	Volume of 1.8 M stock (uL)	Volume of ethanol (uL)
0	0.0	1000
1	33.3	966.7
2	66.7	933.3
4	133.3	866.7
6	200.0	800.0
8	266.7	733.3
10	333.3	666.7
15	500.0	500.0
20	666.7	333.3
30	1000.0	0.0

A.6 Antibiotics

100 mg/mL Ampicillin stock
1 g Ampicillin (PanReac AppliChem)
10 mL dH_2O
Sterile filtered and aliquoted in Eppendorf tubes (1 mL)

50 mg/mL Kanamycin stock
0.5 g Kanamycin (PanReac AppliChem)
10 mL dH_2O
Sterile filtered and aliquoted in Eppendorf tubes (1 mL)

20 mg/mL Chloramphenicol stock
0.1 g Chloramphenicol (SIGMA)
5 mL 99.9 % Ethanol (VWR)
Sterile filtered and aliquoted in Eppendorf tubes (1 mL)

B Stock solutions for Hi+Ye Basis Medium 1

Stock solutions used to make basis medium 1 for Hi+Ye medium are shown in figure 7.1. All stock solutions were borrowed from Vectron Biosolutions AS and were pre-made. The producers of the chemicals are unknown.

Component	g/L	pr 0,5 l
Fe(III) citrate hydrate	6	3
Distilled, ion free water	1000	500

Component	g/L	pr 25 ml
H3BO3	30	0.75
Distilled, ion free water	1000	25

Component	g/L	pr 250 ml
MnCl2*4H2O	10	2.5
Distilled, ion free water	1000	250

Component	g/L	pr 25 ml
EDTA*2H2O (titriplex)	84	2.1
Distilled, ion free water	1000	25

Component	g/L	pr 25 ml
CuCl2*2H2O	15	0.375
Distilled, ion free water	1000	25

Component	g/L	pr 25 ml
Na2Mo4O4*2H2O	25	0.625
Distilled, ion free water	1000	25

Component	g/L	pr 25 ml
CoCl2*6H2O	25	0.625
Distilled, ion free water	1000	25

Component	g/L	pr 250 ml
Zn(CH3COO)2*2H20	4	1
Distilled, ion free water	1000	250

Figure 7.1: Stock solutions used in preparing Hi+Ye Basis medium. All stock solutions were pre-made and borrowed from Vectron Biosolutions AS

C Calculation of Generation Time

For the growth studies conducted (see section 3.1), the generation time for *L. plantarum* and *B. subtilis* was calculated for each set of growth conditions. During exponential growth, a straight line can be obtained by plotting the collected data on a semi-logarithmic plot. A straight line forms due to the cells growing at a constant rate, where the cell mass doubles in a constant time interval [21]. By using the natural logarithm, a general formula for a straight line can be obtained (equation 1). A and B refer to constants, and A represents the slope of the resulting line.

$$lny = Ax + lnB \tag{1}$$

Microsoft Excel has several built in functions useful for processing data obtained in experiments. One of these functions is the ability to conduct linear regression on a data set, where the slope (μ) is calculated. Equation 2 depicts how to calculate the slope of a straight line without the use of Excel.

$$\mu = \frac{lnOD_2 - lnOD_1}{t_2 - t_1} \tag{2}$$

By using Excel, the slopes for all the OD_{600} measurements obtained was calculated. The slope was further used to calculate the generation time (g) for the two species under different growth conditions. Equation 3 demonstrates how to calculate g from the slope, μ.

$$g = \frac{ln(2)}{\mu} \tag{3}$$

C.1 Example calculation

As an example, the generation time of *L. plantarum* grown in MRS is calculated below (equation 4). Microsoft Excel found the slope to be 0.99 when a linear regression was carried out (figure 3.3)

$$g = \frac{ln(2)}{0.99} = 0.70\ h = 42\ min \tag{4}$$

D Molecular Weight Standards for Gel Electrophoresis

Molecular weight standards were used to determine the size of DNA fragments separated using gel electrophoresis. These standards have fragments of known sizes, allowing for a direct comparison with fragments where the size is unknown. The standards used in this study were from NEB (figure 7.2) and Invitrogen (figure 7.3).

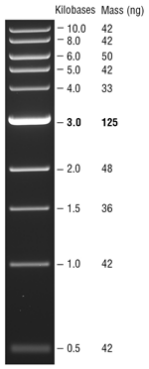

Figure 7.2: 1 kb DNA ladder (NEB) in 0.8% agarose TAE gel and visualized by GelRed staining.

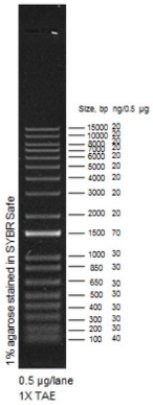

Figure 7.3: 1 kb DNA ladder (Invitrogen) in 1 % agarose TAE gel and visualized by SYBR safe staining.

E Molecular Weight Standards for SDS-PAGE and Western Blots

Molecular weight standards were used to determine the size of proteins separated using SDS-PAGE. These standards have proteins of known sizes, allowing for a direct comparison with proteins where the size is unknown. The standards used in this study were from Bio-Rad (figures 7.4, 7.5 and 7.6).

