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Constitutive expression and anchoring of *Mycobacterium tuberculosis* antigens in *Lactobacillus plantarum*

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Abstract

Tuberculosis is the leading cause of death caused by a single agent worldwide. A new and effective vaccine against this infection is therefore imperative. This study is a part of a larger project where the long-term goal is to create an effective vaccine against tuberculosis using LAB as live vectors. Using LAB as a delivery vector for vaccines is highly desirable because of their GRAS status, their non-pathogenicity, probiotic properties, and their ability to deliver functional proteins to mucosal surfaces. These properties make LAB such as *L. plantarum* an ideal live vector for vaccine delivery.

In this study, a constitutive expression system was constructed by replacing the inducible promoter p_{sppA} used in the pSIP vectors with constitutive promoters derived from *Lactobacillus* spp. Moreover, genes directly related to the inducible system, *sppK* (HK) and *sppR* (RR), were removed in an attempt to reduce the fitness cost of the vector. This study reveals the challenges of constructing a constitutive plasmid for heterologous protein production. *E. coli* TOP10 was utilized as a subcloning vector. The production of AgE6 fusion antigen indicated to elicit a toxic effect in *E. coli* as most of the constitutive promoter constructs only survived when selected for inactive mutants. The toxic effect in *E. coli* indicates that most of the *Lactobacillus* derived promoters were also functional in *E. coli*.

Plasmids with constitutive protein expression which previously promoted antigen production were immobilized by the removal of the *sppK* and *sppR* genes. *sppK* and *sppR* were found to most likely be vital for constitutive protein expression utilizing the SIP system. *L. plantarum* strains harboring the SlpA or PgM promoter produced the most AgE6 anchored on the cell membrane. However, strains harboring the promoter PgM had a significantly higher growth rate. The constitutive AgE6 production is however not comparable to the inducible promoter production of AgE6, and more research is needed. The fluorescent protein mCherry was used to tag the promoters and was successfully cloned downstream of the inducible promoter p_{sppA} and the constitutive SlpA promoter. mCherry did not affect the overall fitness cost in *L. plantarum* and did not lose its ability to fluoresce over time, thus making it a promising candidate for tracking the vaccine through the GIT.

Sammendrag

Tuberkulose er den største årsaken til dødsfall forårsaket av en singulær infeksjon og en ny og effektiv vaksine mot tuberkulose er derfor betydningsfullt. Denne studien er en del at et større prosjekt der langtidsmålet er å lage en ny og effektiv vaksine mot tuberkulose ved å bruke LAB som levende vektor og leverandør av vaksinen. Bruk av LAB er meget gunstig på grunn av deres GRAS status, de er ikke-patogene, har probiotiske egenskaper og har evne til å levere funksjonelle proteiner til slimhinner. Disse egenskapene gjør at LAB, som *L. plantarum*, er ideelle som vaksinevektorer.

I dette studiet ble et konstitutivt ekspresjonssystem laget ved å erstatte den induserbare promotoren p_{sppA} i pSIP systemet med en konstitutiv promotor avledet fra *Lactobasillus* spp. For å redusere Fitness kostnader i vektor ble gener i direkte relasjon til det induserbare system, *sppK* og *sppR*, fjernet. Dette studiet avdekker utfordringene ved å konstruere et konstitutivt ekspresjonssystem i plasmid for heterolog proteinproduksjon. *E. coli* TOP10 ble brukt som en sub-klonings vektor. Produksjonen av AgE6 antigener indikerte å ha en toksisk effekt i *E. coli* fordi bare klon som var selektert for inaktive mutanter overlevde. Den toksiske effekten i *E. coli* indikerer også at de fleste promotorer fra *Lactobacillus* også er funksjonelle i *E. coli*.

Plasmid som konstitutivt transkriberer protein mistet denne funksjonen ved fjerning av sppK og sppR. Dette indikerer at *sppK* og *sppR* er avgjørende også for konstitutiv produksjon av proteiner i pSIP systemet. *L. plantarum* som huser plasmid med SlpA eller PgM promotoren produserte mest AgE6 antigen ankret på cellemembranen, men celler med PgM promoterte antigen hadde en betydelig høyere vekstrate. Selv om disse konstitutive promotorene resulterte i AgE6 produksjon, kan de ikke sammenlignes med antigen produksjonen fra det induserbare systemet og bør forskes videre på. Det fluoriserende proteinet mCherry ble brukt til å tagge promotorer og ble vellykket konstruert nedstrøms fra den induserbare promotoren p_{sppA} og den konstitutive promotoren SlpA. mCherry hadde ikke noe negativ effekt på *L. plantarums* vekst og mistet ikke evnen til å fluorisere over tid, noe som gjør den til en lovende kandidat til å spore vaksinen gjennom mage og tarm i kommende studier.

Abbreviations

BCG	Bacillus Calmette-Guérin
bp	Base pairs
BSA	Bovine serum albumin
dNTP	Deoxyribonucleotide triphosphate
FITC	Fluorescein isothiocyanate
GRAS	Generally Recognized As Safe
GIT	Gastrointestinal tract
HK	Histidine kinase receptor
HRP	Horseradish Peroxidase
LAB	Lactic acid bacteria
NICE	Nisin controlled expression
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase Chain Reaction
RBS	Ribosome binding site
RR	Response regulator
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SD	Standard deviations
SRP	Signal recognition particle
TB	Tuberculosis
QPS	Qualified presumption of safety

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1.0 Introduction

1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) comprise of a group of gram-positive, nonsporulating, non-respiring but aerotolerant cocci or rods. Their major end product during fermentation of carbohydrates is lactic acid and is therefore greatly associated with the food and feed fermentation industry. Because LAB has such a broad physiological definition, it contains around 20 genera. However, the most common LAB are *Aerococcus*, *Cornobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weisella* (Salminen & Wright, 2004; Vinderola et al., 2019). Since LAB are non-respiring, they harvest their energy from substrate-level phosphorylation utilizing the main pathways; glycolysis and phosphoketolase pathway. Both phosphorylation pathways have lactic acid as an end product and requires carbohydrates, making the LAB habitat consist mostly of environments rich in carbohydrates such as plants, mucosal surfaces and gastrointestinal tract (GIT) of mammals (Florou-Paneri et al., 2013; Vinderola et al., 2019).

LAB are considered as probiotic bacteria and are generally recognized as safe organisms (GRAS) (Adams, 1999). LAB may also provide health benefits to mammals acting as a protective agent in the host by serving as a shield from harmful pathogens and partakes in enhancing the immune system (Zielińska & Kołożyn-Krajewska, 2018). Even though there have been cases with LAB-related diseases, this mostly occurs in people with underlying disease and presumably not by ingesting additional probiotics.

1.2 Lactobacillus plantarum

Lactobacillus plantarum is one of the best studied *Lactobacilli*. It has numerous useful properties, is widely engaged in industrial fermentation of food, has a GRAS status and has qualified presumption of safety (QPS) status (Behera et al., 2018).

The genome of *L. plantarum* was sequenced in 2003 by Kleerebezem et al., The genome sequence is predicted to encode 3042 proteins, out of these, 223 proteins were expected to be extracellular proteins and most of them anchored to the cell membrane. 48 out of the extracellular proteins were predicted to inhabit a lipobox motif and thereby anchored by a lipoprotein (section 1.5). The genome sequence revealed a large number of regulatory and transport proteins, including 25 complete proton-linked sugar transport systems. This large variety in proteins may explain why *L. plantarum* is highly adaptable and flexible in its environment. (Boekhorst et al., 2006; Kleerebezem et al., 2003). However, despite *L. plantarum* being largely annotated it still has a significant fraction, about 30%, of genes with unknown function (van den Nieuwboer et al., 2016).

1.3 Bacteria as vectors in medicine

Using live bacteria as vectors for antigen delivery, triggering both the innate and adaptive immune system in the inoculated host, demonstrates an effective alternative for novel vaccine developments. Applying live bacteria as a vaccine is an efficient technique in order to create a long-term immunity against antigens presented by the vector. Two popular vaccine methods using bacteria as vector are attenuated pathogenic bacteria and GRAS food-grade bacteria (Ding et al., 2018).

1.3.1 Pathogenic delivery vectors of antigens

Bacteria as vectors in medicine is preferred, as the delivery efficiency of heterologous proteins is high. Viral vectors using pathogenic viruses applied to deliver functional proteins have an upper hand by having the innate capability of invading mammalian cells and manipulating the host cells to produce specific proteins. However, because of the difficulty of cultivation and possible viral toxins, it may introduce problems as an effective vector in medicine (Collins et al., 2008; *Isolation, Culture, and Identification of Viruses | Microbiology*, n.d.).

Using live attenuated pathogenic bacterial vectors have the ability to deliver strong signals, stimulating the mucosal and systemic immune system by their innate pathogen-associated

molecular patterns (PAMPs). PAMPs include lipopo-lysaccharides, lipoproteins and flagellin, and are recognized by a diverse selection of pathogen recognition receptors (PRRs) in the hosts innate immune system. PAMPs exogenous signals will also stimulate the adaptive immune system via the innate immune system, thus promoting long-term immunity in the host (Tang et al., 2012). Moreover, using live attenuated pathogenic vectors with a heterologous protein antigen delivery system is also a promising vaccine strategy (Ding et al., 2018). Despite the high adjuvancy of live attenuated pathogenic bacterial vectors, it still poses a risk to the inoculated host. Since pathogenic bacteria often have multiple virulence genes, it may be insufficient to remove just one virulence factor, and several changes in the genome may be needed. Moreover, it is unclear how the remaining virulence genes will behave, and the outcome may vary (Pascual et al., 2013).

1.3.2 Food-grade bacteria as delivery vectors of antigens

Lactobacilli as a vector of antigens was first proposed in the 90´s decade, where *L. plantarum* showed significant adjuvancy (Pouwels et al., 1996). Food-grade bacteria as vectors in medicine are highly desirable because of their non-pathogenicity and, in some cases, profits the host. LABs have also proved to efficiently deliver functional proteins to mucosal surfaces. It has been shown that both live and killed LAB strains, after entering the nasal mucosa, may elicit both mucosal and systemic immune responses (Wells, 2011). If singular proteins are applied directly to the mucosal surface, the immunogenicity is generally low. By coupling an antigen to a bacterial vector such as *L. plantarum*, or engineer *L. plantarum* to produce the antigen, the immunogenicity is significantly enhanced (Bermúdez-Humarán et al., 2011). Other features like easy cultivation, high production of recombinant proteins, resistance to low pH in the stomach, surviving the transfer throughout the gastrointestinal tract, adhering to the epithelial cells without colonizing it and no risk of endotoxin shock makes LAB highly versatile and a good vector candidate in medicine (Tagliavia & Nicosia, 2019). *L. plantarum* WCFS1 have shown to induce the expression of different pro-inflammatory cytokines as well as an anti-inflammatory cytokine (van den Nieuwboer et al., 2016).

A new vaccine using food-grade bacterium *L. plantarum* as vector to deliver antigens from *Leptospira borgpetersenii*, one of the most common zoonotic disease in the world, are being

studied. In this particular study, heterologous proteins from *Leptospira borgpetersenii* was fused to a homologous signal peptide of *L. plantarum* using pSIP vectors (section 1.4.1) and anchored to the cell wall, with the intention to elicit an immune response through the nasal mucosa (Suphatpahirapol et al., 2019).

1.4 Heterologous Gene expression in Lactobacillus plantarum

As a lactic acid bacterium, *L. plantarum* is a strong candidate for delivery of immunogens. In regards of expressing recombinant and heterologous proteins, it is important to assess the right expression system to ensure efficient protein expression and avoid stressing the bacteria. Typically, when transforming *L. plantarum* a subcloning bacteria is used as low transformation efficiency is a common limitation for *L. plantarum*. The low number of transformants, or lack thereof after electroporation, is sometimes due to the restriction modification system in the host. The restriction modification protects the host from foreign DNA from bacteriophages as well as plasmid DNA. The restriction modification system consists of a restriction enzyme which cleaves the foreign DNA, and a methyltransferase which protects the host DNA by blocking the restriction enzyme (Spath et al., 2012). A shuttle vector is often used to overcome this.

1.4.1 Inducible heterologous gene expression systems

Inducible expression systems are used for its ability to regulate heterologous gene expression. An inducible system regulates heterologous proteins by a variety of inducer analogues which depends on the expression system in use (Lee & Keasling, 2005). An inducible expression system is preferred when the aim is to overproduce a protein at a certain bacterial density and at a certain point in time. Moreover, an inducible expression have a tendency to exhibit lesser fitness cost thus a higher growth rate, as well as reversibility to some degree and generally a higher expression rate than constitutive expression (Kallunki et al., 2019).

Multiple LABs are producing bacteriocins as a part of their survival mechanisms. Bacteriocins are small, heat-stable bactericidal peptides which may act as an antimicrobial against various microorganisms (Lopetuso et al., 2019). The bacteriocin production is based

on the secretion of a pheromone who's function activates a regulatory system consisting of a histidine kinase receptor (HK) and a cognate response regulator (RR) (Sørvig, Mathiesen, et al., 2005). The stimuli sensed by HK gets transmitted to RR which, in most cases, binds to the associated promoter and induces an overexpression of the cognate bacteriocin (Bhate et al., 2015). LAB strains produce mainly two groups of bacteriocins, class I producing lantibiotics and class II producing non-lantibiotics (Eijsink et al., 2002; Lopetuso et al., 2019). The most well-known class I bacteriocins is nisin, which also function as the inducer by activating the HK, which activates RK and induces a gene expression (Sørvig, Mathiesen, et al., 2005).

The innate system of *Lactococcus lactis* producing nisin have been exploited to develop a regulated plasmid-based nisin-controlled expression (NICE) system in *Lactobacilli* by utilizing its promoter and regulatory genes. This is a powerful system and yields a high protein production when induced. The system includes genes that encode the HK (nisK) and RR (nisR) and the protein production is controlled by the amount of added nisin. The NICE system consists of either a one-plasmid system or a two-plasmid system. In the one-plasmid system, the plasmid harbors the gene of interest and the nisK and nisR gene is integrated into the host's chromosome. This system is limited by the amount of specially designed host strains available. The two-plasmid system consists of one plasmid harboring the nisK and nisR genes and the other plasmid harboring the gene of interest. The two plasmid system is rather laborious (Sørvig et al., 2003) and turned out to be poorly suited to *L. plantarum* (Pavan et al., 2000).

A more recent one-plasmid inducible expression system has been constructed based on the pheromone-like class II bacteriocins sakacin A and sakacin P, called pSIP expression vectors (Sørvig, Mathiesen, et al., 2005). These vectors are built up by cassettes with restriction enzyme sites which permits easy exchange of all components using restriction enzymes and ligation. (Sørvig et al., 2003). The pSIP system has genes encoding HK (*sppK*) and RR (*sppR*). As described earlier, HK gets stimulated by the amount of added pheromone inducer peptide SppIP. The stimuli gets transmitted to RR and stimulates an over-expression of the target protein by activating the cognate promoter. In short, the genes expressed by the inducible promoter are regulated by the cognate inducer (Risøen et al., 2000). The expression system was further optimized for heterologous protein secretion (Mathiesen et al., 2008, 2009).

1.4.2 Constitutive gene expression systems

A constitutive promoter is an unregulated promoter and permits continuous transcription of its associated gene. When the aim is to overproduce a protein *in situ*, it more desirable to utilize a constitutive promoter than an inducible promoter, as a steady production *in situ* requires less invasive strategies as it does not require an inducer and the protein gets expressed continuously. The affinity to the RNA polymerase is a strong determinant for the strength of a promoter, and that affinity is greatly related to the sequence architecture and sequence matches in the -35 box and -10 box in *Lactobacilli*. A single bp introduction or deletion can greatly influence the promoter activity and thus the associated gene transcription (Peirotén & Landete, 2020).

Unlike an inducible promoter, a constitutive promoter does not rely on the activation of other genes such as HK and RR. This makes a plasmid-located constitutive one-plasmid expression system less complicated, with only a promoter upstream of the target gene, replicon determinant and a selection marker as necessary segments. Rud et.al. (2006) have constructed a library of synthetic constitutive promoters based on the Jensen-Hammer approach, where the most powerful constitutive promoters were comparable to native rRNA promoters (Rud et al., 2006). Natural promoters do not harbor every possibility for transcription regulation, and by constructing a synthetic promoter one can fine-tune the gene regulation and optimize protein production. The Jensen-Hammer approach is based on randomizing DNA nearby consensus boxes in natural RNA promoters. Interestingly, the consensus sequence boxes seemed to be almost identical in all prokaryotes (Figure 1.1), the randomized bp surrounding the consensus boxes enabled the construction of promoters with different strengths (Koebmann et al., 2006).

The promoters do not necessarily have the same effect in different organisms. The promoter library constructed for *L. lactis*, and other *lactobacillus* promoters, have been reported to be species-dependent and may vary in activity in *E.coli* and *L. plantarum* (Rud et al., 2006). To find a strong promoter for *L. plantarum*, it is therefore important to select a promoter that is compatible with the host and has the desired pattern of gene expression. To select a promoter compatible to the host, it is therefore most successful to obtain one from the same species. Screening for a compatible constitutive promoter often starts by finding householding genes

being highly constitutively expressed (Peirotén & Landete, 2020), usually RNA promoters when from *Lactobacilli* strains (Rud et al., 2006). Screening for promoters of factors of initiation or elongation can also result in strong constitutive promoters, such as elongation factor Tu CDO33 (P_{tuf33}) from *L. plantarum*. P_{tuf33} showed promising results when expressing reporter green fluorescent protein. It may be advantageous to have a strong constitutive promoter when for example the aim is to severely overexpress antigens in order to elicit an immunogenic response *in situ*.

If the goal is to continuously overexpress a heterologous protein a constitutive expression system has several advantages, the biggest one being not having to be induced and being reliant on fewer genes. However, the constitutive promoter is unregulated and may hinder resources for vital metabolics, which in turn may hamper the bacterial growth. The hampered bacterial growth triggered by constitutively expressed heterologous proteins is thought to be caused by the decrease of the proteome fraction for ribosome associated proteins (Bienick et al., 2014). Moreover, expressing heterologous proteins uncontrollably may have a toxic effect on the producer. The toxic effect may not be singularly due to the plasmid gene transcription, but the translation. The fitness cost of gene translation is associated with the abundance of mRNA compared to available tRNA, amino acids and ribosomes in the cell. For highly expressed heterologous genes, especially AT-rich genes, the codon usage could also be a major cost for the bacterium. Studies have shown that AT-rich genes in *E. coli* can lead to a withdrawal of RNA polymerase (Lamberte et al., 2017). Overexpressing genes could lead to the ribosomes pausing, which in turn can lead to mistranslated proteins, misfolded proteins and a cell growth reduction (Baquero et al., 2019). To overcome this, a weaker constitutive promoter or an inducible promoter may be more desirable if an extreme overexpression of the target protein is not necessary (Peirotén & Landete, 2020).

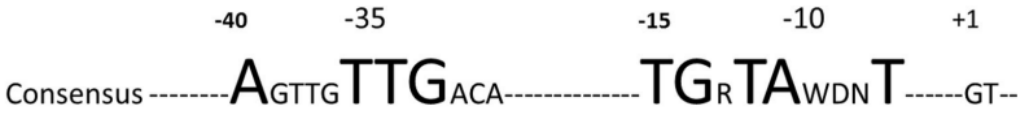

Figure 1.1. Consensus sequence (Rud et al., 2006) from rRNA promoter from *L. plantarum* WCFS1 showing the conserved bases in -40, -35, -15, -10 and +1 boxes. Semi-conserved R: A or G, W: A or T, D: A, G or T, N: A, T, G or C (Peirotén & Landete, 2020).

1.5 Secretory pathway and anchoring of proteins

The cell wall is a common feature in most bacteria, and in gram-positive bacteria the main component in the cell wall is peptidoglycan. Gram-positive bacteria have a relatively thick cell wall due to a thick layer of 20-80 nm peptidoglycan, while gram-negative bacteria have only a thin peptidoglycan layer of 2-3 nm. However, gram-negative bacteria has an additional outer lipid bilayer membrane (Sizar & Unakal, 2020). For proteins to be presented within or outside of the cytoplasmic membrane, the protein must first be targeted to the translocation site. The targeting information often lays within the N-terminal signal sequence, however it can also be found in the mature protein domain (Fekkes et al., 1999).

The known protein secretion pathways in gram-positive bacteria includes the universal pathways for both gram-negative and gram-positive bacteria; the general secretion (Sec) pathway, YidC (Akopian et al., 2013) and twin-arginine translocation (Tat) system. The known secretion systems only found in gram-positive bacteria are flagella export apparatus (FEA), the fimbrilin-protein exporter (FPE), ABC protein exporter, WXG100 secretion systems (Wss) and Sec translocase pathway.