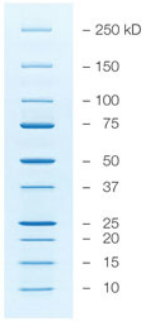

Figure 7.4: The ladder Precision Plus Protein Unstained Standards (Bio-Rad) was used as a molecular weight standard for SDS-PAGE.

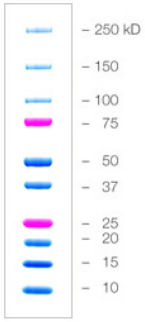

Figure 7.5: The Precision Plus Protein Standards Dual Color (Bio-Rad) was used as a molecular weight standard for SDS-PAGE and the subsequent western blot.

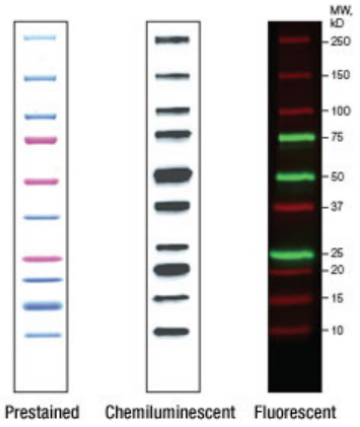

Figure 7.6: The Precision Plus Protein WesternC Protein Standards (Bio-Rad) was used as a molecular weight standard for SDS-PAGE and the subsequent western blot.

F Settings for Measuring GFP and mCherry Activity

mCherry was used as a reporter gene to measure successful induction of its promoter P_m by its activator XylS. In turn, this indicate a functional construct. All 12 pMSI-plasmids contained mCherry, and measurements were carried out directly on culture using TECAN Infinite 200 Pro Multifunctional plate reader. One measurement was made for each culture. Samples of the constructs in both *E. coli* DH5α and *B. subtilis* 168 were examined. The settings used to conduct the measurements are summarized in table 7.2.

In six of the 12 engineered pMSI-plasmids, *GFP* was fused to *xylS* in an attempt to use it as a reporter gene. To gauge the amount of XylS produced, the intention was to measure the fluorescence produced by GFP. The fluorescence was measured using TECAN Infinite 200 Pro Multifunctional plate reader. Measurements for GFP was made on cells resuspended in PBS after being washed in the same solution twice. One measurement was made for each culture. Samples of the constructs in both *E. coli* DH5α and *B. subtilis* 168 were examined. The settings used to conduct the measurements are summarized in table 7.2.

Table 7.2: Settings of the program used on TECAN Infinite Pro 200 for mCherry and GFP measurements. Measurements were carried out on culture for mCherry, and on cells washed and resuspended in PBS for GFP. One measurement was made for each construct.

Parameter	mCherry	GFP
Excitation	584 nm	485 nm
Emission	620 nm	515 nm
Excitation bandwidth	9 nm	9 nm
Emission bandwidth	20 nm	20 nm
Gain	Optimal	Optimal
Number of flashes	10	10
Integration time	20 μs	20 μs
Lag time	0 μs	0 μs
Settle time	0 μs	0 μs

G Primers

The success of a PCR reaction will greatly depend on the primers used. Several parameters can be tuned to optimize the design of the primers used. The typical length of a primer is 17-30 nucleotides long. The length chosen will influence the hybridization rate of the primer to DNA. Longer primers will hybridize at a slower rate. A short primer increase the risk of hybridization off-target, resulting in undesired sequences being amplified. The melting temperature of the primers (T_m) should ideally be within 5°C of each other and the GC content around 50%. The primers should not be complementary either to itself or each other. Complementary segments within a primer increase the chance of formation of hairpins, effectively removing the primer from the reaction. Primer dimers are likely to form if the primers are complementary to each other, removing both primers from the reaction. To avoid hybridization to repetitive segments in the template, long runs of single nucleotides should be avoided [82][63].

Table 7.3: Primers used in this study, their sequence, T_m and template.

Primer	Sequence (5'-3')	T_m	Template
1f-frag1	CCAGTAGATCTTCGTCCACGCCGAGCCCTATGCAGTC	81°C	mCherry fwd
1r-frag1-o(frag2)	CTTTGCTCGCCATAAATTCGCCAGAACCAGCAGCGG AGCCAGCGGATCCAGCCACTTCCTTTTTGCATTG	87°C	mCherry rev
2f-frag2-o(frag1)	GGAAGTGGCTGGATCCGCTGGCTCCGCTGCTGGTTC TGGCGAATTTATGGCGAGCAAAGGCGAAGAACTGTTTAC	88°C	GFP fwd
2r-frag2	CGACGGAATTCTTATTTATACAGTTCATCCATGCCA TGGGTAATGCCCGCCGCGGTCAC	83°C	GFP rev

H Plasmid Maps

Several plasmids were used or constructed during this study. Figures 7.7, 7.8, 7.10, 7.11, 7.12, 7.13 and 7.14 display their respective plasmid maps annotated with genes of interest. The construction of the pMSI-plasmids are summarized in tables 2.6 and 2.7 for constructs with and without GFP, respectively.

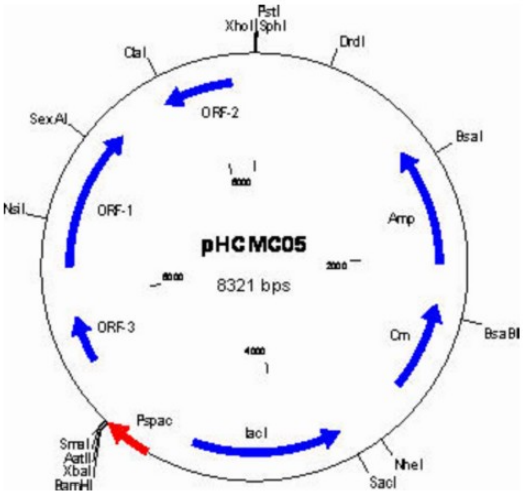

Figure 7.7: Plasmid map of pHCMC05 taken from [58].

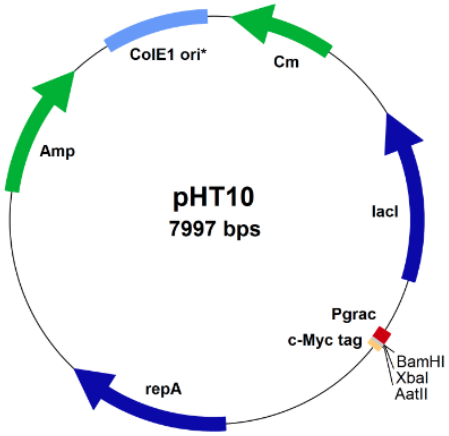

Figure 7.8: Plasmid map of pHT10 taken from [59].

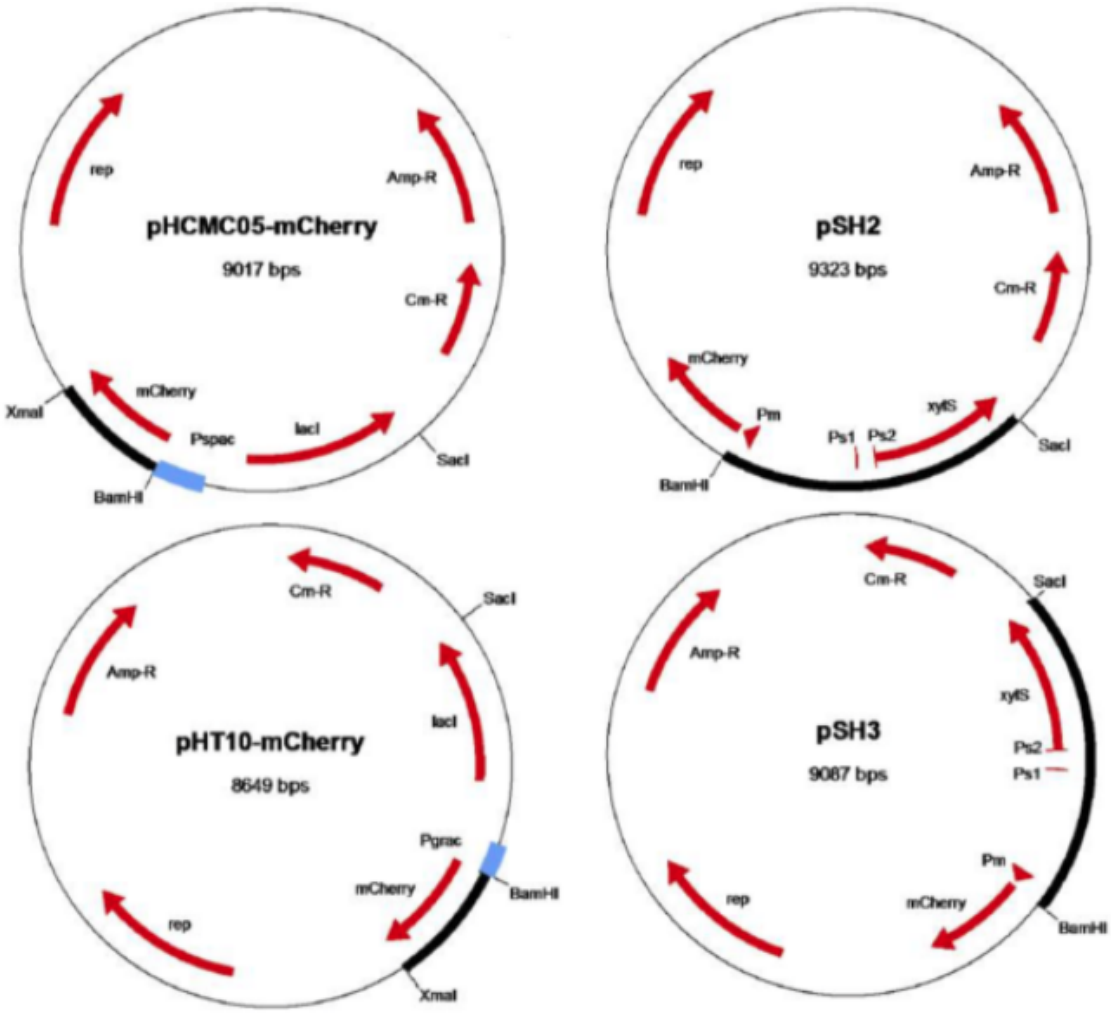

Figure 7.9: Plasmid map of pHCMC05-mCherry, pHT10-mCherry, pSH2 and pSH3 taken from [42].