The Sec translocase pathway is a major pathway for proteins translocated through the cytoplasmic membrane, and plays a key role in further transporting the protein to the periplasmic space in *Lactobacilli* (Rakonjac et al., 2017). The Sec pathway consists of a set of cytosolic and membrane proteins collaborating to translocate proteins. The N-terminal signal sequence will be recognized and bound by a signal recognition peptide (SRP) and the signal-peptide-SRP complex will be directed, in a chaperon-like manner keeping the preprotein unfolded, to the SecYEG translocation channel (Figure 1.2). The signal-peptide-SRP complex will be directed either by FtsY og SecB, depending on whether it is during translation or post-translation respectively. The signal-peptide-SRP complex will bind to the ATP dependent motor protein SecA, which will thread the unfolded preprotein through the SecYEG channel. The SecDF complex participates later in the process, presumably pulling the protein through the channel to the periplasmic side of the membrane (Figure 1.2) (Lycklama a Nijeholt & Driessen, 2012).

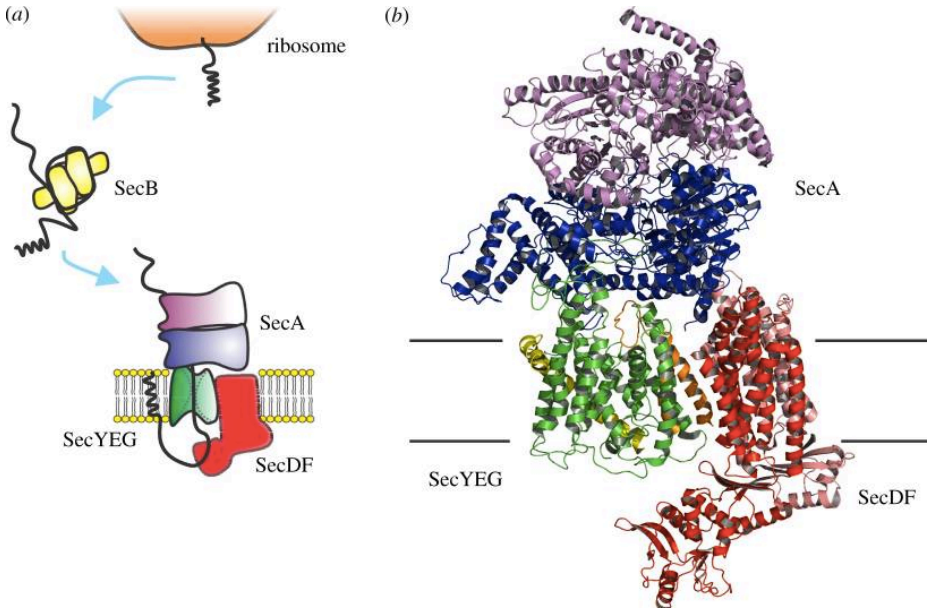

Figure 1.2. Schematic view of post-translational secretion pathway. a) Preproteins synthesized by a ribosome are bound to SecB and directed to the translocation channel SecYEG. Here, the SecB-preprotein complex are bound to the motor protein SecA. When ATP binds, SecA facilitates the translocation through the SecYEG channel. In later stages of the translocation, the heterodimer SecDF is thought to have an accessory role by pulling the protein through to the periplasmic side (Lycklama a Nijeholt & Driessen, 2012).

When heterologous secreted proteins are released and diluted or captured in the mucosal environment, they may be degraded and attacked by proteases and nucleases (Neutra & Kozlowski, 2006) and the activity may weaken by low pH and bile salts.
This can be overcome by administrating a high antigen dosage and anchoring the protein to the cell membrane. By choosing the right anchor the protein may be protected but still mediate the desired host response. Moreover, studies have shown that liposomes and lipid anchors may improve the adjuvancy (Tandrup Schmidt et al., 2016). Gram-positive bacteria have only one cell membrane and is therefore a desired vector for secreting and anchoring antigens because the protein must only be translocated over one membrane (Michon et al., 2016). Gram-positive bacteria have a variety of anchoring mechanisms to the cell-membrane. The proteins can be anchored either covalently or noncovalently. In gram-positive bacteria and *Lactobacilli*, there are four main surface anchor mechanisms; lipoprotein anchor, transmembrane anchor, LysM-domain or LPxTG peptidoglycan anchor (Kleerebezem et al., 2010). In this study, proteins were covalently attached to the bacterial surface by a lipoprotein anchor.

Lipoproteins are transported via the Sec pathway and are the second largest predicted membrane-anchored group in the *Lactobacilli* exoproteomes (Kleerebezem et al., 2010).

Lipoprotein synthesis seems to be a highly conserved pathway in prokaryotes and controlled by two factors: the signal peptide structure and a lipobox. The lipobox is vital for prolipoprotein recognition. The prolipoprotein has a signal-peptide directed export and must be processed by the enzyme prolipoprotein diacylglycerol transferase (Lgt) to become covalently anchored to the cell membrane (Figure 1.3). The signal-peptide consists of a positively charged N-region, a hydrophobic region in the center and a cleavage C-region. Lgt adds diacylglycerol into the thiol (sulfur analogue of alcohol) of a highly conserved cysteine in in the lipobox motif at the cleavage motif (Taylor et al., 2006) L-x-x-C, lipidating the protein (Kleerebezem et al., 2010). SpaseII cleaves the signal peptide at the N-terminal from the conserved cysteine, making the cysteine the N-terminus of the mature lipoprotein (Taylor et al., 2006). The cleavage attaches the mature lipoprotein to the membrane via thioether linkage and is covalently bound to a phospholipid in the cell membrane (Figure 1.3).

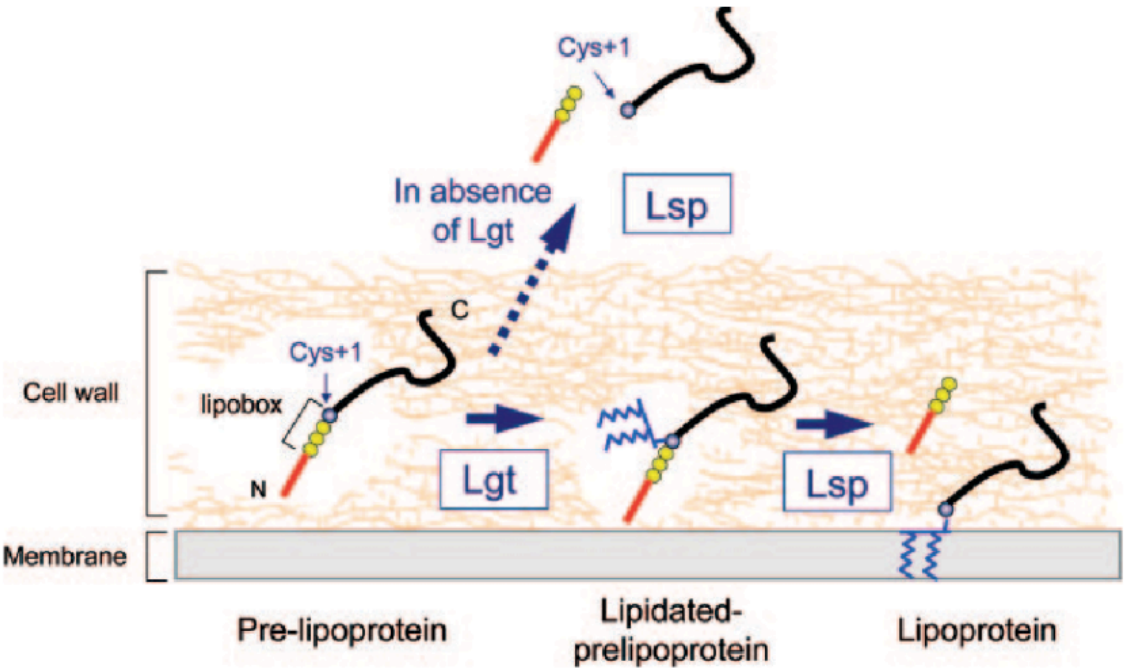

Figure 1.3. Schematic of lipoprotein processing for Gram-positive bacteria. Without Lgt, the protein cannot anchor the cell membrane and gets extruded from the cell. Lgt perform lipidation and Lsp (lipoprotein-specific signal peptideII : SpaseII) cleaves the peptide, anchoring it to the membrane (Portillo & Cossart, 2007).

LABs characteristics are ideal for homologous and heterologous protein expression systems, including membrane proteins. This includes the fact that gram-positive bacteria does not, in contrary to gram-negative bacteria, contain endotoxins in their membrane (García-Fruitós, 2012). The choice of which anchoring mechanism to employ depends on the aim and the

target protein. If the desire is to display an antigen on the surface of a gram-positive vector in order to induce an immune response, and to protect it against harsh conditions and avoid degradation of the antigen in exempli gratia (e.g.) the mucosal layers, a lipoprotein anchor is a good candidate. To surface-display a heterologous protein using a lipoprotein-anchor, the heterologous protein sequence must be fused to a lipoprotein downstream of the lipobox (Michon et al., 2016).

1.6 Tuberculosis

According to the World Health Organization's (WHO) Global Tuberculosis report 2019, a total of 1.5 million people died from Tuberculosis (TB) in 2018, and is one of the top 10 deaths worldwide and the leading cause of death from a single agent. About a quarter of the world's population is infected with TB, and about 10 million of these fell ill each year. Multidrug-resistant TB is an increasing public health crisis and in 2018, 186 772 cases of drug resistant TB was detected. TB is a rather robust bacterium and patients normally need six months of antibiotic treatment to be cured, which also contributes to the increasing antibiotic resistance. The treatment for patients with resistant TB can take up to 20 months.

TB is caused by *Mycobacterium tuberculosis* and spreads through aerosol droplets and usually infects the lungs by entering the alveolar passage. The typical first contact here is the macrophages where they can get phagocytosed, which in turn triggers an immune response through their chemokines. When cellular immunity develops, the bacilli loaded macrophages are killed and forms a caseous center of a granuloma. All though *M. tuberculosis* are postulated to be inhibited to multiply due to the acidic environment and low concentrations of oxygen inside the caseous tissue, some bacilli have the ability to stay dormant for decades (Smith, 2003).

TB is most common in poor countries, people with poor living conditions and immuno-suppressive diseases like HIV/AIDS. The most efficient method to avoid disease is by vaccination, and the only available vaccine to date is the Bacille Calmette-Guérin (BCG). However, BCG does not prevent primary infection nor does it prevent reoccurrence of latent pulmonary infection, which is the main source of bacilli spread (*Pulmonary Tuberculosis*, n.d.). There is an urgent need for new and more efficient vaccines against *M. tuberculosis*.

According to WHO in 2019, there were 14 novel vaccine candidates in clinical trials. Recently, the M72/AS01$_E$ vaccine was found to elicit an immune response that was sustained for three years, including protection against latent TB infections. The M72/AS01$_E$ vaccine compound is an recombinant fusion protein from *M. tuberculosis* antigens paired with the AS01$_E$ adjuvant system (Tait et al., 2019).

When choosing antigens for a vaccine, it is important to choose antigens with a T-cell reactivity. The most commonly used antigens are of the antigen 85 and ESAT-6 family due to their virulence with high immunogenicity and T-cell reactivity (Kuczkowska et al., 2016). This study uses *L. plantarum* to present the fusion-protein comprised of Ag85B and ESAT-6, abbreviated to AgE6.

1.7 Aim of this study

This study is a part of a larger project with the objective to produce a mucosal administrative vaccine against *Mycobacterium tuberculosis* using LAB as a live vector. This thesis had three goals. (1) Substitute the inducible promoter P_{sppA} with constitutive promoters to constitutively express the fusion antigen AgE6. (2) Remove the genes *sppK* (HK) and *sppR* (RR), which are associated with the inducible system in order to reduce the fitness cost during heterologous protein production in host bacterium *Lactobacillus plantarum*. (3) Tag the inducible and constitutive promoters with mCherry in order to measure promoter ability and to ensure the ability to follow *L. plantarum* through e.g. the GIT in future studies.

pSIP vectors harboring an inducible promoter have been used in previous studies, in which antigen expression is induced by the pheromone SppIP. In this study, pSIP plasmid vectors with constitutive expression were constructed to in order for recombinant *L. plantarum* to constitutively produce the fusion antigen AgE6. The starting point of all plasmid construction in this study was derived from the pSIP401 vector (Table 2.8), and the AgE6 production and localization in the host cell were characterized. As one goal was to constitutively express the antigens, making the vaccine vector able to continuously produce antigens without an inducer peptide, plasmid genes were removed to relieve any metabolic stress. The *sppK* and *sppR*

genes were thought to only have a purpose with an inducible system. As constitutive heterologous protein expression can reduce the bacteria growth rate, removing these genes were thought to relieve the overall fitness cost of heterologous gene expression (section 1.4.2). To be able to measure promote activity in *L. plantarum* they were tagged with the fluorescent protein mCherry. By constructing vectors harboring mCherry, one could also follow the vaccine vector through the GIT in future studies.

The experimental work was carried out in the following steps:

- Construction of constitutive plasmids for AgE6 expression
- Removing *sppK* and *sppR* in constitutive and the inducible plasmids.
- Investigation of bacterial growth, AgE6 production and cell localization in *L. plantarum* by western blotting, flow cytometry and confocal laser scanning microscopy
- Tagging the promoters with mCherry and measure the relative fluorescence

2. Materials

2.1 Lab equipment

Table 2.1. Shows Laboratory equipment utilized and suppliers of these.

Laboratory equipments	Supplier
Cryovials, 1.5 mL	Sarstedt
Disposable cuvette, 1.5 mL	Brand
Electroporation cuvette, Gene Pulser®, 0.2 cm	Bio-rad
Eppendorf tube, 1.5 and 2.0 mL	Axygen
Falcon 2059 Polypropylene Round Bottom tube, 14 mL	Becton Dickinson
FastPrep® tube	Fisher scientific
Glass beads	Sigma
Lysing matrix 2 tubes, 2 mL	MP Biomedicals
Microplates for fluorescent based assays, 96-well	Thermo Scientific
Microwell plate, 96 wells	Thermo Scientific
PCR tube, 0.2 mL	Axygen
Pipetboy comfort	Integra
Serological pipette, 5, 10 and 25 mL	Sarstedt
Slides and cover slip, Menzel-gläser	Thermo scientific
Sterile filter, 0.20 µM in pore size	Sarstedt
Syringe, 10-60 mL	Plastipac

Various glassware	
Water bath	Julaba
1 mm cuvette	Bio-Rad
Ultrospec 10 Cell Density Meter	Amersham Biosciences
Varioskan™ LUX multimode microplate reader	Thermo Scientific
QuBit fluorometer	Thermo fisher scientific

2.2 Software

Table 2.2. Shows software used to produce results and its suppliers.

Software	**Supplier**
AzureSpot Analysis Software (??)	Azure biosystems
CLC DNA Main Workbenck 7	Qiagen
MacsQuantify™ Software	Miltenyi Biotec
pDRAW32	www.acaclone.com
Zen Software	Zeiss
FlowJo	www.flowjo.com

2.3 Chemicals

Table 2.3. Shows chemicals utilized and its suppliers.

Chemicals	Supplier
Ammonium citrate tribasic, C6H17N3O7	VWR
Ampicillin, C16H19N3O4S	Sigma-Aldrich
Brain-Heart-Infusion (BHI)	Oxoid
De Man, Rogosa, Sharpe (MRS)	Oxoid
Ethylenediaminetetraacetic acid (EDTA), C10H16N2O8	Merck
Erythromycin, C37H67NO13	Merck
D-(+)- Glucose, C6H12O6	VWR
Disodium phosphate, Na_2HPO_4	Merck
Potassium dihydgrogen phosphate, KH_2PO_4	Merck
Glycerol 85%, C3H8O3	Merck
Glycine, C2H5NO2 85%	Duchefa Biochemie
Magnesium Chloride, MgCl2	Merck
Magnesium Phosphate, MgSO4	Sigma
Polyethylene glycol, PEG1450	Aldrich
SeaKem® LE Agarose	Lonza
Sodium Acetate, C2H3NaO2	Sigma-Aldrich
Sodium Chloride, NaCl	Merck
Sodium hydroxide, NaOH	Merck
Potassium Chloride, KCl	Merck
Super Optimal broth with Catabolite repression (S. O. C.)	Invitrogen
Tween-20	Sigma-Aldrich
Tris-base, $C4H_{11}NO_3$	Sigma
BSA	Sigma
NucleoSpin® Plasmid Kit	MACHEREY-NAGEL, Düren, Germany

2.4 Proteins, enzymes and DNA

Table 2.4. Shows different proteins, enzymes and DNA utilized, and its suppliers.

Protein, enzymes and DNA	Supplier
The BenchMark™ Protein Ladder	Invitrogen
FastDigest® Green Buffer	Thermo scientific
FastDigest® Restriction enzymes	Thermo scientific
Bgl11	Thermo scientific
Eco31I	Thermo scientific
NdeI	Thermo scientific
New England biolabs, Neb, Restriction enzymes	New England biolab inc (NEB)
Bgl11	NEB
Nde1	NEB
Sal1	NEB
Hind111	NEB
Bsa1	NEB
Apa1	NEB
Cla1	NEB
Neb Buffer	
1.1 10X buffer	NEB
2.1 10X buffer	NEB
3.1 10X buffer	NEB
Cutsmart® 10X buffer	NEB
Mung bean nuclease + 10Xbuffer	NEB
T4 polymerase + buffer	NEB
Inducer peptide SppIP	CASLO
RED Taq DNA Polymerase Master Mix	VWR
Q5® Hot Start High-Fidelity DNA Polymerase	NEB
ElectroLigase®	NEB
ElectroLigase® Reaction Buffer	NEB
5X In-Fusion HD Enzyme Premix	Takara Bio

DNA-standards	
Quick-Load® Purple 1 kb DNA Ladder	NEB
100 bp DNA ladder	NEB
Q5® Hot Start High-Fidelity DNA Polymerase	NEB

2.5 Primers

Table 2.5. Shows the primers used in this study.

Name	Sequence
pJET1.2_F	TCTCGGTACCTCGCGAATGC
pJET1.2_R	ATCTGCAGTCGACGGGCC
SekF	GGCTTTTATAATATGAGATAATGCCGAC
SekR	CCTTATGGGATTTATCTTCCTTATTCTC
SeqAg85_R	CCCATTGATGGACTTGGAAC
SeKEry_F	ACTAGGGTTGCTCTTGCA
SlpA8287_F	CTGATTACAAAGGCTTTAAGCA
slpD_F	TGATAAGATATCGTTGTAGCATA
Tuf34_F	ATCTGTTTACAAACATTACCAGTATC
Tuf_Inf_F	TTACAGCTCC AGATCTGATC AGGAAATTAA AATTGGTC

Tuf_Inf_R	GTTTTGAAATTCATATGTAAAATCTCCTTGTTTTCAAGAATTAC
Cherry_F	GGAGTATGATTCATATGAGCAAAGGAGAAGAAGATAAC
Cherry_R	CTGTAATTTGAAGCTTTTATTTGTAAAGCTCATCCATTCCGC

Table 2.6. Description of primers used in this study.

Name	Relevant characteristics
pJET1.2_F	Reverse primer that binds to the pJET vector, used to amplify promoters to insert to pSIP vectors.
pJET1.2_R	Forward primer that binds to the pJET vector, used to amplify promoters to insert to pSIP vectors.
SekF	Forward primer for sequencing of inserted promoters.
SekR	Reverse primer for sequencing of inserted promoters.
SeqAg85_R	Sequence reverse primer of antigen Ag85.
SeKEry_F	Forward primer that binds to erythromycin resistance gene.
Slpa8287_F	Forward primer that binds to promoter gene SlpA8287.
slpD_F	Forward primer that binds to promoter gene slpD.
Tuf34_F	Forward primer that binds to promoter gene Tuf34.
Tuf_Inf_F	In-fusion forward primer to amplify promoter gene Tuf34 for insertion to pSIP vector.
Tuf_Inf_R	In-fusion reverse primer to amplify promoter gene Tuf34 for insertion to pSIP vector.
Cherry_F	In-fusion forward primer to amplify the gene mCherry for insertion to pSIP vector.
Cherry_R	In-fusion forward primer to amplify the gene mCherry for insertion to pSIP vector.

2.6 Bacterial strains

Table 2.7. The bacterial strains used in this study and its source

Bacterial strain	Source
Escherichia coli TOP10	Invitrogen
Lactobacillus plantarum WCFS1	(Kleerebezem et al., 2003)
NEB® 5-alpha Electrocompetent *E. coli*	New England Biolab inc.