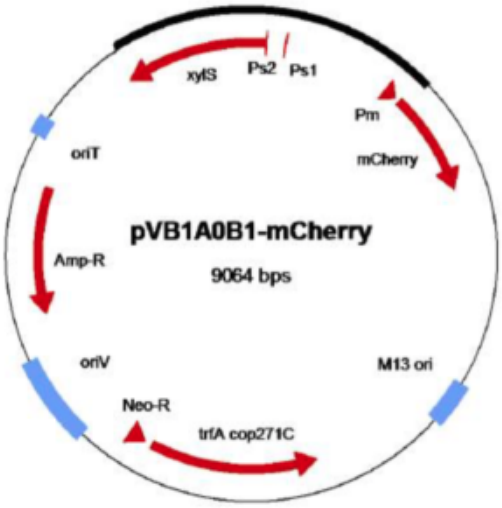

Figure 7.10: Plasmid map of pVB-1A0B1-mCherry taken from [42].

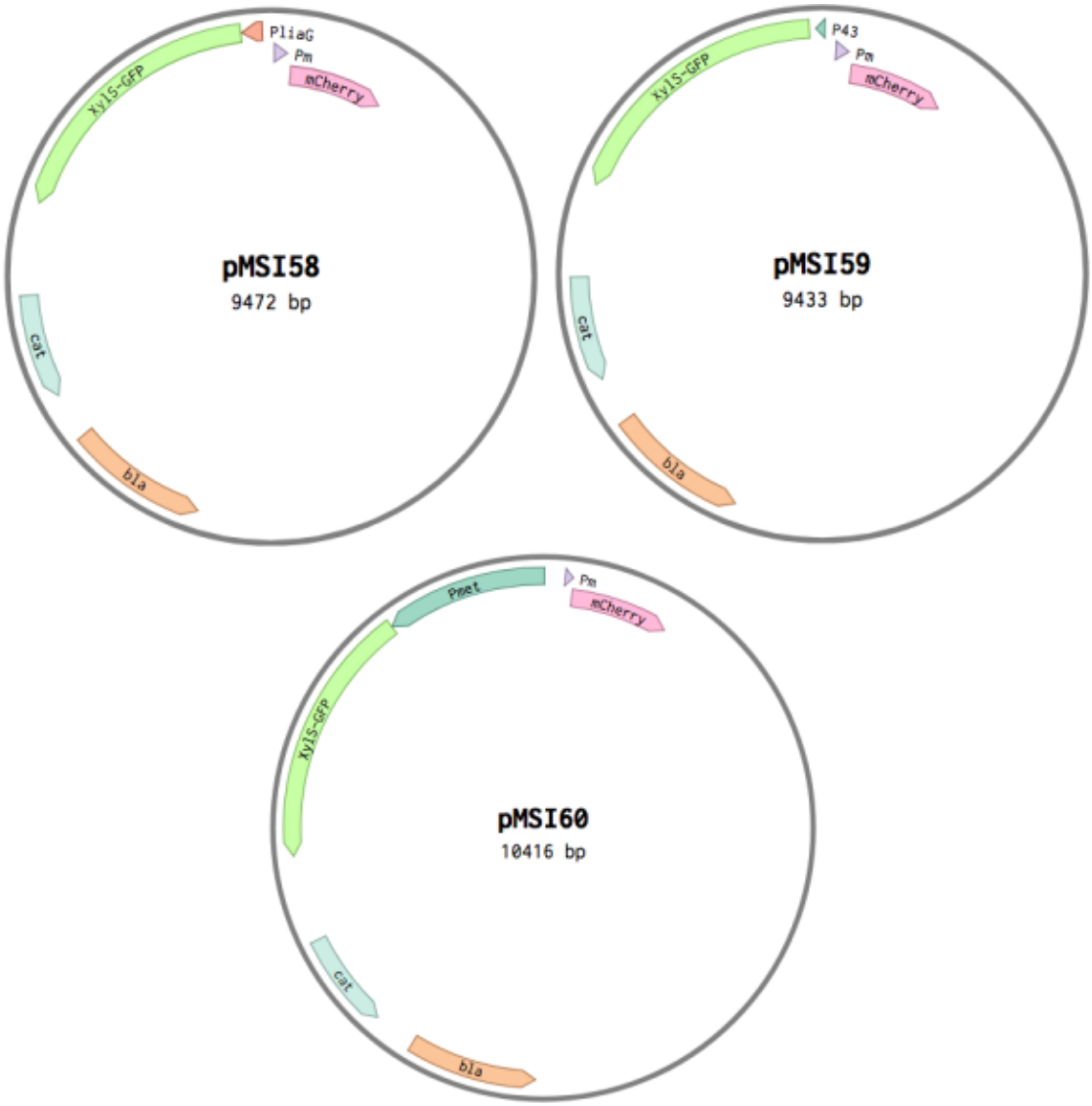

Figure 7.11: Plasmid maps of pMSI58-60 generated using Benchling.