2.7 Plasmids

Table 2.8. Plasmids used in this study.

Plasmid name	Source	Relevant descriptions
pLp_1261_Ag85-ESAT6-DC	(Kuczkowska et al., 2016)	pSIP 400 derivate, p256, harboring inducible promoter P_{sppA} and Ag85B-ESAT6 (AgE6) antigen fused to N-terminal lipoprotein derived from Lp_1261.
pJET1.2_SlpA8287	Genscript	pJET vector harboring constitutive promoter slpA8287
pJET1.2_SlpD	Genscript	pJET vector harboring constitutive promoter SlpD
pJET1.2_Tuf34	Genscript	pJET vector harboring constitutive promoter Tuf34
pSIP_SlpA_1261_Ag85-ESAT6-DC	Kamilla Wiull	pSIP 400 derivate from harboring constitutive promoter SlpA.
pSIP_Pgm_1261_Ag85-ESAT6-DC	Kamilla Wiull	pSIP 400 derivate from harboring constitutive promoter Pgm.

2.8 Mediums and agars

Table 2.9. Mediums and corresponding agars used in this study.

Medium	Agar plates
Brain-Heart-Infusion (BHI) 18.6 g BHI dissolved in dH_2O to 0.5 L Sterilized in CertoClav at 121 °C for 10 min.	BHI broth supplemented with 1.5 % (w/v) agar, mixed thoroughly and sterilized in CertoClav at 121 °C for 10 min. The media was allowed to cool down to 50°C before adding appropriate antibiotics, poured into sterile agar plates and solidified before being stored at 4°C.
De Man, Rogosa, Sharpe (MRS) 26 g MRS broth dissolved in dH2O to 0.5 L Sterilized in a CertoClav at 121 °C for 10 minutes.	MRS broth supplemented with 1.5 % (w/v) agar, mixed thoroughly and sterilized in CertoClav at 121 °C for 10 min. The media was allowed to cool down to 50°C before adding appropriate antibiotics, poured into sterile agar plates and solidified before being stored at 4°C.
MRSSM medium MRS + 0.5 M sucrose + 0.1 M MgCl2 to 40 mL, mixed and sterilized by filtrating it through a 0.2 µm pore size filter. The medium was then stored in 1.5 mL Eppendorf tubes at -20°C	
GM17 medium 18.63 g M17 dissolved in dH2O to 0.5 L Sterilized in CertoClav at 121 °C for 10 min. The medium was allowed to cool down to 50°C before adding 0.5% sterile glucose.	GM17 broth supplemented with 1.5 % (w/v) agar, mixed thoroughly and sterilized in CertoClav at 121 °C for 10 min. The media was allowed to cool down to 50°C before adding appropriate antibiotics, poured into sterile agar plates and solidified before being stored at 4°C.
SGM17	

GM17 medium + 0.5 M sterile sucrose.

Super Optimal broth with Catabolite repression, S.O.C. Premade by manufacturer.

2.9 Buffers and solutions

Phosphate Buffered Saline (PBS)

8 g/l NaCl 0.2 g/l KCl 1.44 g/l Na2HPO4 0.24 g/l KH2PO4

TPBS

PBS 0.1 % (w/v) Tween

3.0 Methods

3.1 Bacterial cultivation

Escherichia coli was grown in BHI. When in liquid medium, it was incubated at 37 °C while being vigorously shaken. *Lactobacillus plantarum* was grown in MRS. When in liquid medium it was cultivated without shaking at 37 °C.

In conjunction with Table 3.1, ampicillin or erythromycin was used as a selection marker for bacteria harboring pJET or pSIP derivates, respectively.

Table 3.1. Shows appropriate amounts of different antibiotics when applied to different bacteria.

Antibiotica	Agar agar – *E. coli*	Liquid medium - *E.coli*	Agar agar- Lactic acid bacteria	Liquid medium - Lactic acid bacteria
Erythromycin	200-300 ug/mL	200 ug/mL	5-10 ug/mL	5-10 ug/mL
Ampicilin	100 ug/mL	200 ug/mL	5-10 ug/mL	5-10 ug/mL

3.2 Bacterial stock

A glycerol stock 87% (v/v) was made to store bacteria over a long period of time at -80°C.

Materials

Bacterial culture

Sterile 87% glycerol

1.5 mL cryovial.

Procedure

1 mL of bacterial culture incubated over night was added to a cryovial. 300 µL of 87% glycerol was added, inverted two to four times and stored at -80°C. To cultivate this bacterium later; a small amount of the glycerol stock was picked with a sterile toothpick and added to an appropriate medium with corresponding antibiotic and cultivated overnight.

3.3 DNA and plasmid isolation

To isolate DNA from cultivated bacteria, NucleoSpin® Plasmid Kit protocol 5.1 or 5.2 were used, depending whether it was high or low copy, following the manufacture`s procedure.

3.4 DNA digestion

DNA was digested by one or more restriction enzymes with appropriate buffers. Vector and insert were digested with the same enzymes, creating compatible sticky ends and ligated. Vector digested and ligated without insert resulted in incompatible ends, and either mung bean nuclease or T4 polymerase were used to create blunt or compatible ends before ligation.

Materials

Restriction enzyme

DNA: 1 µg

10X buffer

dH_2O

Procedure

DNA, 5 µL 10X buffer and dH_2O were first mixed, and supplemented with desired restriction enzymes and the solution was carefully mixed to a total volume of 50 µL. The mixture was then incubated for at least one hour, for up to two hours, at appropriate temperature. The temperature depended on the enzymes and buffer used. After incubation, it was loaded on to an agarose gel to separate the DNA fragments.

3.4.1 Mung bean nuclease

Mung bean nuclease was used to blunt sticky ends before ligation. This method removes single stranded overhangs on both the 5´ and the 3´.

Materials

1X mung bean nuclease reaction buffer
1 U mung bean nuclease per ug DNA
dH_2O: to 50 µL Mung bean nuclease
SDS: 0,01%

Procedure

Due to low concentration of DNA, the nuclease was diluted to get the correct concentration. Mung bean reaction buffer 10X was diluted to 1X by adding 10 µL to 90 µL water.
All components, except SDS, were added to 1.5 ml Eppendorf tubes and incubated at 30 °C for 30 minutes.
To stop the reaction, 0,01% SDS was added.

3.4.2 T4 polymerase

T4 polymerase was used to blunt sticky ends before ligation. This method removes single stranded overhangs at 3´or fill in on 5´.

Materials

1X 2.1 buffer
dNTP
Digested DNA
dH_2O: to 25 µL
EDTA

Procedure

2.5 µL 1X 2.1 buffer, 0.2 µL dNTP and 12 µL digested DNA were mixed, dH_2O was added to adjust the volume to 25 µL, excluding EDTA. The mixture was incubated at 12 °C for 15 minutes. Subsequently, 5 µL EDTA was added and the mixture was incubated at 75 °C for 20 minutes to stop the reaction. This was ligated with T4 quick ligase and either transformed to *E. coli* TOP10 or stored at -20°C.

3.5 Ligation

3.5.1 Quick ligase

Chemically competent cells were transformed with a plasmid ligation using New England biolabs protocol for quick ligation (M2200).

Materials

Quick ligase reaction buffer (2X):
Insert/vector molar ratios: 3:1
Quick ligase: 1µL
Adjust volume to 20µL with dH_2O

When removing the *sppK* and *sppR* genes, 1µL of vector was used when ligating the plasmid.

Procedure

10 µL Quick ligase reaction buffer (2X), insert, vector, 1 µL Quick ligase and dH_2O were mixed, adding the enzyme last to the reaction, by centrifuging briefly. The mixture was incubated at room temperature for five minutes. Subsequently, the ligated vectors were transformed into competent cells, or stored at -20°C.

3.5.2 Electroligase

Electrocompetent cells were transformed with a plasmid electroligation using New England biolabs protocol for cloning with electroligase® (M0369)

Materials

H_2O to 5 µL

Electroligase reaction buffer ®

Electroligase ®

Procedure

Electroligase reaction buffer and electroligase were gently tapped to mix before use. Vector and insert (insert/vector ratio: 3:1) were mixed together with dH_2O to 5µL. Subsequently, 5 µL electroligase reaction buffer and 1 µL electroligase was added to the mixture by pipetting up and down several times. The ligation mixture was incubated at room temperature from 30-40 minutes. The mixture was then inactivated by incubating at 65°C for 15 minutes, then chilled on ice or stored at -20°C.

3.5.3 In-fusion cloning

The In-fusion cloning kit was used for direct cloning of DNA into a linearized vector. The in-fusion enzyme is able to recognize 15 bp overlaps at each end of the vector, which needs to be homologous to 15 bp at the ends of amplified PCR products which is achieved by using designed in-fusion primers (Figure 3.1). When designing in-fusion PCR primers, it is essential that the 5′ end has 15 bases homolog to 15 bases at the end of desired linearized vector, and the 3′end of the primer must be specific to the desired insert.

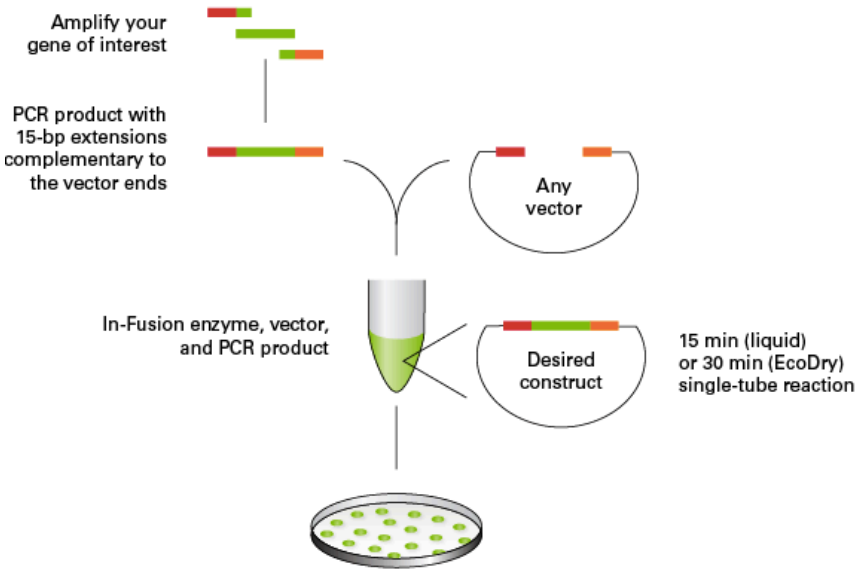

Figure 3. 1. A simplified illustration of In-fusion cloning procedure. The gene to be inserted is amplified using designed primers with a 15 bp overhang homolog to the vector (red and orange overhang). The vector is linearized using appropriate restriction enzymes. A reaction mixture containing the simplified insert the linearized vector is incubated at 50 °C for 15 minutes and subsequently transformed. The illustration is taken from TaKaRa bio In-Fusion® HD Cloning Kit User Manual.

Materials

5X In-fusion HD enzyme premix

Linearized vector

Purified PCR fragment

dH_2O to 10 µL.

Procedure

To determine the amount of linearized vector and insert needed for each reaction, the In-Fusion® Molar Ratio Calculator from TaKaRa Bio was used.

The insert and vector were mixed with 2 µL 5X in-fusion HD enzyme premix and the volume was adjusted to 10 µL and mixed by briefly centrifuging. The reaction was then incubated at 50 °C for 15 minutes, and then placed on ice or stored at –20°C.

For reactions with a larger total volume than 7 µL of insert and vector, 4 µL of 5X in-fusion HD enzyme premix was used, and volume was adjusted to 20 µL with dH_2O.

3.6 Gel electrophoresis

3.6.1 Agarose gel electrophoresis

Agarose gel electrophoresis separates DNA fragments based on its size. A 1 kb DNA ladder or 100 bp ladder with known fragment sizes were used as reference.

Materials

SeaKem® LE Agarose
1x TAE Buffer
peqGREEN
Loading buffer
DNA ladder

Procedure

To make 1.2% gel, 6 g SeaKem® LE Agarose powder was dissolved in 500 mL 1x TAE buffer and sterilized at 120°C for ten minutes in a CertoClav, and later stored at 50°C. To prepare a gel, 60 mL agarose solution and 2.5 µL peqGREEN were mixed and poured into a molding tray with combs of desired size. When the gel had solidified, combs were removed, and the gel was transferred to an electrophoresis tray and filled with 1x TAE buffer. The DNA ladder, along with appropriate amounts of loading buffer added to each of the DNA samples, were carefully applied to separate wells. The gel was run at 90 volts, and depending on the DNA fragment size, between 25-60 minutes.

3.6.1.1DNA purification from agarose gels

Protocol from NucleoSpin® Gel and PCR Clean-up by MACHEREY-NAGEL was utilized to purify digested DNA from agarose gel and PCR products.

3.6.2 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-page) separates denatured proteins based on the length of the polypeptide chain. SDS has a significant protein-denaturing effect, and by adding detergent lithium dodecyl sulphate (reducing agent, a SDS analoug) and LSD sample buffer, the disulfide bonds critical for protein folding are cleaved. This results in protein chain lengths that are proportionate to its negative charge (*The Principle and Method of Polyacrylamide Gel Electrophoresis (SDS-PAGE) | MBL Life Science -JAPAN-*, n.d.). To determine the molecular weight of the proteins, kDa, a protein standard was used.

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Materials

Mini-PROTEAN® TGX Stain-FreeTM Precast Gels, 10 or 15 wells
NuPAGE® LDS Sample Buffer (4X)
NuPAGE® Reducing agent (10X)
TGS Buffer
MagicMarkTM XP Western Protein Standard

Procedure

7.5 µL NuPAGE® LDS Sample Buffer (4X) a 3 µL nd NuPAGE® Reducing agent (10X) were mixed to form a 2x working solution. 2 µL protein solution was added to the mixture and the solution was boiled for ten minutes. Mini-PROTEAN® TGX Stain-FreeTM Precast Gel was set up in the electrophoresis chamber before adding TGS buffer. 10 µL of the protein standard (MagicMarkTM XP Western Protein Standard) was added to a well, followed by the heat-treated protein samples, often applied with one empty well between them to avoid cross-contamination. The gel was run at 280 volts for 18 minutes, then placed in dH_2O awaiting further analysis.

3.7 Determination of DNA concentration

After agarose gel purification, the concentration of the eluted DNA was determined using Qubit Fluorometer.

Materials

Qubit® dsDNA HS Reagent

Qubit® dsDNA HS Buffer

DNA eluate

Method

A solution of 197 µL Qubit® dsDNA HS Duffer and 3 µL fluorescent Qubit® dsDNA HS Reagent was mixed per DNA eluate. 197 µL of the mixture was added to an Eppendorf tube together with 3 µL of DNA eluate and mixed thoroughly. The sample was incubated at room temperature for one minute and placed in the Qubit Fluorometer to measure absorbance at 260 nm.

3.8 Preparation of electrocompetent Lactobacillus plantarum WCFS1

When transforming bacteria, is important that they are able to uptake free DNA. Although this happens in nature, it is imperative to create artificially competent cells to make them more susceptible for new DNA. Bacteria can either be chemically competent or electrocompetent.

Unlike chemically competent cells, electrocompetent cells do not require membrane bound DNA. The principle of electroporation is to apply a transient electrical field in order to create a transient movement of negatively charged DNA, allowing the DNA to penetrate the cell membrane. Glycine is a cell wall weakening agent making cells more electrocompetent. Glycine works by reducing the peptidoglycan bonds and loosening up the cell wall by replacing the L-alanine bridges, making the cells more permeable for plasmids during electroporation.

Materials

MRS medium

MRS + 1% glycine

30% w/v PEG1450

MRS + 0.5 M Sucrose + 0.1 M $MgCl_2$

Procedure

L. plantarum from glycerol stock was grown overnight in 10 mL MRS at 37°C. A serial dilution, 10^{-1}-10^{-10} of the overnight culture was made by using 1 mL culture in 10 mL MRS + 1% glycine. The diluted cultures were further incubated overnight at 37°C. 1 mL of the overnight culture with OD_{600} of 2.5 ± 0.5 was further diluted in 20 mL MRS + 1% glycine. This culture was grown in 37°C until reaching the logarithmic phase OD600 of 0.7 ± 0.07, and then placed on ice for ten minutes. The culture was then centrifuged at 5000 x g for five-ten minutes at 4°C, discarding the supernatant. The pellet was resuspended in 5 mL of freshly made, ice cold 30% PEG_{1450}. Additional 20 mL more of PEG_{1450} was added, the tube inverted and placed on ice again for ten minutes. The resuspension was centrifuged at 5000 x g for five to ten minutes at 4°C, discarding the supernatant. The pellet was resuspended in 400 µL 30% w/v PEG_{1450} and 40 µL was distributed into ice cold Eppendorf tubes and immediately frozen at -80°C

3.9 Transformation in competent cells

3.9.1 Transformation in chemically competent cells

In this thesis, chemically competent *E. coli* TOP10 were used, as they provide a high transformation efficiency of 1 x 10^9 cfu/µg plasmid DNA. The cells are treated with a salt solution to facilitate plasmid attachment to the cell wall and promote DNA passage. When the TOP10-ligation mixture undergoes a heat shock, the cell membrane pores opens and further allows the DNA to enter.

Materials

Ligation mix (quick ligase)

E. coli TOP10 competent cells

S.O.C, super Optimal broth with Catabolites repression medium.

BHI agar plates

Procedure

1. Vials containing 50 µL *E. coli* TOP10 competent cells were thawed on ice, and vials containing the desired ligation mix were briefly centrifuged and placed on ice.
2. Once *E. coli* TOP10 were thawed, the cells were pipetted into a falcon tube.
3. 5 µL of each ligation were subsequently pipetted directly into the *E. coli* TOP10 cells and tapped gently four or five times.
4. The reaction was incubated for 30 minutes on ice.
5. The cells were then heat shocked in a water bath at 42°C for exactly 30 seconds, and subsequently placed on ice for minimum 2 minutes.
6. 250 µL S.O.C medium was added to the tube and incubated in a microcentrifuge rack at 37°C for an hour.
7. 100-150 µL from each transformation were spread on BHI agar plates with appropriate antibiotics and incubated at 37°C overnight. The leftover S.O.C culture was kept in room temperature overnight and could be spread the following day.

3.9.2 Transformation in electrocompetent cells

3.9.2.1 Transformation in electrocompetent Lactobacillus plantarum WCFS1

When transforming electrocompetent *L. plantarum* cells, the solution must be completely free of salts.

Materials

Electrocompetent lactobacillus

Plasmid DNA/ electroligation reaction

GenePulser® electroporation cuvette 0.2 cm

MRRSM medium
MRS agar plates with appropriate antibiotics

Procedure

The electroporation parameters were adjusted on the Gene Pulser II from Biorad. The tension was set at 1.5 kV, capacitance to 25 mF and resistance to 400ω.

1. 40 µL competent *L. plantarum* was thawed on ice.
2. 5 µL of plasmid DNA/electroligation reaction were added to the competent cells.
3. The cell-DNA/transformation mix was immediately transferred to an ice-cold electroporation cuvette and tapped to prevent air bubbles.
4. The cuvette(s) was placed in the electroporation handle and given the tension pulse.
5. Immediately after the tension pulse, 950 µL ice-cold MRRSM was added to the cuvette and resuspended with the cells. The transformation solution was then transferred to a sterile Eppendorf tube.
6. The solution was incubated at 37°C for a minimum of two hours before spreading 100-200 µL on agar plates and incubated at 37°C for up to four days.

3.9.2.2. Transformation in electrocompetent E. coli

When transforming electrocompetent *E. coli* cells, the solution must be substantially free of salts. The transformation was conducted according to the protocol by NEB (New England Biolabs. Electroporation protocol C3020)

Materials

Electrocompetent *E. coli*
NEB® 10-beta/Stable Outgrowth Medium, prewarmed to 37°C
Pre-warmed BHI agar plates, 37°C
Electroporation cuvettes (1 mm)

Procedure

The electroporation parameters were adjusted on the Gene Pulser II from biorad. The tension was set at 2.0 kV, capacitance to 25 mF and resistance to 200ω.

1. 25 µL electrocompetent *E. coli* was thawed on ice and mixed gently by finger flicking.
2. 1 µL of plasmid DNA/ electroligation solution were added.
3. The cell/DNA transformation mix was immediately transferred to an ice-cold electroporation cuvette and tapped to prevent air bubbles.
4. The cuvette was placed in the electroporation handle and given the tension pulse.
5. Immediately after the tension pulse, 950 µL NEB® 10-beta/Stable Outgrowth Medium was added to the cuvette and resuspended with the cells. The transformation solution was then transferred to a sterile Eppendorf tube.
6. The solution was incubated and shaken vigorously at 37°C for a minimum of one hour before spreading 100-200 µL on agar plates and incubated at 37°C for up to two days.