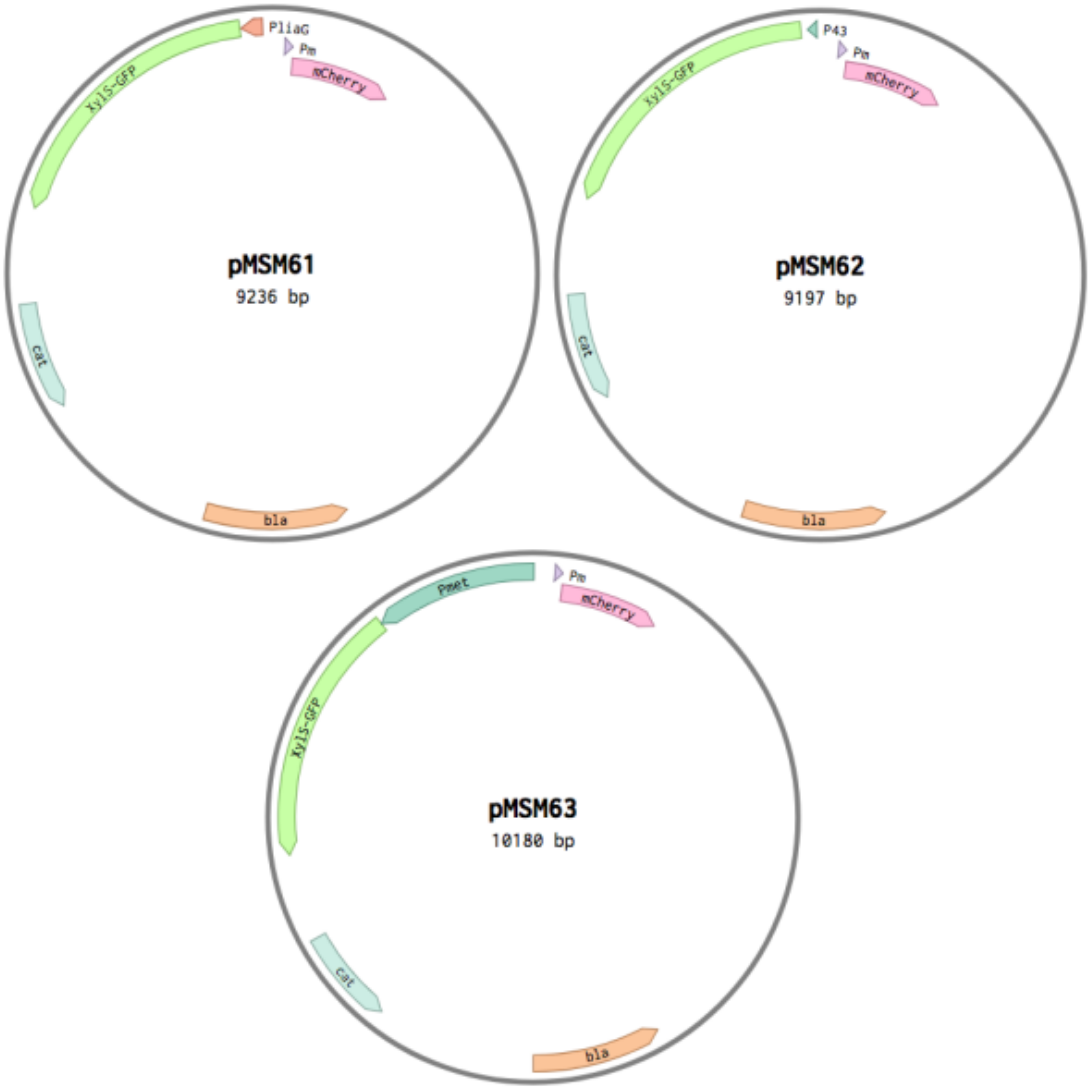

Figure 7.12: Plasmid maps of pMSI61-63 generated using Benchling.

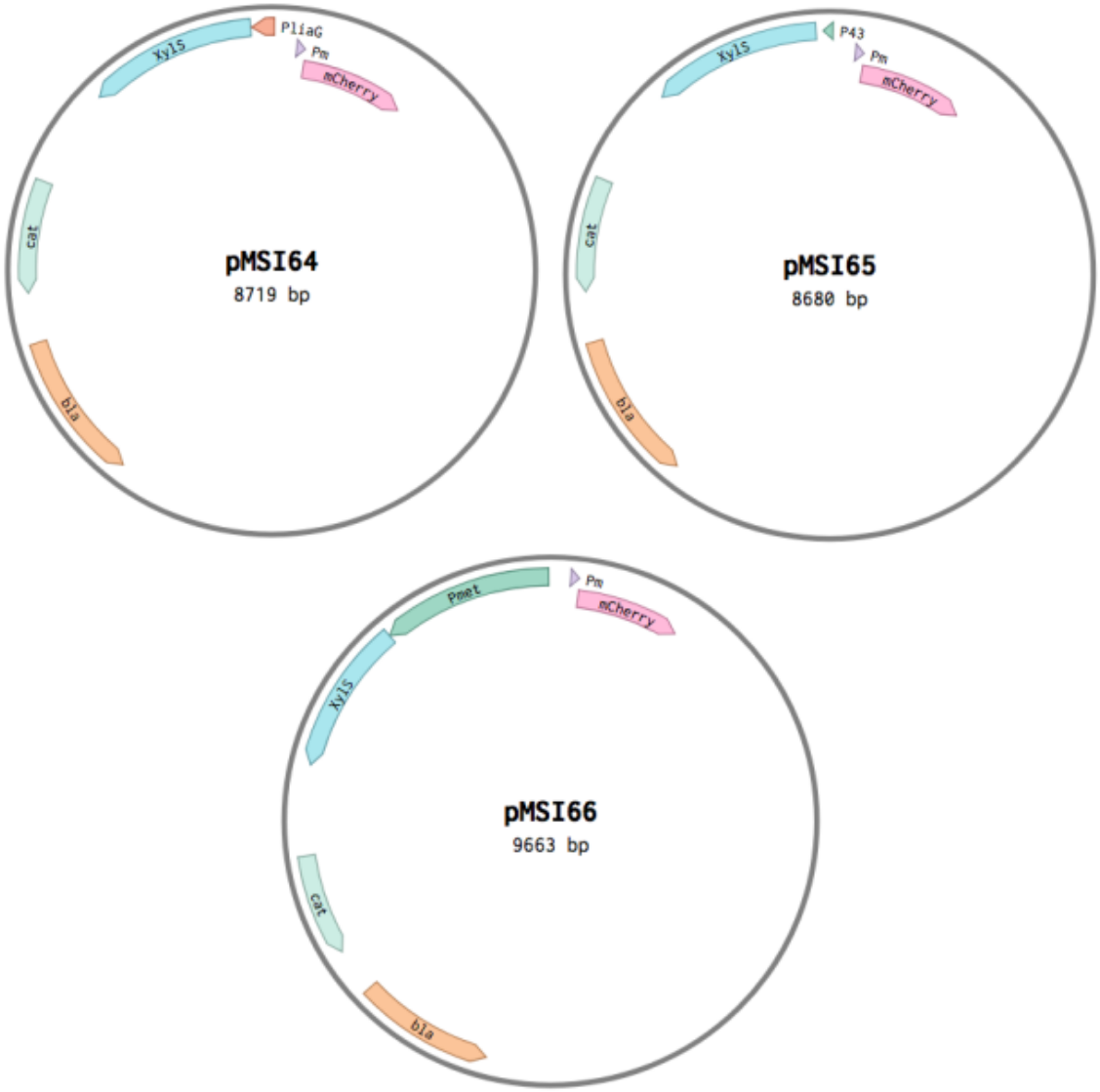

Figure 7.13: Plasmid maps of pMSI64-66 generated using Benchling.

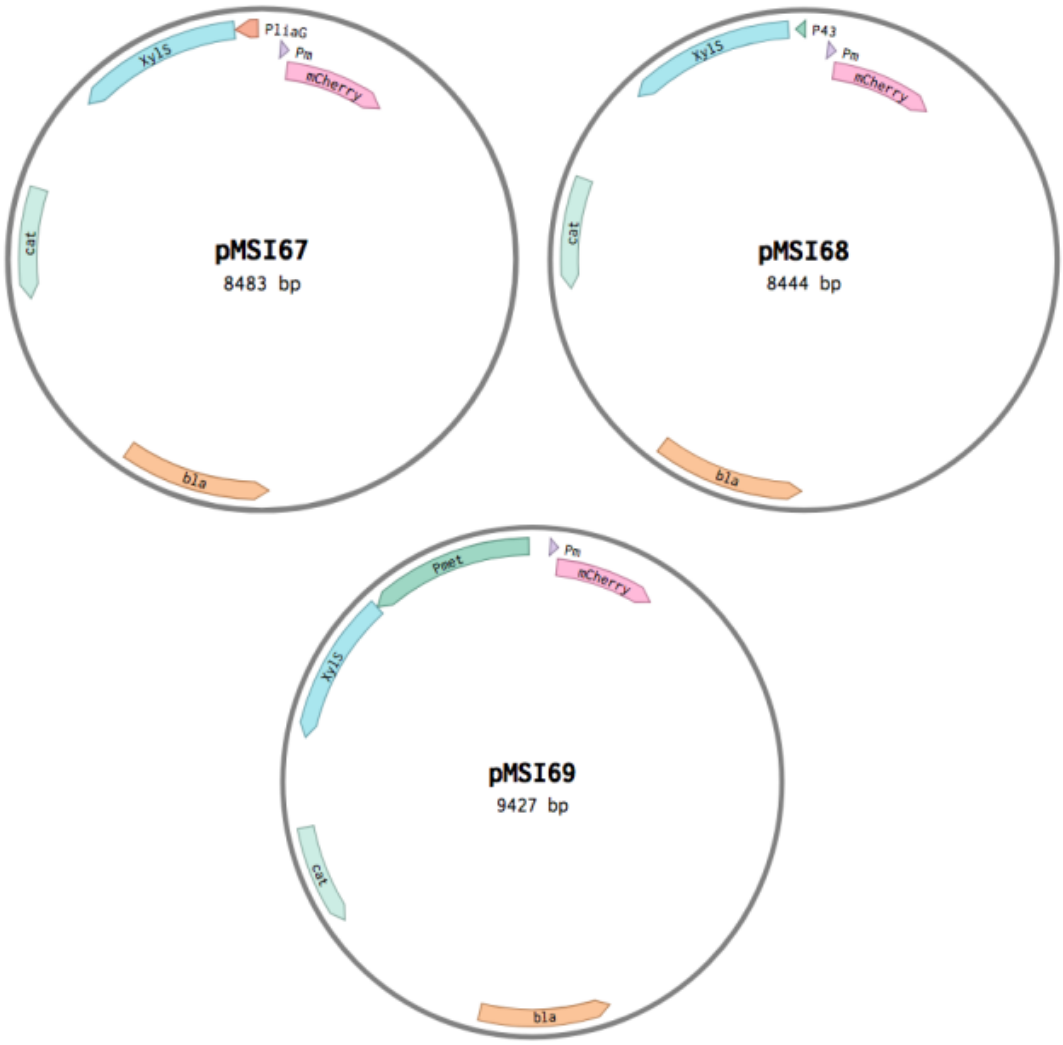

Figure 7.14: Plasmid maps of pMSI67-69 generated using Benchling.