3.10 Polymerase Chain reaction

PCR is a method used for an exponential amplification of specific DNA fragments. The PCR cycles include *heating* to separate the DNA strands, *annealing* to bind specific primers to target DNA, and *extension* to extend the DNA strands by the DNA polymerase incorporating dNTPs.

3.10.1 Q5 ® Hot Start High-Fidelity 2x Master Mix

When running Q5® Hot Start High-Fidelity 2x Master Mix PCR, the protocol created by the manufacturer was used. The components of Q5 PCR were added in a PCR tube (Table 3.2).

Table 3.2. Overview of Q5® Hot Start High-Fidelity 2x Master Mix components

Components	50 µL reaction	Final concentration
Q5® Hot Start High-Fidelity 2x Master Mix	25 µL	1X
10 µM Forward primer	2.5 µL	0.5 µM
10 µM Reverse primer	2.5 µL	0.5 µM
Template DNA	variable	<1 µg
dH_2O	To 50 µL	-

The reaction was put in a thermal cycler applying Q5 program (Table 3.3).

Table 3.3. Overview of the Q5 cycling program.

Step	Temperature °C	Time	Cycles
Initial denaturation	98	30 seconds	1
Denaturation	98	10 seconds	25-35
Annealing	50-72*	30 seconds	25-35
Elongation	72	30 seconds/500 bp	25-35
Final elongation	72	2 minutes	1

*The annealing time depends on the specific primers used; usually 3°C lower than primer with the lowest Tm without tail.

3.10.2 VWR Red Taq Polymerase Master Mix

Red Taq PCR was used for colony PCR to confirm colonies harboring a desired plasmid. To prepare colony PCR, the colony was picked with a sterile toothpick to a sterile PCR tube and microwaved for one minute to ensure cell lysis. The components for Red Taq PCR was then added (Table 3.4).

When running VWR Red Taq Polymerase Master Mix PCR, the protocol created by the manufacturer was used.

Table 3.4. Overview of Red Taq PCR components

Components	50 µL reaction	Final concentration
Red Taq DNA Polymerase 2x Master Mix	25 µL	1X
10 µM Forward primer	1 µL	0.2 µM
10 µM Reverse primer	1 µL	0.2 µM
Template	Variable	<1 ug
dH_2O	To 50µL	-

The reaction was put in a thermal cycler applying Red Taq program.

Table 3.5. Overview of the Red Taq cycling program.

Step	Temperature °C	Time	Cycles
Initial denaturation	98	2 minutes	1
Denaturation	98	10 seconds	25-35
Annealing	50-65*	30 seconds	25-35
Elongation	72	30 seconds/500 bp	25-35
Final elongation	72	5 minutes	1

*The annealing time depends on the specific primers used; usually 3°C lower than primer with the lowest Tm without tail.

Both Q5® Hot Start High-Fidelity 2x Master Mix and VWR Red Taq polymerase master mix PCR products were run on agarose gels, either to purify DNA fragment or confirm the presence of one.

3.11 Sanger sequencing

Materials

400-500 ng DNA

Primer

dH_2O to 11 µL

Procedure

Plasmid DNA was sent to Eurofins and sequenced by GATC biotech sanger sequencing to verify correct recombinant sequences. Usually, each plasmid was divided to two Eppendorf tubes: one containing 2.5 µL forward primer and one containing 2.5 µL reverse primer. Each tube was labelled with a unique barcode and sent to Eurofins for Sanger sequencing. The results from Eurofins were analyzed using CLC DNA Main Workbench 7.

3.12 Sample preparation for harvesting L. plantarum for analysis

Plasmids harboring the inducible promoter sppA requires the presence of the inducer peptide SppIP to initiate a gene expression (Sørvig et al., 2003). When different bacterial cultures harboring different promoters were grown with the purpose of analyzing the protein expression, *L. plantarum* cultures harboring both pLp1261_Ag85B-ESAT-6-DC and pEv (empty vector) were induced by SppIP when the OD_{600} was 0,1-0,15. All cultures, including cultures harboring a constitutive expression system, were harvested three or six hours after the induction of SppIP.

3.12.1 Cultivation and harvesting of bacteria

Materials

MRS medium

Inducing peptide SppIP

Antibodies

Procedure

1. *L. plantarum* strains were grown at 37°C overnight in MRS medium containing appropriate antibiotics.
2. The overnight *L. plantarum* cultures were diluted to an OD_{600} of 0.13-0.15. in 10 mL prewarmed MRS medium.

3. The cultures were incubated at 37°C until it reached an OD_{600} of 0.3 and cultures harboring the inducible plasmids (pLp1261_Ag85B-ESAT-6-DC and pEv) were induced with SppIP (25 ng/mL).
4. The *L. plantarum* strains were further incubated for 3 or 6 hours. Both the strains harboring inducible and constitutive plasmids were harvested by centrifugation at 5000x g for 10 minutes at 4°C.

3.13 Detection of antigens in L. plantarum

3.13.1 Western blot

Western blot analysis uses the protein separation from SDS-page procedure and gel described in section 3.6.2 to visualize and, to some degree, quantify specific proteins using antibodies. In this case the desired protein was the AgE6 antigen. The proteins were blotted to a nitrocellulose membrane. Next, the membrane got treated with a blocking solution, TPBS/1%BSA to prevent nonspecific reactions with the antibody and membrane. The membrane is then incubated with the primary antibody, which recognizes the epitope of the antigen and binds to it, and any unbound primary antibodies are subsequently washed away with a washing solution. The membrane is incubated a second time with a secondary antibody which only binds to the primary antibody. The secondary antibody is conjugates with horse radish peroxidase (HRP) with oxidizes luminol, thus making a detectible light and visualization of existing antigen.

3.13.1.1 Blotting with iBlot™ Dry Blot System

When blotting proteins from SDS-page gel to a nitrocellulose membrane, iBlot™ Dry Blot System was used.

Materials

iBlotTM Dry Blotting system

TPBS, pH 7.4

Procedure

After the SDS-page was run, the gel was placed in dH_2O for five minutes. A gel transfer stack was assembled (Figure 3.2) placing the SDS-page gel on top of a membrane. Any air bubbles were removed using a blotting roller. All components were assembled and blotted at 30 volts for seven minutes. After blotting, the membrane was placed in TBS awaiting next step in western blot procedure; antibody hybridization.

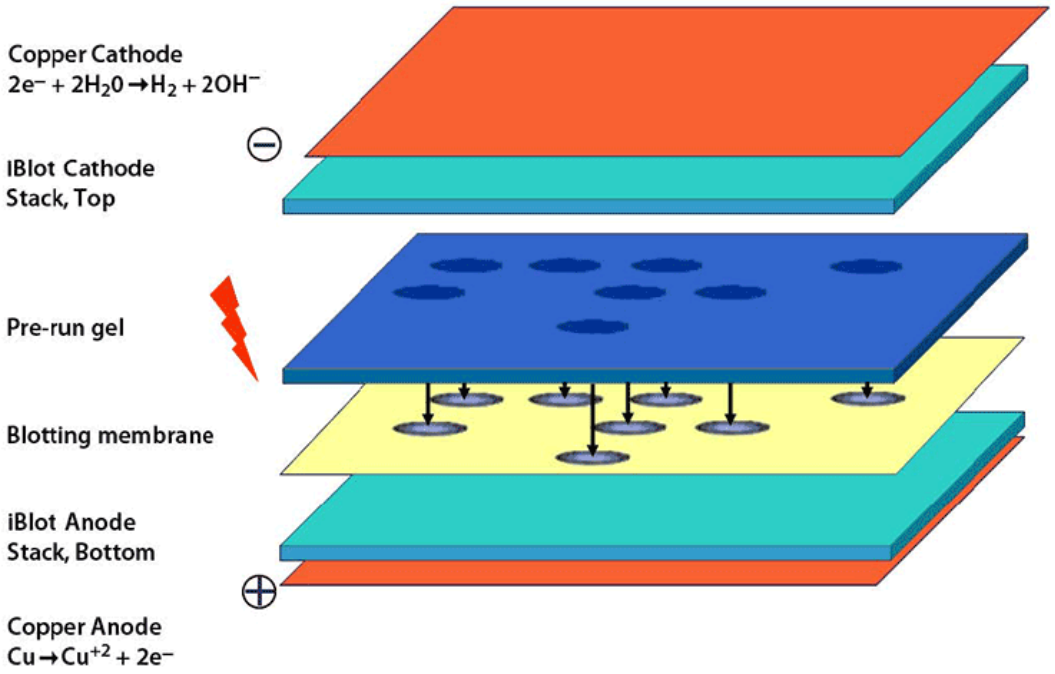


Figure 3.2. Assembly of the components of the iBlotTM Dry Blot System. The iBlot system consists of a botting stack and a top stack, anode and cathode respectively, with the pre-run gel and nitrocellulose membrane in the middle. On top of the bottom stack is the blotting membrane, and the pre-run gel on the top stack. The figure is taken from the iBlot® Dry Blotting system manual.

3.13.1.2 SNAP i.d.® immunodetection

To perform hybridization of the antigens on a membrane, the SNAP i.d.® immunodetection system was used. The system uses a vacuum to pull the added components through the membrane.

Materials

SNAP i.d.® Protein detection system

TPBS, PBS + 0.1% tween-20

TPBS/ 3% BSA, blocking solution

Primary antibody: anti-ESAT6

Secondary antibody: HRP-Goat Anti-Rabbit IgG

Procedure

1. First, a blot holder was saturated with dH_2O, the membrane was placed in the blot holder with the blotted protein side facing down. A filter was placed on top of the membrane, the blot holder closed and rolled over with a blot roller to remove any air bubbles.
2. The blot holder with the membrane and filter paper was placed in the SNAP i.d.® Protein detection system.
3. 30 mL of the TPBS/0.5%BSA blocking solution was poured over, 10 mL at the time, while the vacuum pulled the liquid through.
4. 3 mL TPBS/ 3% BSA and 3 µL primary antibody were mixed, added to the membrane, and incubated for ten minutes.
5. The membrane was then washed three times with 10 mL TPBS, having the vacuum pull the liquid through.
6. 3 mL TPBS/ 3% BSA and 0.2 µL secondary antibody were mixed, added to the membrane, and incubated for ten minutes.
7. Step 5 was repeated.
8. The membrane was removed from the blot holder awaiting antigen detection using Chemiluminescent.

3.13.2 Chemiluminescent

Materials

SuperSignal® West Pico PLUS Chemiluminescent Substrate

Luminol/Ehancer

Stable Peroxide Buffer

Procedure

A substrate working solution was prepared by mixing 5 mL Luminol/Enhancer and 5 mL Stable Peroxide Buffer. The membrane was put in this solution and covered with light cancelling foil and incubated for five minutes. Azure c400 was used to visualize and capture the membrane and any antigens bound to the antibodies.

3.14 Detection of surface antibodies

3.14.1 Flow cytometry

Materials

PBS

PBS/2%BSA

Primary antibody, Anti Ag85

Secondary antibody, Anti-Rabbit IgG-FITC

Procedure

1. The bacterial cultures were harvested with an OD_{600} of 1, then washed with 750 µL PBS and centrifuged at 5000 x g for three minutes.
2. The washed pellet was stored at 4 °C overnight before continuing preparation for flow cytometry.
3. 50 µL PBS/2%BSA was mixed with 0.2 µL primary antibody per cell pellet and 50 µL was portioned and resuspended in each pellet.
4. The resuspended cell pellets were incubated at 30 minutes at room temperature, then centrifuged at 5000 x g for one minute and the supernatant was discharged.
5. The cell pellets were washed three times with 500 µL PBS/2%BSA and centrifuged twice at 5000 x g.
6. 50 µL PBS/2%BSA was mixed with 0.3 µL secondary antibody per cell pellet and 50 µL was portioned and resuspended in each pellet.
7. The resuspended cell pellets were incubated at 30 minutes in room temperature wrapped in light cancelling foil avoiding UV exposure.
8. The cells were centrifuged at 5000 x g for one minute and the supernatant pipetted off while avoiding light exposure.

9. The cell pellets were washed four times with 600 µL PBS/2%BSA and centrifuged at 5000 x g for two minutes.
10. The cell pellets were then resuspended in one mL PBS, while still avoiding light exposure.
11. When analyzing the cells in flow cytometry, the cell-PBS solutions were further diluted by carefully mixing 100 µL cell-PBS solution with 900 µL PBS. 15 µL of the final dilutions were used to perform the flow analyses on MacsQuant® Analyser and MacsQuantify™ software was performed.

3.14.2 Confocal laser scanning microscopy

Materials

For materials, see section 3.14.1

Procedure

Recombinant *L. Plantarum* was cultivated and induced as described in *sample preparation for L. plantarum* and then followed the procedure described in section 3.14.1 Flow cytometry, excluding step 11. The cell-PBS samples were stored at 4°C avoiding light exposure. Then the cells were applied to Leica TCS SP5 Confocal laser scanning microscope using Zen software.

4.0 Results

The goal of this study was to construct three new vectors to promote expression of the fusion-protein comprised of Ag85B and ESAT-6, abbreviated to AgE6. The pSIP vector pLp_1261Ag85B-ESAT-6-DC (Kuczkowska et al., 2016) was utilized as the backbone of the plasmid and the initial step was to remove and replace the inducible promoter P_{SppA} with a constitutive promoter; SlpA8287, SlpD or Tuf34 (Table 4.1). The gene product by the various promoters were anchored to the cell surface using the lipoprotein anchor derived from *Lp_1261* gene in *L. plantarum*. To potentially increase antigen expression, plasmids with deleted genes (*sppK* and *sppR*) involved in the inducible expression system were constructed, thus making the plasmid smaller. Plasmids harboring the inducible promoter P_{SppA}, along with previously constructed plasmids harboring the constitutive promoters SlpA and PgM were used to analyze protein production (Appendix 7.1.4 and 7.1.5).

Plasmids previously constructed with the fusion antigen AgE6 have shown to elicit antigen specific immune response after nasal and oral immunization (Kuczkowska et al., 2016). In this study the AgE6 gene were translationally fused downstream of the lipoprotein anchor *Lp_1261* gene in all plasmid constructs. The present study is a part of a project where the objective is to develop a new vaccine against *M. tuberculosis*, and optimization of antigen production in a live-vector carrier is important. The pSIP plasmid used as a starting point in this study already harbors *Lp_1261*, AgE6 and the inducible promoter P_{SppA} along with regulatory genes HK (*sppK*) and RR (*sppR*) (section 1.4.1). However, because one of the main objectives of the vaccine is to produce the antigens *in situ*, and in this study *in vitro,* a constitutive promoter is preferred (Peirotén & Landete, 2020). The promoter sequence SlpA8287, SlpD and Tuf34 were ordered from GenScript and delivered in a pJET1.2 cloning vector (Table 2.8). By digesting the promoter from the pJET1.2 vector, and digesting the inducible plasmid pLp1261 (Table 2.8) using restriction enzymes and ligation, new constitutive plasmids were constructed.

Table 4.1. Overview of the origin species of the constitutive promoters used in this study.

Promoter	Origin species
SlpA	*Lactobacillus acidophilus* ATCC4356
PgM	*Lactobacillus acidophilus* NCFM
SlpA8287	*Lactobacillus brevis* ATCC 8287 (Vidgrén et al., 1992)
SlpD	*Lactobacillus brevis* ATCC14869 (Jakava-Viljanen et al., 2002)
Tuf34	*Lactobacillus buchneri* CD034 (Tauer et al., 2014)

Table 4.2 shows the full pSIP construct names and the abbreviations that will be used going further in this study.

Full construct name	Constructed	Abbreviation
pLp1261_Ag85B-ESAT-6-DC	A production of Ag85B-ESAT-6 (AgE6) with the Lp_1261 lipoprotein anchor (Kuczkowska et al., 2016)	pLp1261
pSIP_SlpA8287_1261_Ag85_ESAT-6-DC	In this study	pSIP_SlpA8287
pSIP_Tuf34_1261_Ag85_ESAT-6-DC	In this study	pSIP_Tuf34
pSIP_SlpD _1261_Ag85_ESAT-6-DC	In this study	pSIP_SlpD
pSIP_SlpA_1261_Ag85_ESAT-6-DC	Made by Kamilla Wiull	pSIP_SlpA
pSIP_PgM_1261_Ag85_ESAT-6-DC	Made by Kamilla Wiull	pSIP_PgM
pSlpA_1261_Ag85_ESAT-6-DC	In this study	pSlpA
pPgM_1261_Ag85_ESAT-6-DC	In this study	pPgM
pSlpD _1261_Ag85_ESAT-6-DC	In this study	pSlpD

4.1 Construction of constitutive vectors

The main goal of this study was to constitutively express the fusion-protein AgE6 using constitutive promoters, and several strategies were examined. The first step was to isolate and amplify the promoters from the cloning vector pJET. Because the target promoters were small in size, the concentration after digestion and fragment isolation was low, and were therefor PCR amplified using primers (Table 2.5). The amplified promoter sequences and the pLp1261 vector (Kuczkowska et al., 2016) were then digested and linearized to remove the inducible P_{sppA} using restriction enzymes. SlpA8287 and Tuf34, along with pLp1261, were digested with *BglII* and *NdeI* (Figure 4.1). SlpD along with pLp1261 was digested with restriction enzymes *BglII* and *SalI*. The target sequences were then run on agarose gel to cut out and purify the DNA fragment. The promoters were subsequently ligated into the pLp1261 vector to construct; pSIP_SlpA8287_1261_Ag85_ESAT-6-DC, pSIP_Tuf34_1261_Ag85_ESAT-6-DC and pSIP_SlpD_1261_Ag85_ESAT-6-DC (Table 4.2)

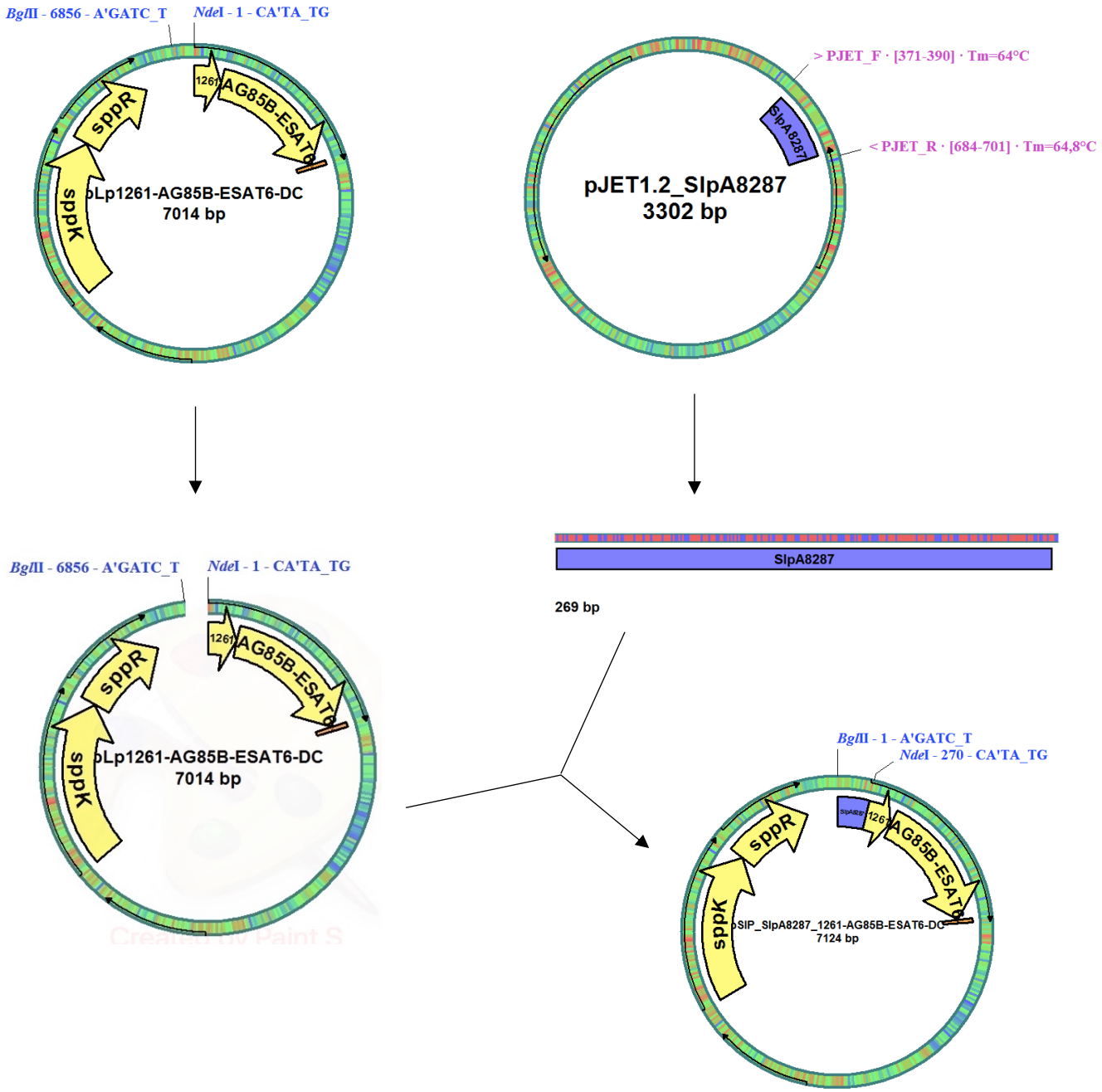


Figure 4.1. Plasmid construction strategy with constitutive promoters. The pJET vectors were amplified using primers PJET_F and PJET_R(Table 2.5) and digested using restriction enzymes (*BglII/NdeI*) (*BglII/SalI*) to linearize DNA insert to a linearized pSIP vector pLp1261. The schematic overview of the PJET plasmids show the genes SlpA8287 promoter (puple) and the gene for ampilicin resistance (black arrow). pSIP_SlpA8287_1261-Ag85B-ESAT6-DC shows the genes: promoter SlpA8287 (purple), lipo-anchor 1261 (yellow), the fusion antigen Ag85B-ESAT6 (yellow), the next gene is represented with a black arrow before *sppK* and *sppR* and represents the gene for erythromycin resistance.