I Codon-Optimized Genes

Several of the genes included in the pMSI-constructs had been codon-optimized for *B. subtilis*. The genes were *mCherry*, *XylS* and *XylS-GFP*. The codon-optimization was done by Vectron Biosolutions AS, and their sequences are given in figures 7.15, 7.16 and 7.17.

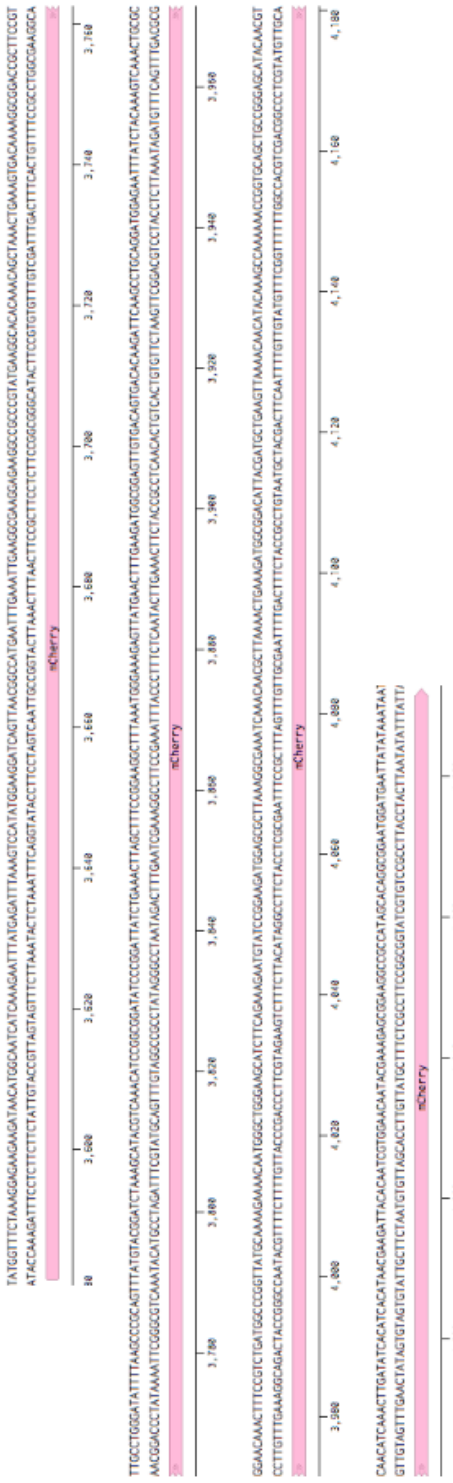

Figure 7.15: Nucleotide sequence of the codon-optimized *mCherry*. The pink arrow indicates the direction of the gene. The figure was generated using Benchling.

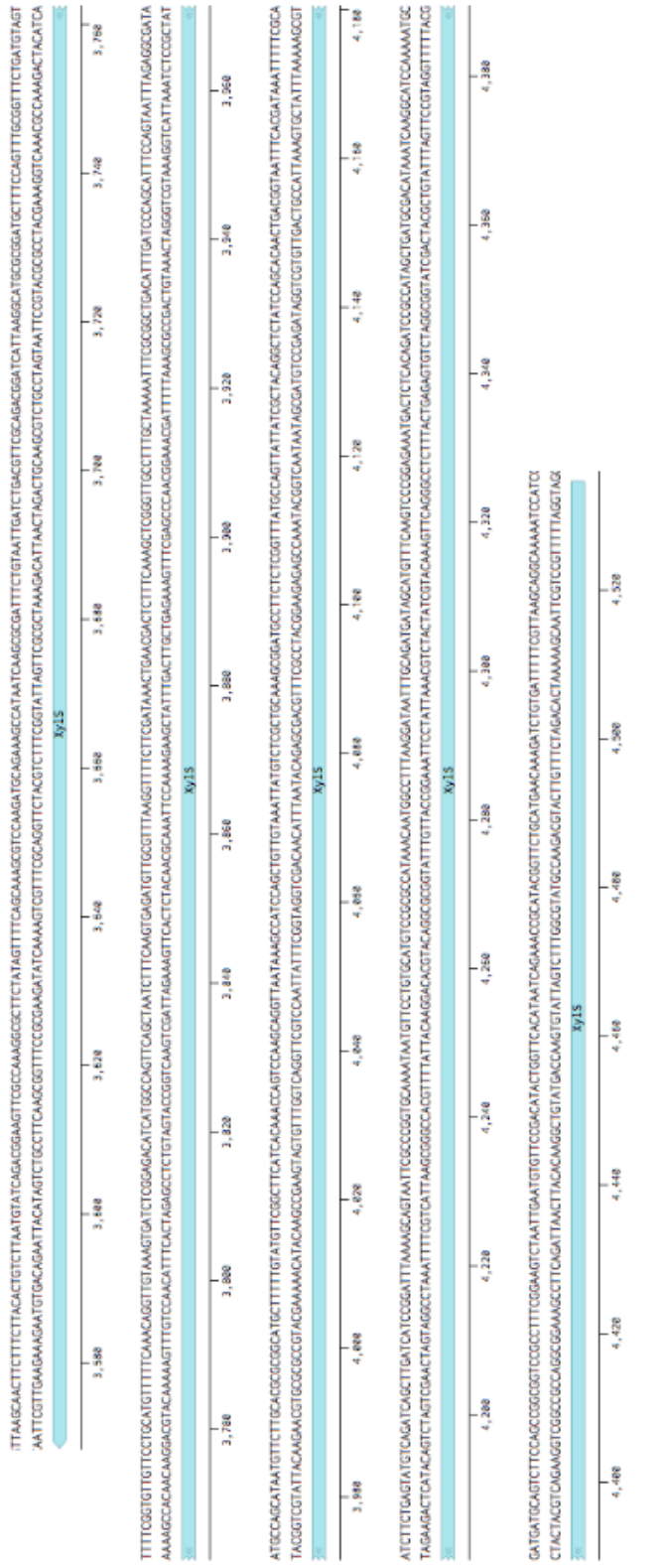

Figure 7.16: Nucleotide sequence of the codon-optimized *xylS*. The blue arrow indicates the direction of the gene. The figure was generated using Benchling.

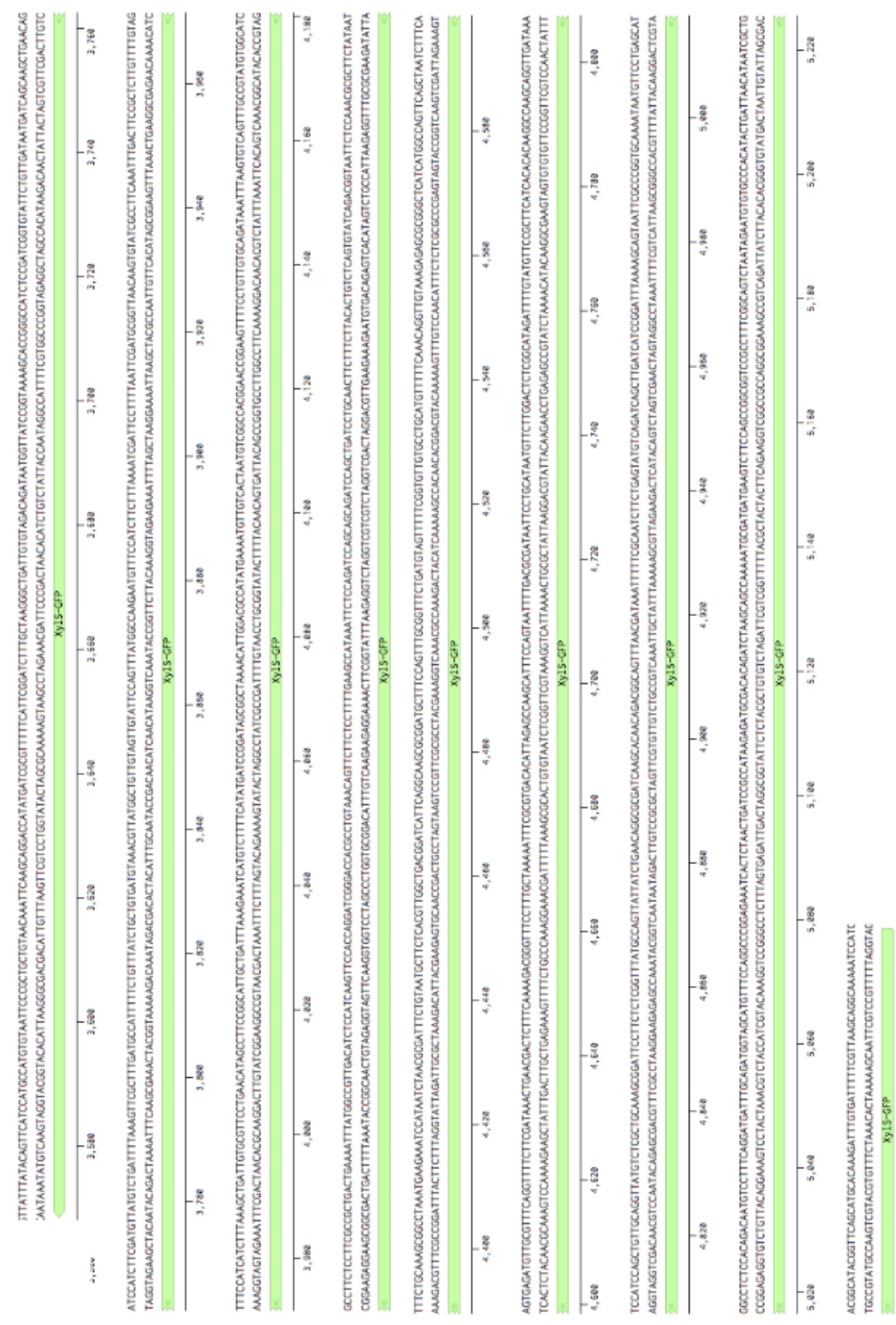

Figure 7.17: Nucleotide sequence of the codon-optimized *xylS-GFP*. The green arrow indicates the direction of the gene. The figure was generated using Benchling.