The ligation mixture was transformed into competent *E. coli* TOP10 cells and screened using colony PCR. The PCR products were applied to an agarose gel to screen for correct clones. For each plasmid construct that potentially harbored a constitutive promoter (Table 4.1), and transformed into bacteria, frequently resulted numerous colonies. However, colony PCR revealed that most of the colonies harbored religated plasmids and clones with the correct promoter were rare (Figure 4.2). Figure 4.2 shows one transformation where one out of six colonies potentially harboring SlpA8287 after colony PCR, at the correct band size of 560 bp.

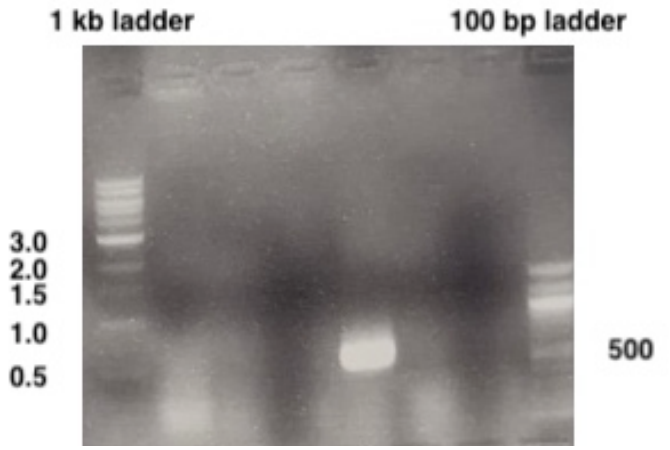


Figure 4.2. Agarose gel after colony PCR. Depiction of *E. coli* TOP10 clones harboring pSIP_SlpA8287 by applying the specific primer SlpA8287 and the unspecific primer SeqAg85_R (Table 2.6). A band, seemingly at the correct size at 560 bp, can be observed in well five.

After continuous cloning, three pSIP_SlpA8287 clones with correct band sizes were sent to sequencing. All colonies had deleted large portions of the promoter sequence (Appendix 7.1.1.1). The common denominator in all three clones was the absence of a ribosome binding site (RBS). Due to the sequence deletion and lack of RBS, the antigens would not be translated and the SlpA8287 clones were not used any further in this study. The cause of the multiple religated *E. coli* TOP10 colonies was conjectured to be incomplete digestion, and restriction enzymes from different producers (NEB and ThermoFisher) were used as well as altering the digestion period. The agarose gel percentage was also altered throughout the study due to small promoter size, with no prevail.

To construct pSIP_SlpD proved to be challenging because of the difficulty isolating the promoter due to its small size. It was also challenging performing ligation as the concentration of the SlpD promoter dropped drastically when it was digested, and dropped even further when isolated from agarose gel. This was approached by a significant increase of pJET1.2_SlpD plasmid concentration during digestion, and a significant increase of digested SlpD promoter in multiple wells when the SlpD sequence was purified on agarose gel. After multiple transformations in *E. coli* TOP10, only one colony had the correct band size after colony PCR. This plasmid was isolated and sent to sequencing. After blasting the original SlpD sequence against the SlpD sequence from the *E. coli* TOP10 colony, it showed a point mutation in the pribnow box -10 position (Table 4.3). Despite the point mutation, pSIP_SlpD was further transformed to *L. plantarum*. Due to the high number of religation of plasmids in transformed *E. coli* TOP10, it was believed to be caused by incomplete digestion, and restriction enzymes from different producers (NEB and ThermoFisher) were used, as well as altering the digestion period.

Table 4.3. Promoter consensus sequence in -10 box and the -10 box sequence in promoter SlpD. The consensus sequence from the synthetic promoter library constructed by Rud.et.al (2006) shows the conserved bases in bold, and the semi-conserved bases; R=A or G; W=A or T; D=A, G or T; N=A, G, T or C (Rud et al., 2006).

	-10 box
Consensus	**TA**WDN**T**
Original sequence (SlpD)	TAAGAT
Mutant (SlpD)	TAAGAG

A point mutation from thymine to guanin in the reserved region of the - 10 box in the promoter SlpD (Table 4.3). SlpD does not have a -35 box but two -10 boxes in its promoter sequence (Appendix 7.1.2) (Jakava-Viljanen et al., 2002).

4.1.1 Construction of the constitutive promoter Tuf34

Tuf34 (Appendix 7.1.3) was also a promising promoter due to its origin species being a *Lactobacillus* strain and the gene is found upstream of a gene coding for translation elongation factor (Peirotén & Landete, 2020; Tauer et al., 2014). The pSIP_Tuf34 plasmid was constructed using the same approach as to SlpA8287 and SlpD, ligated and transformed to *E. coli* TOP10. The result was a great number of colonies after every transformation. However, no colony gave a correct band size after colony PCR. An important note is that some very weak bands were observed after colony PCR (Figure 4.3). These plasmids were sent to sequencing and control digested by *ClaI*, which have one unique site in the Tuf34 promoter. All colonies proved to be religated.

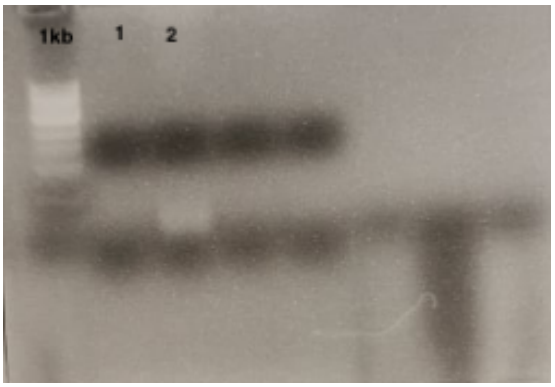


Figure 4.3. Agarose gel after colony PCR of *E. coli* TOP10 colonies potentially harboring pSIP_Tuf34. A weak band around 400-500 is observed in well 2, expected band size for primers used (SekF and SeqAg85_R were 622 bp).

Due to all colonies being religated, other methods of ligation and transformation were executed. The initial step in the process was to interchange restriction enzymes from different producers (NEB and ThermoFisher) as well as the digestion period as incomplete digestion seemed to also be a challenge here due to the multiple religated *E. coli* TOP10 colonies. Simultaneously, new methods were applied to construct pSIP_Tuf43. Table 4.4 show all methods applied to attempt construction of the constitutive pSIP_Tuf34 plasmid. The general thought is that incomplete digestion was an issue, which was tried to be overcome using different restriction enzymes and digestion time with no prevail.

Table 4.4. An overview of all methods applied to construct the constitutive pSIP_Tuf34 plasmid.

Methods of pSIP-Tuf34 construction

Approach	Results
pSIP_Tuf34 palsmid construction using In-Fusion cloning (section 3.5.3). The Tuf34 gene was amplified using designed primers (Table 2.6) and inserted into a digested *(BglII/NdeI*) linearized pLp1261 vector. This method was applied multiple times using different molar ratios.	When the Tuf34 amplicon was run on agarose gel two inseparable bands was observed, only one band was expected. This yielded no clones containing the Tuf34 promoter.
Gradient PCR to separate the two bands from agarose gel (from 48 ºC - 58ºC).	The temperature gradient did not alter the result, but the agarose bands seemed stronger at higher temperatures.
The electroligase protocol was used (section 3.5.2). The Tuf34 promoter was amplified using PCR, and the DNA fragment was subsequently digested with restriction enzymes (*BglII/NdeI)*. The fragment was then ligated into the vector pLp1261 using electroligase and transformed into electrocompetent *E. coli* and *L. plantarum.*	Electroligase gave many transformants. However, after colony PCR, no correct band size was observed, and all of the colonies were religated.
In-Fusion ligation mixture was tried transformed into electrocompetent *E. coli*.	No colonies.

The pSIP_SlpA8287 plasmid was not used onwards in this study due to the severe deletions of the promoter (Appendix 7.1.1.1). Despite applying multiple methods to construct pSIP_Tuf34 no correct clones were detected and construction of pSIP_Tuf34 was attempted multiple times throughout the entirety of this study. Moving forward, only the constitutive plasmid pSIP_SlpD (Appendix 7.2.2) was used in the present study, alongside already constructed constitutive plasmids pSIP_SlpA and pSIP_PgM.

4.2 Deletion of sppK and sppR genes

As this study's main goal was to construct plasmids using constitutive promoters and compare the protein production to the inducible system, unnecessary genes related to the inducible system from the pSIP vectors were removed. The genes *sppK* and *sppR* were deleted in the pSIP_SlpA, pSIP_PgM and pSIP_SlpD plasmids using restriction enzymes, and the plasmids were ligated after removal (figure 4.4). First, the restriction enzymes *ApaI* and *BglII* were used to digest the plasmid to remove *sppK* and *sppR*. However, *ApaI* did not digest properly and was replaced with *BsaI* and the plasmids were digested. Since *BsaI* and *BglII* does not make compatible sticky ends, the ends had to be blunted using Mung Bean nuclease (section 3.4.1) This resulted in unusually low plasmid concentrations and Mung bean nuclease was replaced with T4 polymerase (section 3.4.2) which removes 3´overhangs and fills in 5´overhangs blunting the ends. This yielded correct colonies in *E. coli* TOP10. The plasmids pSlpA, pPgM and pSlpD (Appendix 7.2) (Figure 4.4) was successfully constructed and transformed into *L. plantarum*. Plasmids with deleted *sppK* and *sppR* had the "SIP" removed from its name, thus referred to as for example pSlpA (table 4.2).

A) pSIP_SlpA B) pSlpA

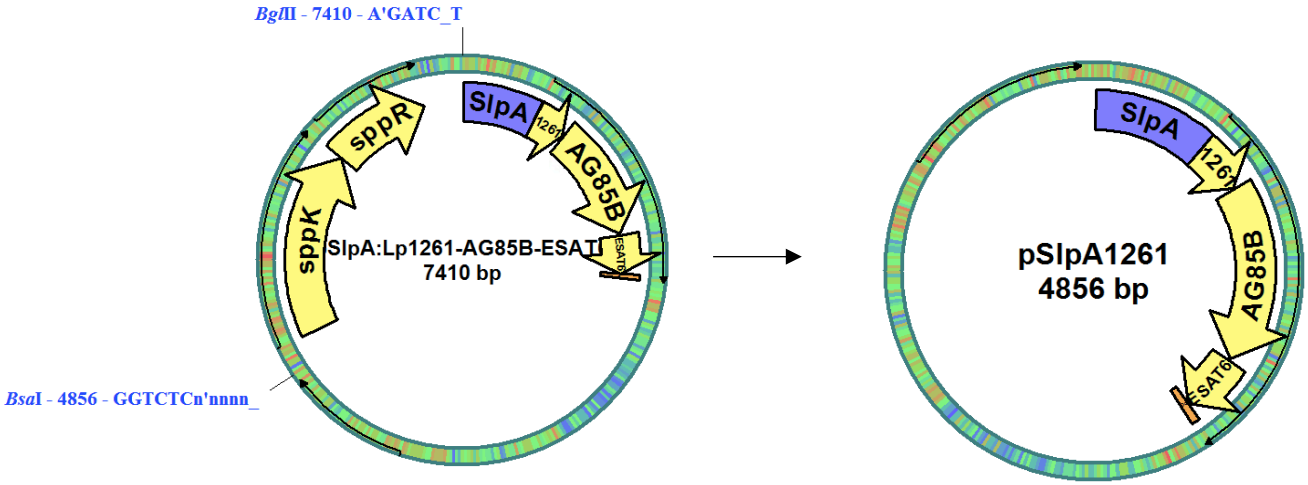


Figure 4.4. Removing the *sppK* and *SppR* genes from plasmids harboring constitutive vector.
A) pSIP_SlpA was digested with the restriction enzymes *BsaI* and *BglII* to remove *SppK* and *SppR*. B) After T4 blunting and ligation of the plasmid pSlpA. *SppK* and *SppR* were removed, but the promoter SlpA gene (purple), anchor 1261 (yellow), fusion protein AgE6 (yellow) and erythromycin resistance gene (black arrow right before SlpA) were intact.

4.3 Growth curve analysis of L. plantarum harboring different plasmids

Production of heterologous proteins may significantly hamper bacterial growth, especially when it is constitutively expressed as it can hinder resources for vital metabolic activities. Moreover, the heterologous expression can exhibit a toxic effect on the bacterial cells and cause a reduction in growth rates (Bienick et al., 2014). To analyze this, overnight cultures of *L. plantarum* harboring plasmids with different promoters were diluted to an OD_{600} of 0.15 in pre-warmed MRS. The bacterial dilutions were then grown until they reached an approximate OD_{600}=0.3. 200 µL of bacteria were transferred to a 96-well microtiter plate. Bacteria harboring the inducible promoter were induced with 25 ng/mL SppIP before being transferred to individual wells. OD_{595} was measured every 5 minutes for 15 hours by a MultiSkan FC microplate reader. As a reference, *L. plantarum* carrying pEV (empty vector; lacks P_{sppA} expressed genes) was also applied to the microtiter plate. Standard deviations, SD, were calculated by measuring growth in three independent preparations from the same *L. plantarum* strains. SD are included for every strain excluding MRS blank.

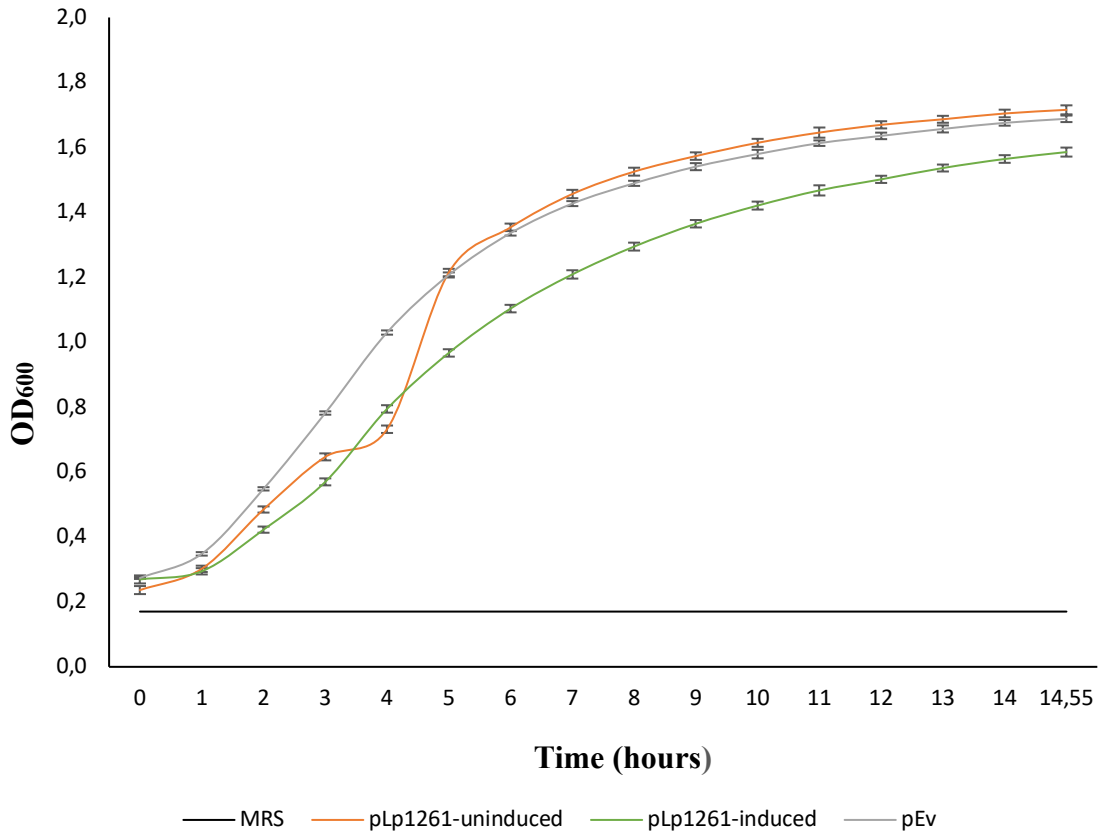


Figure 4.5. A schematic overview of growth in *Lactobacillus plantarum*, measured in OD_{600}, between cultures harboring the inducible pLp1261 plasmid over 15 hours, one culture was induced with SppIP and one was not (induced, uninduced). Cultures harboring pEv and MRS blank are used as reference. Standard deviations are included for each graph excluding MRS blank.

Figure 4.5 shows that induced cultures of *L. plantarum* harboring pLp1261 seems to slightly hamper bacterial growth compared to uninduced pLp1261 and pEv (Figure 4.5).

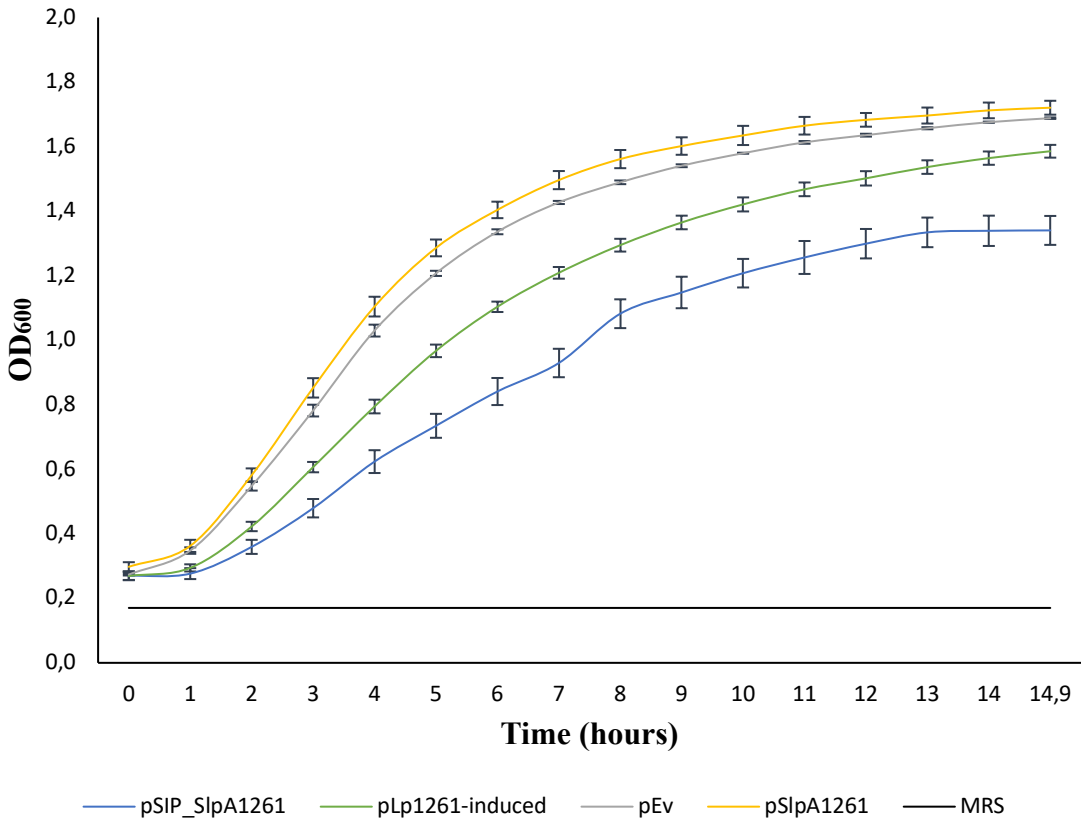


Figure 4.6. A schematic overview of growth in *Lactobacillus plantarum* cultures, measured in OD_{600}, between cultures harboring pSIP_SlpA (dark blue line) or pSlpA (yellow line) over 15 hours. Cultures harboring pEv, pLp1261 induced and MRS blank are used as reference. Standard deviations are included for each graph excluding MRS blank.

L. plantarum cultures harboring pSlpA seems to have no hampered growth compared to pSIP_SlpA, pLp1261 induced and pEv, indicating low to no production of heterologous proteins (Figure 4.6)

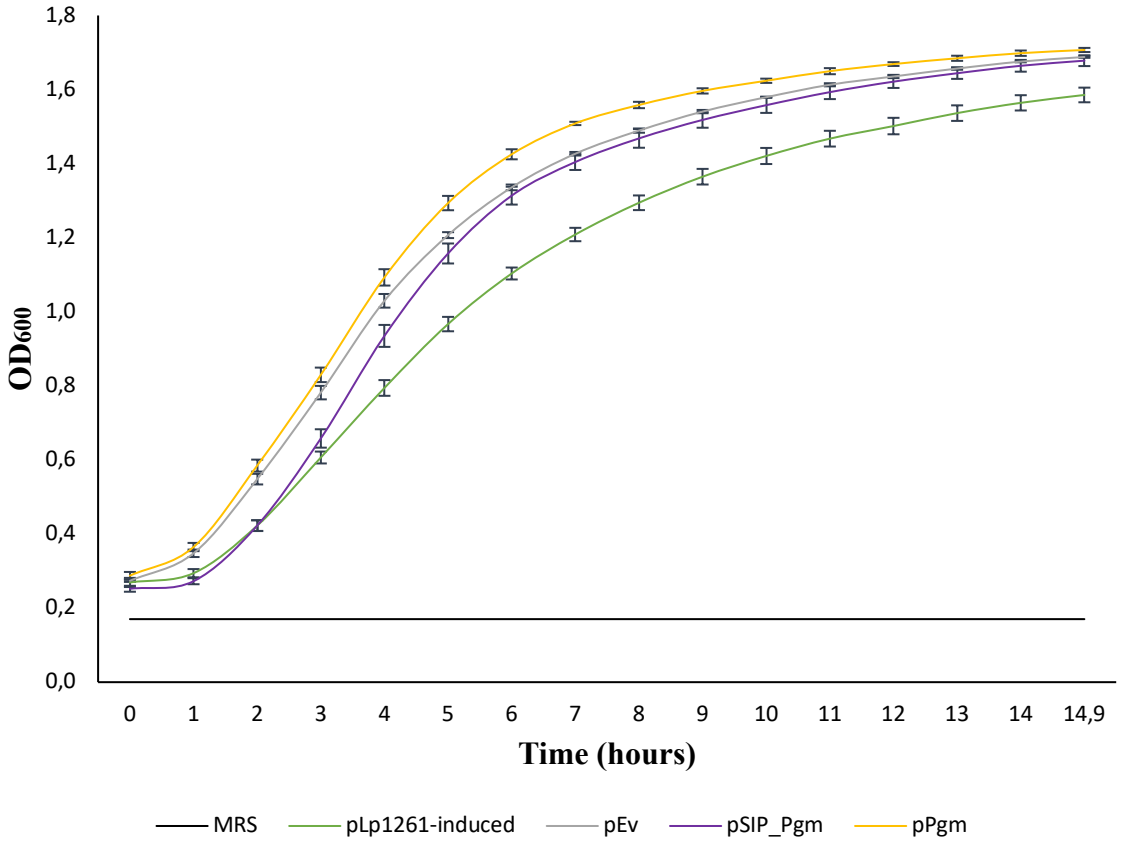


Figure 4.7. A schematic overview of growth in different *Lactobacillus plantarum* cultures, measured in OD_{600}, between cultures harboring pSIP_pgM (purple line) and pPgM (yellow line) over 15 hours. Cultures harboring pEv, pLp1261 induced and MRS blank are used as reference. Standard deviations are included for each graph excluding MRS blank.

L. plantarum cultures harboring pPgM seems to have no hampered growth compared to pSIP_PgM, pEv and pLp1261 induced, indicating low to no production of heterologous proteins (Figure 4.7).

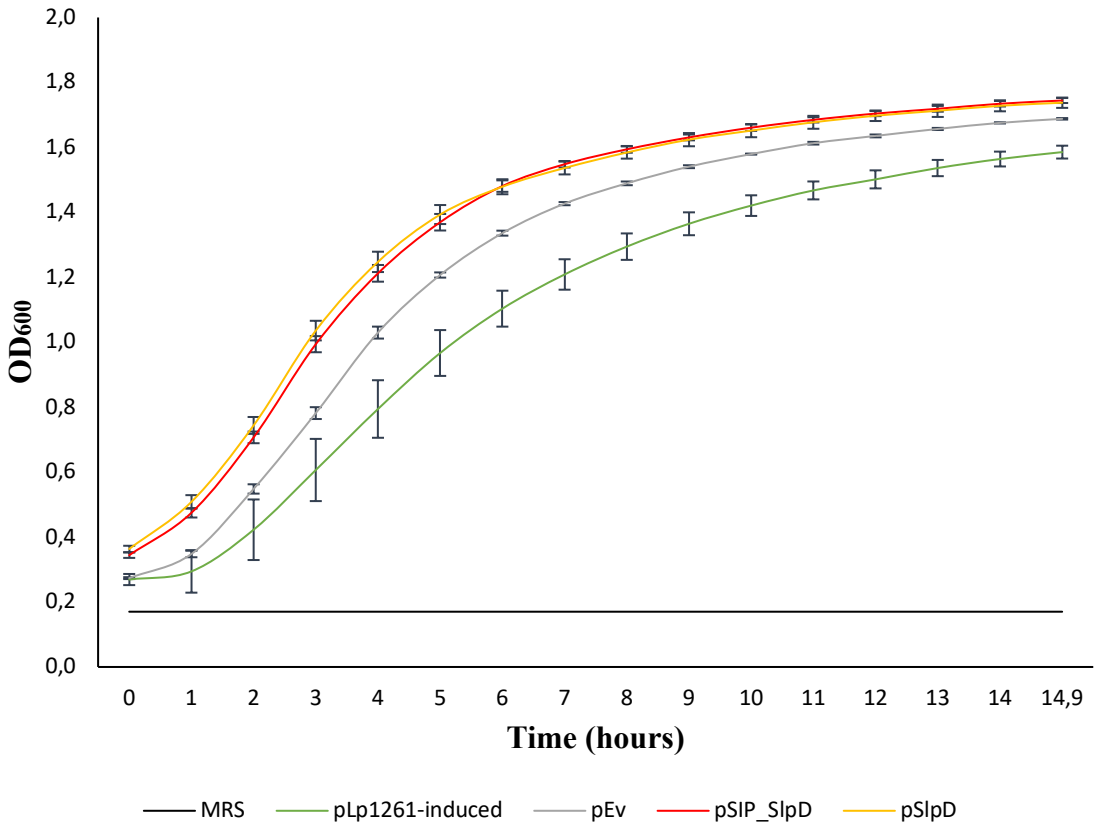


Figure 4.8. A schematic overview of growth in different *Lactobacillus plantarum* cultures, measured in OD_{600},, between cultures harboring pSIP_SlpD (red line) and pSlpD (yellow line) over 15 hours. Cultures harboring pEv, pLp1261 induced and MRS blank are used as reference. Standard deviations are included for each graph excluding MRS blank.

L. plantarum cultures harboring pSIP_SlpD and pSlpD both seem to exceed cultures harboring pEv and pLp1261-induced in growth, indicating low to none heterologous protein production (Figure 4.8).

The growth curves (Figure 4.6 to 4.8) indicates that *L. plantarum* harboring plasmids constructed without the *sppK* and *sppR* genes (pSlpA, pPgM and pSlpD) surpass pEv in growth indicating no heterologous protein production.

4.4 Western blot analysis of antigen production

L. plantarum cultures harboring different promoters were analyzed by western blot in order to investigate antigen production of AgE6 using antibody specific detection (section 3.13). *L. plantarum* harboring pEv was included as a negative control and the inducible pLp1261 plasmid were used as a positive control. The bacterial cultures were cultivated and harvested as described in section 3.12.1 and prepared as described in section 3.13. The volume of harvested cells depended on the measured OD_{600}, making sure an equal number of cells were harvested from each culture.

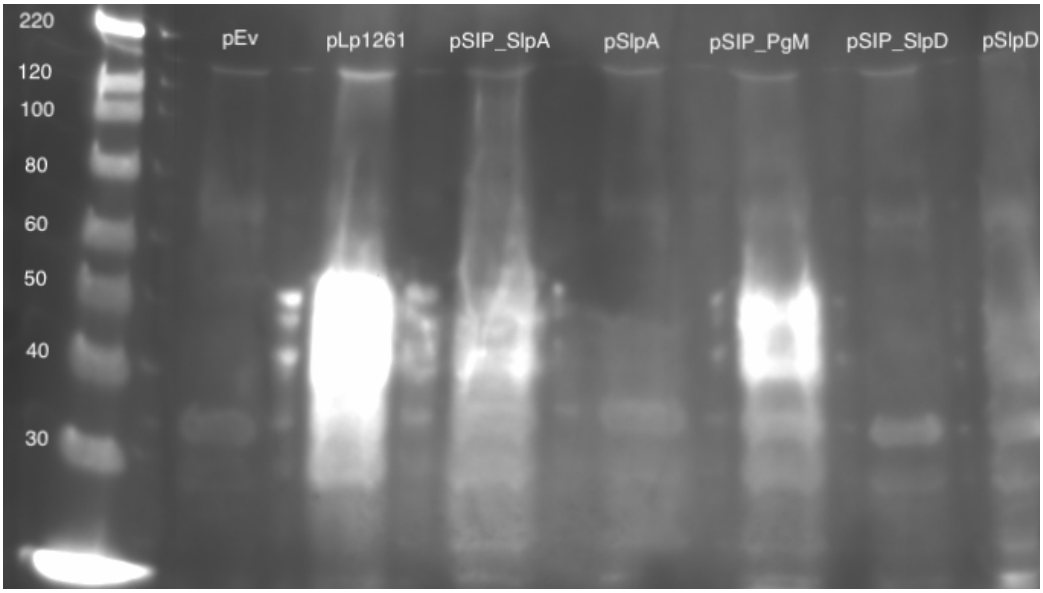


Figure 4.9. Western blot from *L. plantarum* cultures harboring different promoters. *L. plantarum* cultures harboring promoters pEv, pLp1261, pSIP_SlpA, pSlpA, pSIP_PgM, pSIP_SlpD and pSlpD. The cultures were harvested three hours after the induction of the inducible plasmids (pEv and pLp1261). The western blot analysis displays the expression of AgE6 antigens collected from cell-free protein extracts from *L. plantarum* and shows the expected band size for the fusion-protein antigen AgE6 at 48,4 kDa (Kuczkowska et al., 2019). *pPgM is not present

Figure 4.9 shows cell-free protein extracts from recombinant *L. plantarum* cultures harboring plasmids with different promoters. The cell-free protein extracts of bacteria with the promoter pEv, pSlpA and SlpD showed no antigen signal, while pLp1261 have produced seemingly the most antigen, followed by pSIP_PgM. Figure 4.9 shows slight antigen detection in pSlpD; however, this amount cannot compare to the antigen production in cells harboring the pLp1261 plasmid, pSIP_PgM or pSIP_SlpA. Several bands can be seen and is likely due to unspecific binding of the secondary antibody.

4.4.1 Growth curve analysis of antigen-producing L. plantarum

The western blot analysis (figure 4.9) illustrates which plasmids in *L. plantarum* cultures that produce the antigen AgE6. Using the positive results from the western blot, a new growth analysis was assembled to visualize how the cultures harboring the antigen-producing plasmids grew compared to each other. pSlpD Showed some AgE6 production, but as the antigen production appeared to be trifling; cultures harboring pSlpD was not included in the new growth analysis.

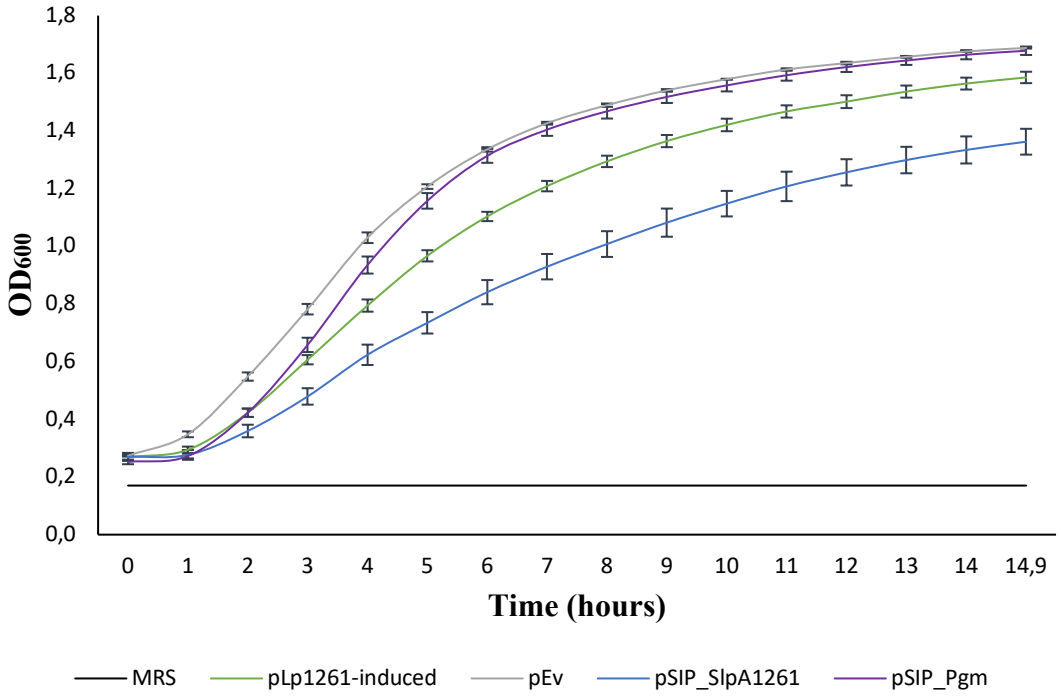


Figure 4.10. A schematic overview of growth in different *Lactobacillus plantarum* cultures, measured in OD_{600}, between cultures harboring pLp1261 induced (green line), pSIP_SlpA (blue line) and pSIP_PgM (purple line) over 15 hours. Cultures with pEV (grey line) and MRS blank (black line) are used as reference. Standard deviations are included for each graph excluding MRS blank.

Figure 4.10 shows that pSIP_SlpA seems to grow much slower and at a slower rate than the rest, and *L. plantarum* cultures harboring pSIP_PgM seems to compare in growth rate with cultures harboring pEv.

4.5 Detection of surface-level antigens of L. plantarum using flow cytometry

Flow cytometry of *L. plantarum* cultures was executed to inspect whether the antigen AgE6 was located on the surface of the bacterial wall and the degree of fluorescence indicates level of expression. The data from flow cytometry is, in this study, represented in a histogram (Figure 4.11 to 4.13). The number of sampled bacterial cells are represented by the y-axis and the relative fluorescence emitted from the bacteria is represented by the x-axis.

Heterologous *L. plantarum* cultures harboring the inducible plasmid pLp1261 and pEv, and the constitutive plasmids harboring the promoters pSIP_PgM, pSIP_SlpA or pSIP_SlpD/ p_PgM, p_SlpA and p_SlpD ,with and without the *sppK* and *sppR* genes respectively, were cultivated and harvested, hybridized and analyzed according to section 3.14.1. Both bacterial strains harboring inducible and constitutive promoters were harvested at two different points; two and six hours after induction.

4.5.1 Flow cytometry three hours after induction of the inducible plasmids

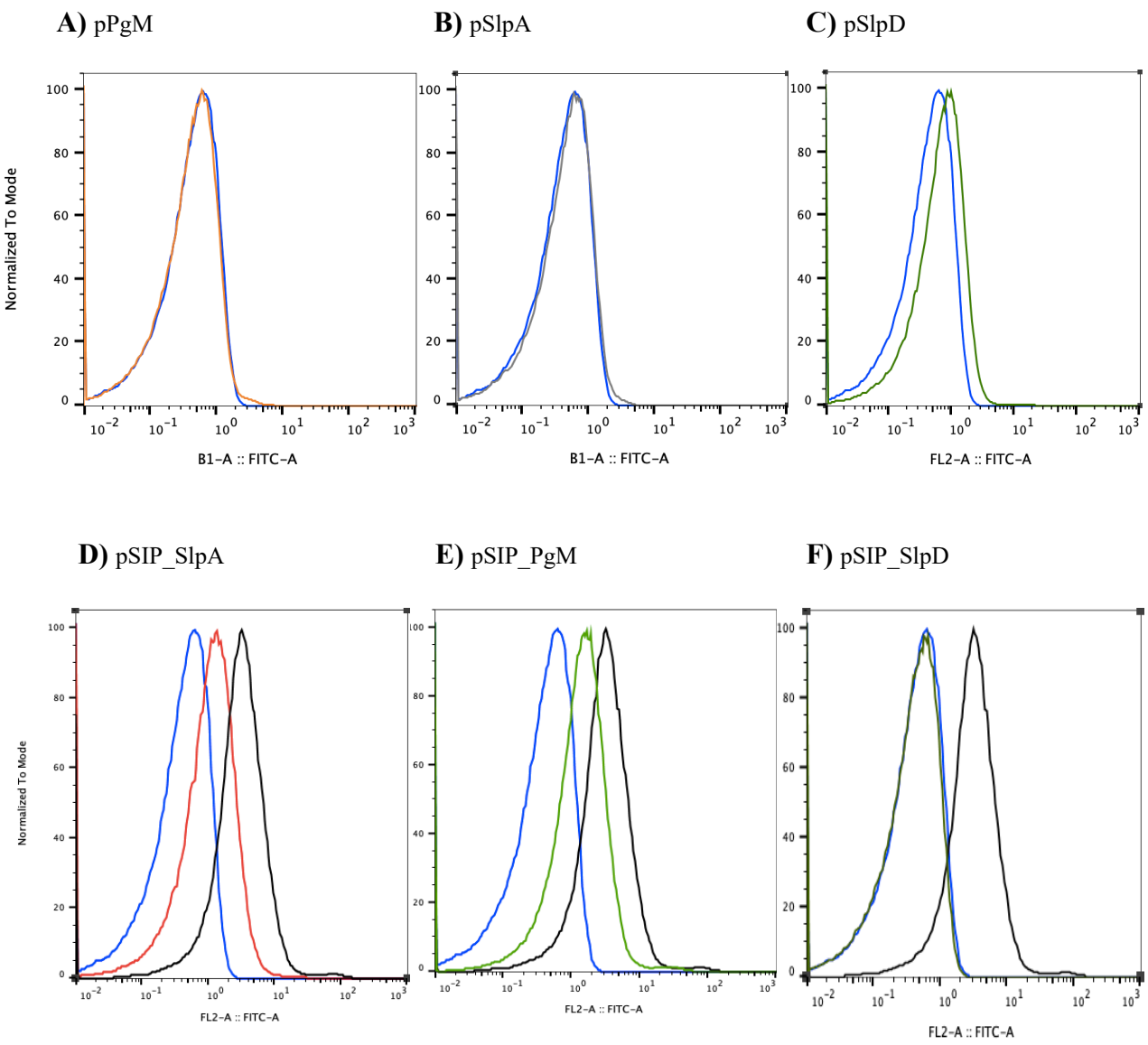


Figure 4.11. Flow cytometry analysis of FITC stained recombinant *L. plantarum* harvested three hours after induction. Histogram A-C shows *L. plantarum* harboring promoters without *sppK* and *sppR* including pEv (blue) as negative control. A) *L. plantarum* harboring pPgM (orange), B) *L. plantarum* harboring pSlpA (grey), C) *L. plantarum* harboring pSlpD (green). Histogram D-F shows *L. plantarum* harboring promoters with *sppK and sppR*, included both pEv as a negative control and pLp1261 as positive control. The recombinant *L. plantarum* cultures harboring different promoters were harvested three hours after induction. Bacteria harboring pEv serves as a negative control with no fluorescent signal, bacteria harboring the positive control pLp1261 is represented with a black line. The relative fluorescence can be seen in the x-axis plotted against the number of events represented by the y-axis. The y-axis was normalized and smoothed during analysis using the MacsQuantifyTM Software.

No fluorescent signal was detected from pEv and can be used as a true negative control.

Figure 4.11 A) and B) shows no fluorescent signal was detected from pPgM or pSlpA.

Interestingly, a small shift on the x-axis to the right can be overserved in pSlpD in 4.11 C), indicating that bacteria harboring SlpD without *sppK* and *sppR* are somewhat producing and representing the antigen on its surface. The histogram in figure 4.11 D) shows *L. plantarum* harboring the promoter pSIP_SlpA with *sppK* and *sppR* and has a clear shift to the right from the negative control. However, the pSIP_SlpA culture still appear to represent less surface antigens than the positive control pLp1261. Histogram E) (figure 4.11) shows *L. plantarum* harboring the promoter pSIP_PgM and has a clear shift to the right from the negative control however cultures harboring pSIP_PgM appear to represent less surface antigens than the positive control pLp1261 as well. Histogram F) (figure 4.11) shows *L. plantarum* harboring the promoter pSIP_SlpD and appear to have no shift on the x-axis and can be compared to pEv.

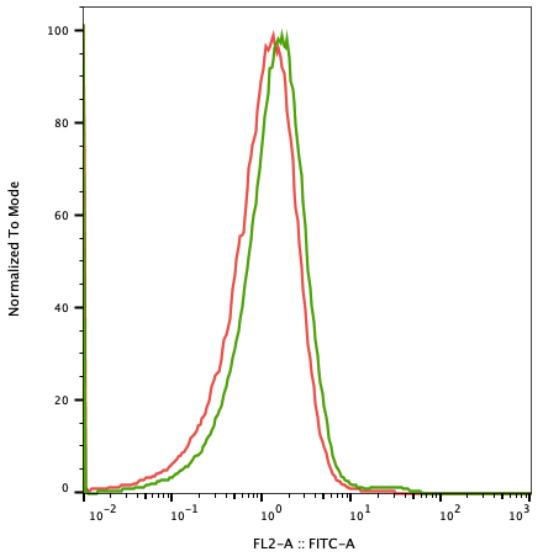


Figure 4.12. Flow cytometry analysis of FITC stained recombinant *L. plantarum* harvested three hours after induction, comparing cultures harboring the promoter pSIP_SlpA (red) and pSIP_PgM (green). A marginal shift can to the right can be observed for cultures harboring pSIP_PgM compared to cultures harboring pSIP_SlpA. The relative fluorescence can be seen in the x-axis plotted against the number of events represented by the y-axis. The y-axis was normalized and smoothed during analysis using the MacsQuantifyTM Software.

As observed in figure 4.11 D) and E), cultures harboring the promoter pSIP_SlpA and pSIP_PgM seems to be the strongest constitutive promoters in this study. When pSIP_SlpA and pSIP_PgM are compared, it appears that *L. plantarum* cultures harboring the pSIP_PgM promoter represents marginally more antigens on the cell surface compared to cultures harboring pSIP_SlpA when analyzed three hours after induction (figure 4.12).

4.5.2 Flow cytometry six hours after induction of the inducible plasmids

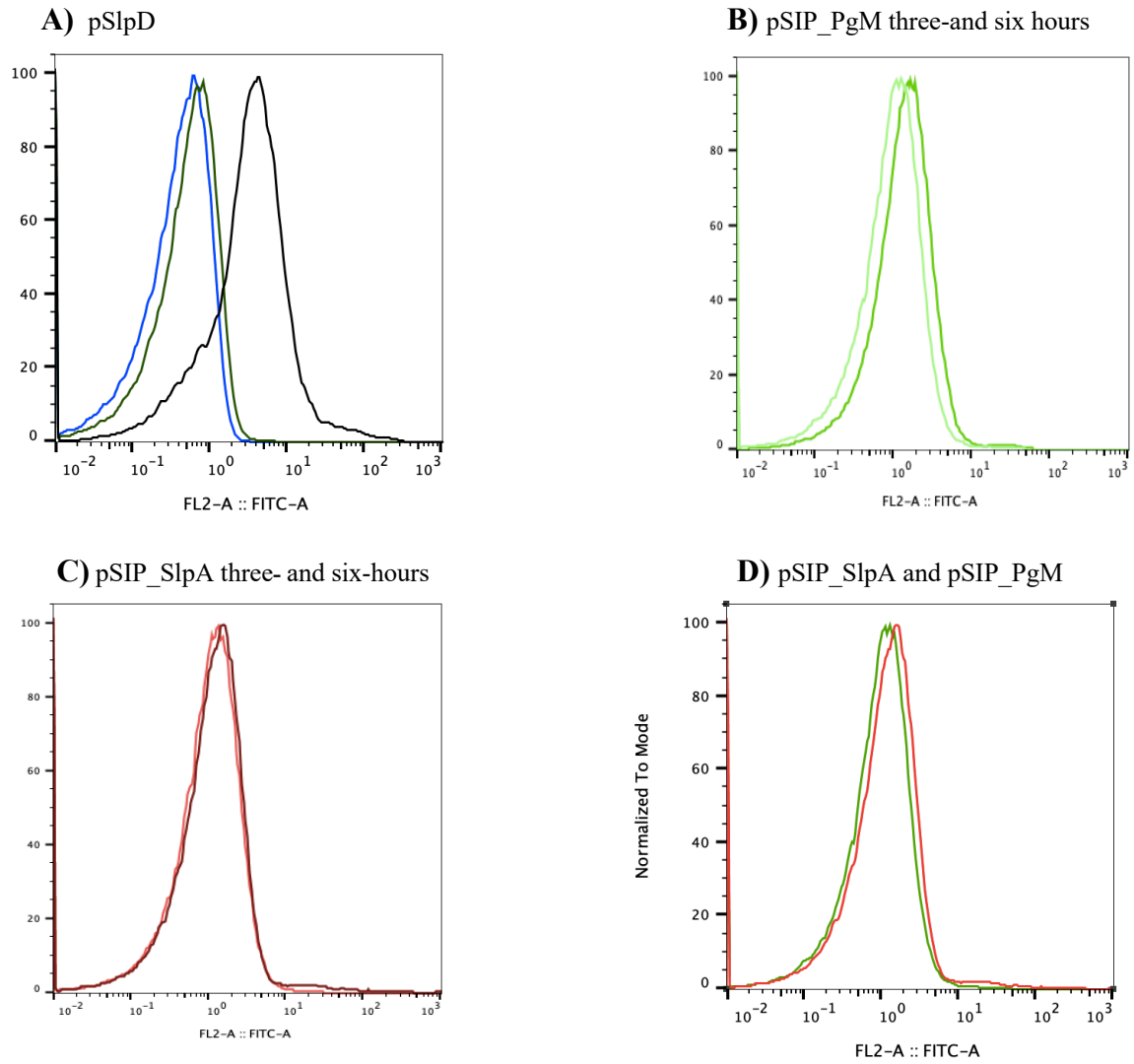


Figure 4.13. Flow cytometry analysis of FITC stained recombinant *L. plantarum* harboring different promoters. Recombinant *L. plantarum* harboring plasmids A) pSlpD (dark green line) six hours after induction, B) pSIP_PgM three and six hours after induction plotted against each other (light green and dark green lines respectively) C) pSIP_SlpA three and six hours after induction plotted against each other (light red and dark red lines respectively) and D) pSIP_SlpA and pSIP_PgM plotted against each other six hours after induction (red and green lines respectivley). Bacteria harboring pEv is represented with a blue line, has no fluorescent signal and functions as a negative control. Bacteria harboring pLp1261 is represented with a black line and functions as a positive control. The relative fluorescence can be seen in the x-axis plotted against the number of events represented by the y-axis. The y-axis was normalized and smoothed during analysis using the MacsQuantifyTM Software.

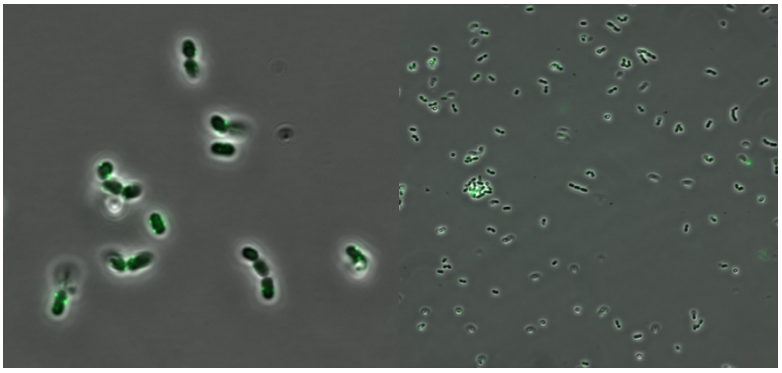
Figure 4.13 shows that the fluorescent signal does not change significantly with the increased incubation time, indicating approximately the same antigen production on the surface of bacteria harvested three and six hours after inducing the inducible plasmids.

Another approach was also tested where the aim was to harvest the different bacterial strains once every hour over eight hours and then after 24 hours following inducing the inducible plasmids. However, due to very many harvested cultures it was believed that the antibodies were overexposed to light during hybridization, and no positive results were obtained during flow cytometry analysis.

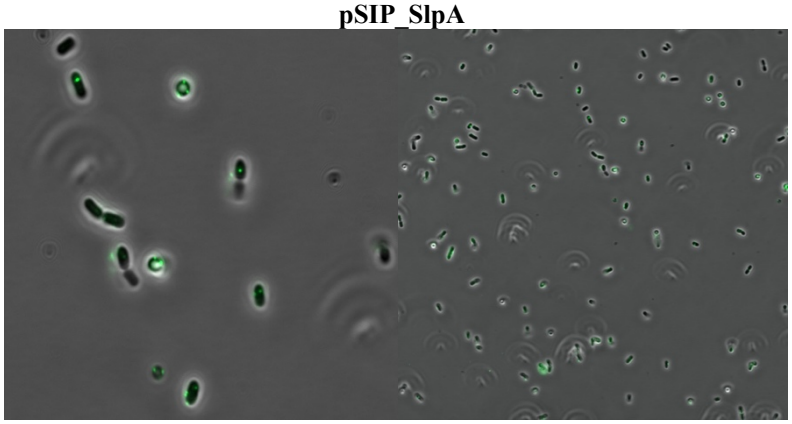
4.6 Detection of Antigen on the Surface of L. plantarum with Immunofluorescent Microscopy

Immunofluorescent microscopy was utilized to confirm the presence of antigens located on the bacterial surface. The cells analyzed by immunofluorescent microscopy was stained as described in section 3.14.1 Flow cytometry.

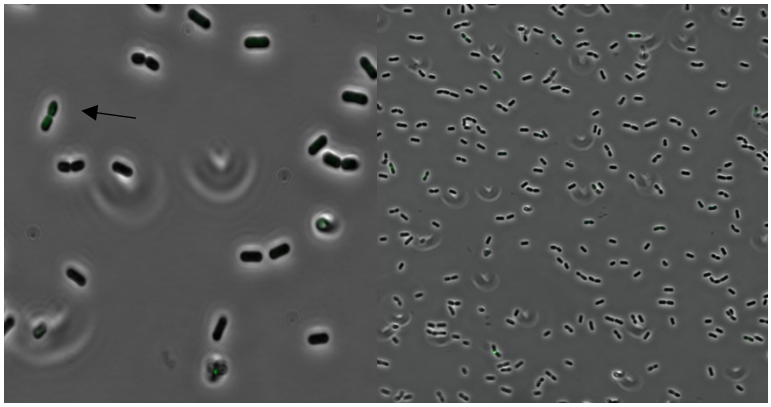
pLp1261



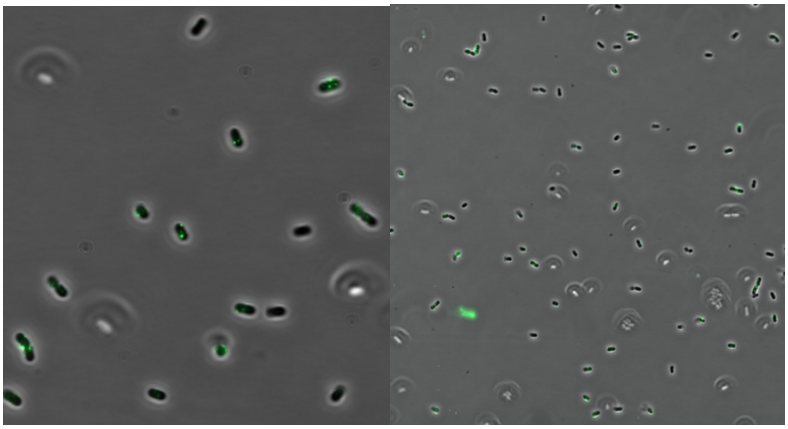
pSIP_SlpA



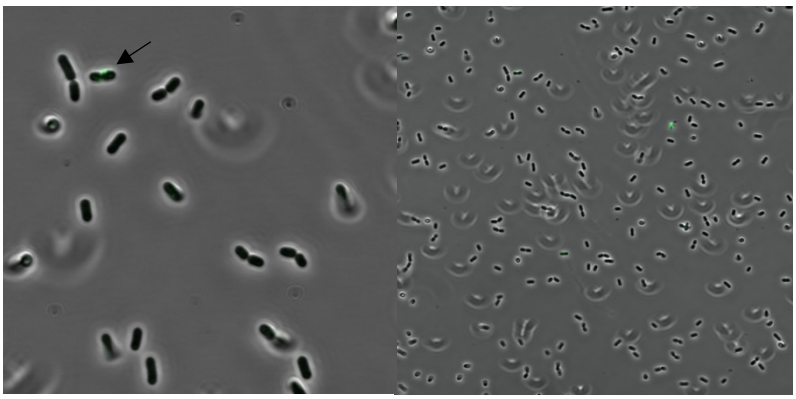
pSlpA



pSIP_PgM



pPgM



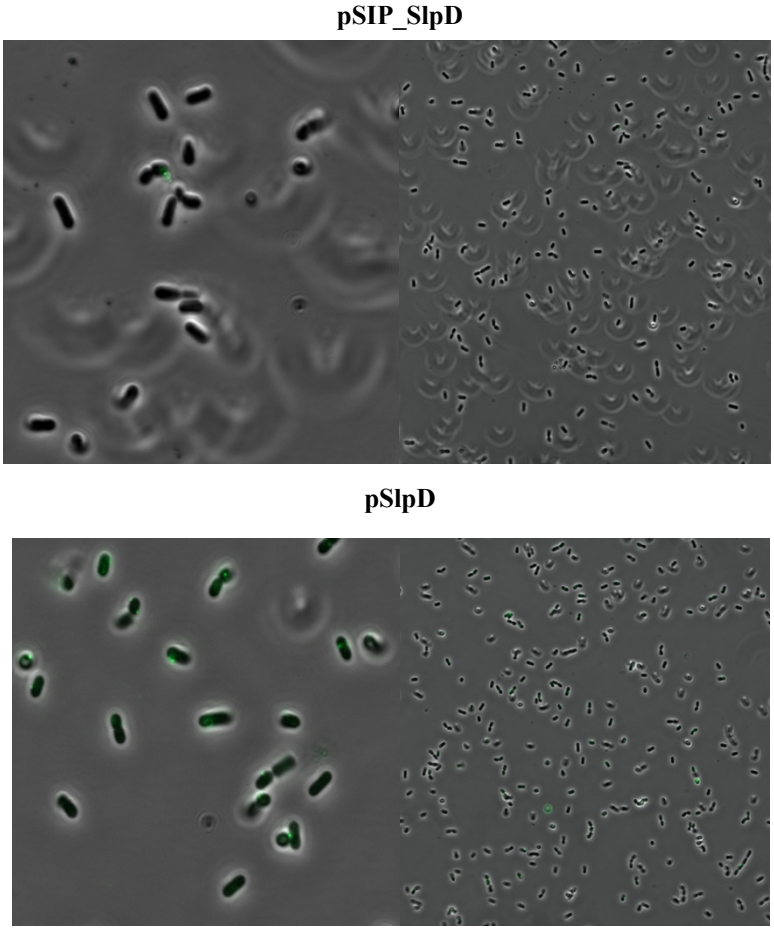


Figure 4.14. Immunofluorescent microscopy of FITC stained recombinant *L. plantarum*, cultures harboring pSIP_SlpA and pSIP_PgM have produced AgE6 (indicated by green fluorescent light). pSlpA and pPgM shows an insignificant amount of fluorescent cells. *L. plantarum* harboring pSIP_SlpD also show no green fluorescent cells, however cultures harboring pSlpD show a slight number of green fluorescent cells. Arrows indicates fluorescent antigens on the cell surface where fluorescence is scarce.

As expected, Figure 4.14 shows that only *L. plantarum* cultures harboring pSIP_SlpA, pSIP_PgM and pSlpD emit green fluorescent signals indicating the presence of AgE6 as shown by flow cytometry (Figure 4.11).

4.7 Analyzing promoter activity utilizing mCherry

The fluorescent mCherry protein was constructed downstream of the promoter using In-Fusion cloning in order to measure and compare the activity of the promoter over 15 hours. The mCherry gene was amplified and the vector was digested with restriction enzymes (*NdeI/HindIII)*. The pSIP_mCherry plasmid was constructed using In-Fusion cloning protocol and transformed into chemically competent *E. coli* and subsequently *L. plantarum.* The lipo-anchor Lp_1261 and fusion-protein AgE6 were replaced with the mCherry gene. Constructing constitutive plasmids harboring mCherry proved to be a challenge as the inducible promoter showed constitutive qualities in *E. coli* (Figure 4.16 A), and new approaches were applied.

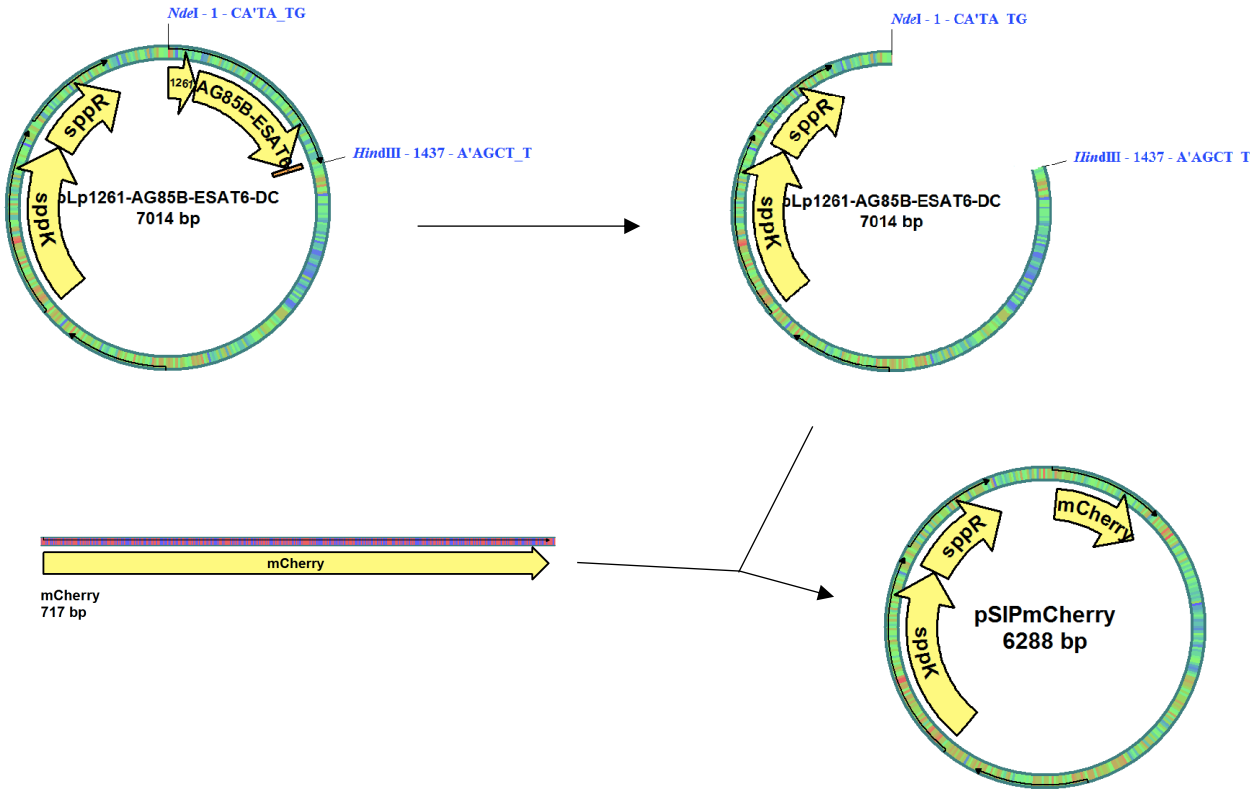


Figure 4.15. Plasmid construct of pSIP_mCherry. The vector pLp1261 were digested and linearized using the restriction enzymes *NdeI* and *HindIII* and ligated with the mCherry gene (yellow). The anchor and antigens are removed, while the gene for erythromycin resistance (black arrow) *sppK* and *sppR* (yellow) remain.

4.7.1 Construction of mCherry plasmids

As visualized in Figure 4.15; mCherry was first inserted into a digested linearized inducible pSIP vector (Kuczkowska et al., 2016) using In-Fusion cloning and transformed into chemically competent *E. coli* TOP10. This transformation yielded cherry-red colonies (Figure 4.16. A). The attempts to construct a constitutive expression of mCherry are shown in Table 4.5.

Table 4.5. An overview over different approaches to construct pSIP_SlpA_mCherry plasmid.

pSIP_SlpA_mCherry construction

Approach	Result
In-fusion amplified mCherry was inserted into a digested (*NdeI/HindIII)* pSIP_SlpA plasmid using In-Fusion protocol. The plasmid was transformed into *E. coli* TOP10 and *L. plantarum.*	No colonies.
In-fusion amplified mCherry was digested (*NdeI/HindIII*) to remove the In-Fusion overlaps and ligated into a linearized pSIP_SlpA vector using quick ligase and transformed into *E. coli* TOP10.	No correct clones after colony PCR.
pSIP_mCherry was digested with restriction enzymes (*NdeI/HindIII*) and the mCherry gene was purified from agarose gel. The linearized mCherry gene was then ligated into a digested (*NdeI/HindIII*) and linearized pSIP_SlpA vector using electroligase and transformed directly in *L. plantarum.*	Cherry-red colonies (Figure 4.16 C).

The method which yielded cherry-red colonies with the SlpA promoter (Table 4.5) was then tried to construct pSIP_PgM_mCherry. Because of limited time this was only tried twice and yielded no correct clones as cloning ligation mixtures directly into *L. plantarum* is particularly challenging.

A) pLp_mCherry in *E. coli* **B)** pLp_mCherry in *L. plantarum* **C)** pSIP_SlpA_mCherry in *L. plantarum*

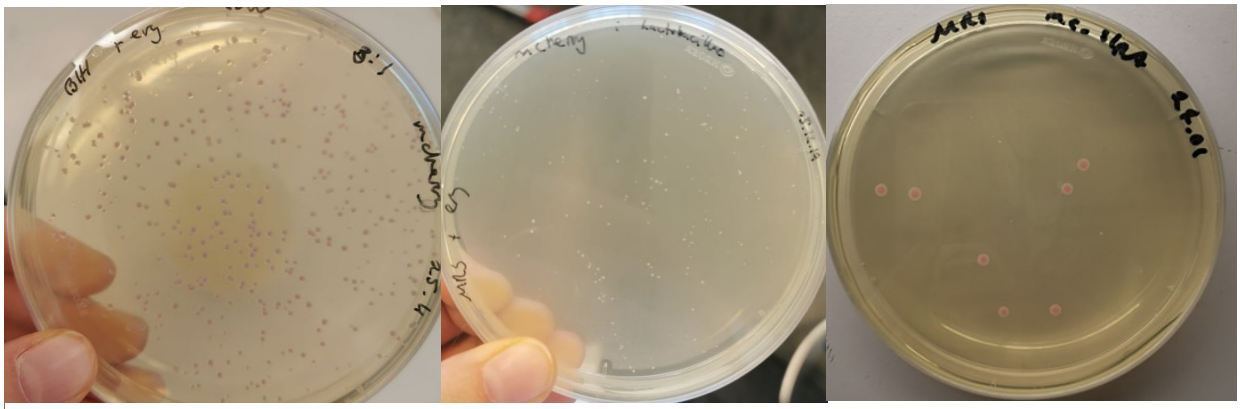


Figure 4.16. Recombinant bacteria harboring mCherry. A) shows *E. coli* harboring mCherry expressed by the inducible promoter $P_{sppA,}$ the colonies are cherry-red without being induced. B) shows *L. plantarum* harboring mCherry expressed by the inducible promoter P_{sppA}, C) shows *L. plantarum* harboring mCherry expressed by the constitutive promoter SlpA, expressing cherry-red colonies.

Figure 4.16 A) *E. coli* TOP10 harboring mCherry downstream of the inducible promoter showed cherry-red colonies when the colonies were not induced. B) shows *L. plantarum* harboring the same plasmid as *E. coli* TOP10 and displays white colonies C) shows *L. plantarum* harboring plasmids constructed with mCherry downstream of the constitutive promoter SlpA and displays cherry-red colonies. Figure 4.16 indicates that the inducible P_{sppA} only promotes constitutive expression of mCherry in *E. coli* TOP10, and not in *L. plantarum*.

4.7.2 Growth curve analysis of L. plantarum harboring mCherry

Preparation of *L. plantarum* strains harboring pSIP_mCherry and pSIP_SlpA_mCherry is described in section 3.12. *L. plantarum* cultures harboring pEv is used as a negative control. Standard deviations, SD, were calculated by measuring growth in three independent preparations from the same *L. plantarum* strains. SD are included for every group, excluding MRS blank.

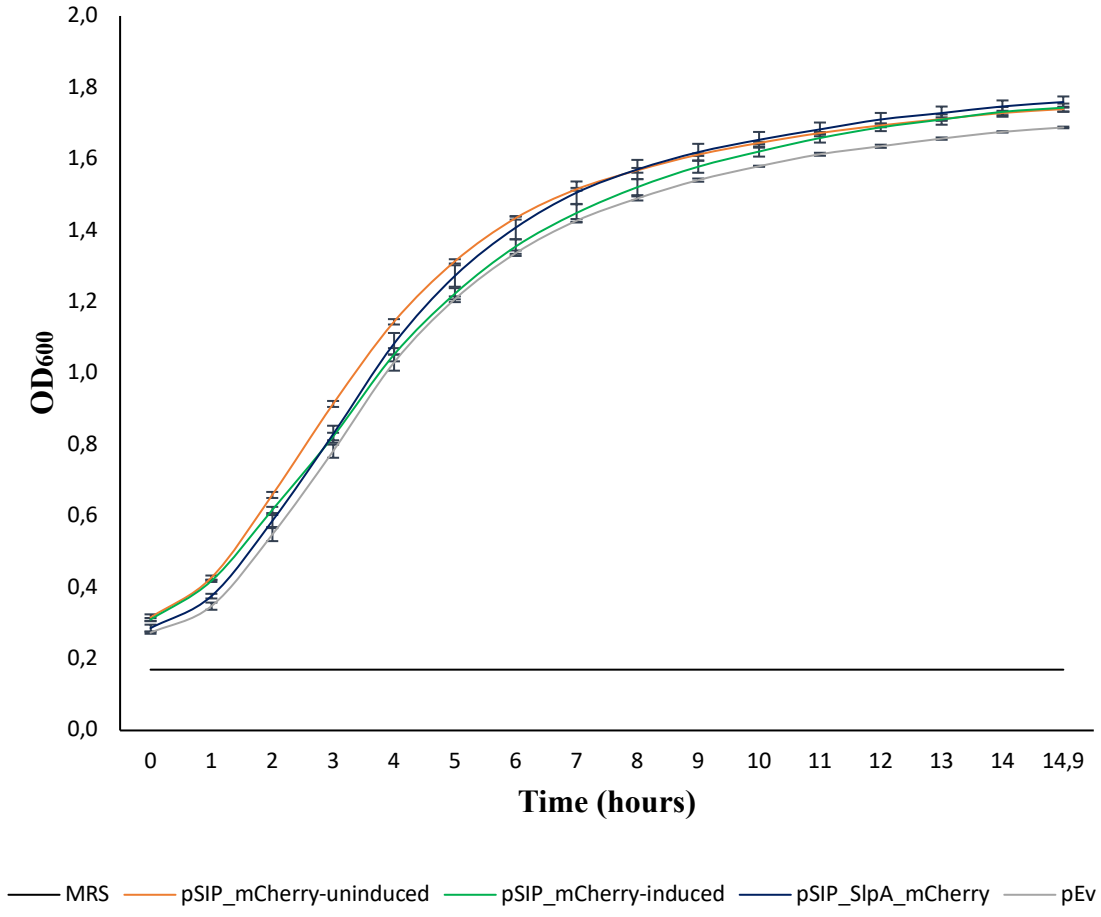


Figure 4.17. A schematic overview of growth in different *L. plantarum* cultures, measured in OD_{600}, between cultures harboring pSIP_mCherry induced and uninduced or pSIP_SlpA_mCherry over 15 hours. Cultures with pEV and MRS blank are used as reference. Standard deviations are included for each culture excluding MRS blank.

Figure 4.17 shows that *L. plantarum* cultures harboring pSIP_SlpA_mCherry, pSIP_mCherry induced and uninduced grow very similar, and referring to the standard deviations it appears they have no significant deviations from each other in growth rate.

4.7.3 Detecting promoter activity by measuring relative fluorescence

To detect promoter activity, mCherry was used as a protein tag and the fluorescence measured over 15 hours were presumed to be related to how active the promoter is. The preparation of cells was done as described in section 3.12 and transferred to a black microtiter plate. Firstly, the fluorescence of cultures harboring induced and uninduced pSIP_mCherry and pSIP_SlpA_mCherry were analyzed. Then the fluorescence from *E. coli* TOP10 harboring induced and uninduced pAIP_mCherry were analyzed. The wavelength was set to 587 and measured in relative fluorescent unit (RFU).

The promoters represented in *L. plantarum* colonies are the inducible SppA promoter and the constitutive promoter SlpA. The promoter represented in *E. coli* colonies is the inducible SppA promoter. Standard deviations are included for every test group.

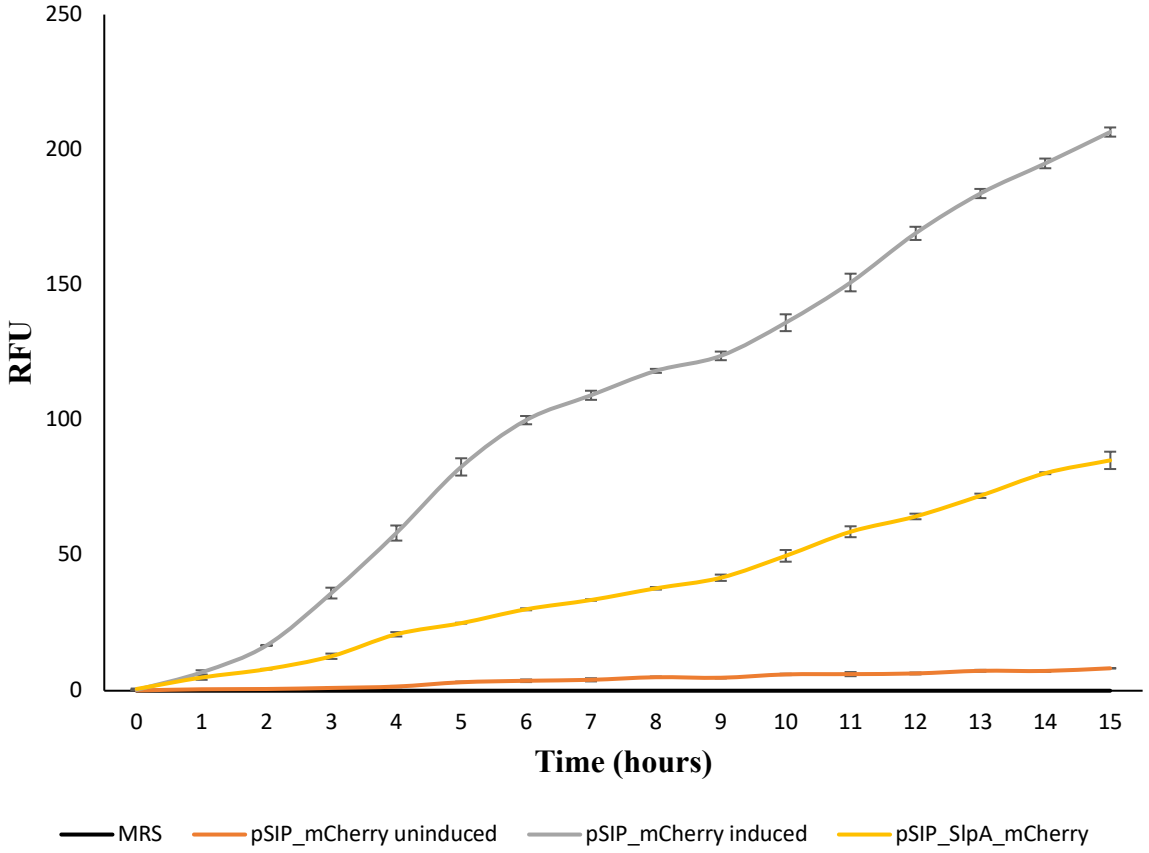


Figure 4.18. A schematic overview of fluorescence RFU in *L. plantarum* harboring plasmids constructed with mCherry downstream of the inducible promoter and the constitutive SlpA promoter. MRS blank is used as reference and standard deviations are included for all cultures excluding MRS blank.

Figure 4.18 shows *L. plantarum* culture harboring mCherry downstream of the inducible promoter and the constitutive SlpA promoter. The fluorescence of the different cultures was measured from the different cultures over 15 hours. Cultures harboring pSIP_SlpA seems to emit less than cultures harboring the induced inducible promoter and was comparable to the growth curve shown in Figure 4.10.

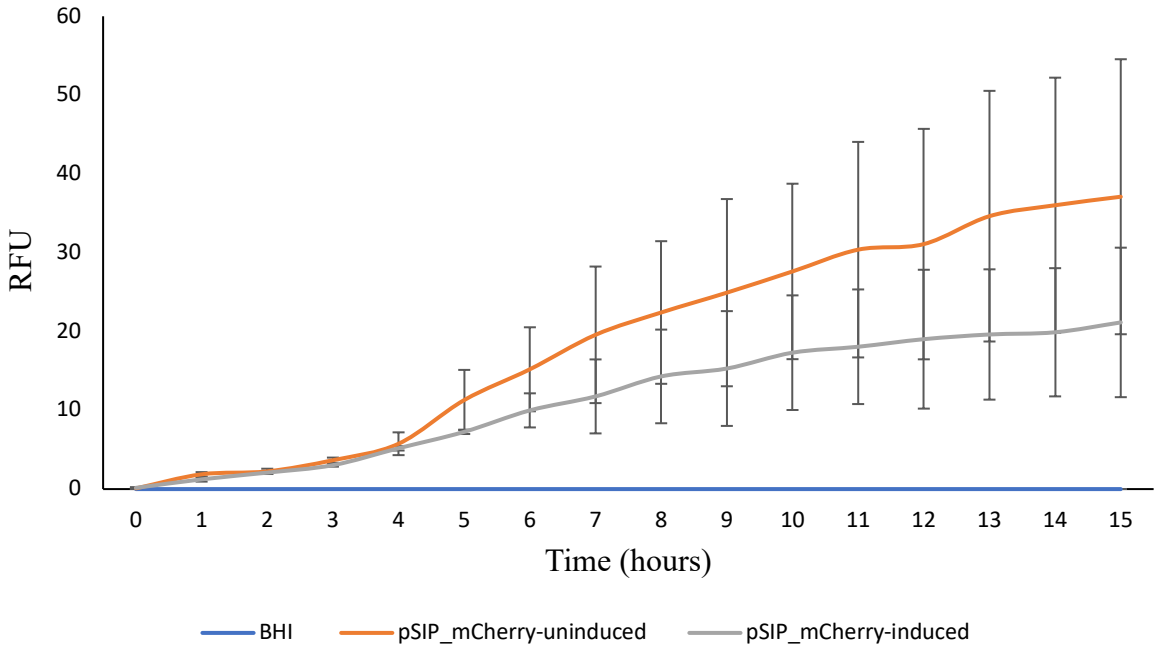


Figure 4.19. A schematic overview of fluorescence in *E. coli* TOP10 harboring plasmids constructed with mCherry downstream of the inducible promoter. One culture was induced with SppIP, the other was not (uninduced). BHI blank is used as reference and standard deviations are included for all cultures excluding BHI blank.

Figure 4.19 illustrates *E. coli* TOP10 cultures pSIP_mCherry emit fluorescence, in contrary to the initial idea that the pSIP system does not function in *E. coli*. Another noteworthy observation is that *E. coli* harboring the uninduced pSIP_mCherry seems to emit marginally more RFU than the induced culture. Compared to figure 4.18, *E. coli* TOP10 harboring plasmids constructed with mCherry downstream of the inducible promoter still emit a lot less than *L. plantarum* harboring the same plasmids. The standard deviations for *E. coli* TOP10 harboring plasmids constructed with mCherry are also much larger than for *L. plantarum* (figure 4.19).

5.0 Discussion

5.1 Construction of constitutive vectors

When the aim is to overproduce the antigens AgE6 *in situ* it would be beneficial to utilize a constitutive promoter instead of an inducible promoter. A constitutive production requires less invasive strategies as it does not require an inducer and the protein gets expressed continuously (Peirotén & Landete, 2020). To construct vectors for constitutive expression of AgE6, three different promoters, SlpA8287, SlpD and Tuf34, were chosen to replace the inducible P_{sppA}. The promoters used in the present study are active promoters from different bacterial species within the *Lactobacillus* genus. The promoters were chosen to be constructed into the pSIP system which originally was optimized for heterologous inducible gene expression (Sørvig, Mathiesen, et al., 2005).

In this study, the cassette system (section 1.4.1) was utilized to construct constitutive plasmids and some challenges were met during the digestion and linearization phase causing multiple religated vectors. During the digestion phase incomplete digestion can occur when too much or too little enzyme is used. Altering the volume of restriction enzyme could benefit the digestion. Incomplete digestion could also occur if contaminants were present in the DNA sample and inhibited the restriction enzymes. There was no evidence of contamination. However, by isolating the DNA and adding the components for digestion utilizing sterile technique, this supposition could be rejected.

After transformation into *E. coli* TOP10, the promoters SlpA8287 and SlpD had deletions and substitutions in their sequences (Appendix 7.1.1.1 and 7.1.2). No correct promoter sequences were identified and thus the constitutive plasmid constructions were not successful. The RBS was deleted on all SlpA8287 promoters and they would not have been able to recruit ribosomes for translation. The point mutation in the -10 box in SlpD was at a highly reserved area and a single bp introduction or deletion can greatly influence the promoter activity and thus the associated AgE6 transcription (Peirotén & Landete, 2020). The promoter Tuf34 was not successfully cloned in any of the ligation attempts and it is thought that these three promoters elicited a toxic effect in *E. coli*. The unsuccessful cloning of Tuf34

may be because it is too strong of a promoter in *E. coli*. It is imaginable that the difficulty of constructing a pSIP plasmid with different strong constitutive promoters occurred when they were transformed into the bacterial host, as *E. coli* TOP10 colonies survived only when selected for inactive mutants. Perhaps the strong promoters resulted in an overexpression of AgE6 and were toxic in *E. coli*, as constitutively expressing heterologous proteins is well known to exhibit a toxic effect on the host (Peirotén & Landete, 2020). To overcome this, weaker promoters could be utilized. However, there is a possibility that the AgE6 production would not be able to compete with the inducible system. Another approach could be to choose promoters from the same bacteria that SlpA and PgM originated from; *Lactobacillus acidophilus*, as these were previously successfully cloned into *E. coli* TOP10. It could also prove profitable to construct plasmids with new promoters from wild type *L. plantarum* in the future, as the promoters SlpA8287 and SlpD originated from *Lactobacillus brevis* and Tuf34 from *Lactobacillus buchneri* appeared to be active and subsequently toxic in *E. coli*. Alternatively, strong constitutive promoters could be transformed directly into *L. plantarum* by using non-methylated DNA. Spath et al., (2012) successfully developed a direct cloning approach for *L. plantarum* CD033 using non-methylated DNA and using LAB origin of replication (Spath et al., 2012). Another approach could be to transform the plasmids in to a subcloning bacteria within the same genus as *L. plantarum* instead of going via *E. coli* TOP10, as it is possible that these promoters are less toxic in strains similar to *L. plantarum*. Species like *Lactobacillus curvatus* and *Lactobacillus sakei* could be of interest, as the replicon 256_{rep}, which was used to construct the shuttle vector in this study, has these species in its host range (Sørvig, Skaugen, et al., 2005). A known LAB to be an efficient subcloning strain is *Lactococcus lactis* (Karlskås et al., 2014), and could be an alternative to *E. coli* as it might be more accepting of the constitutive promoters and antigen used in this study. However, the plasmid replicon used in this study is not compatible with *L. lactis*, although by utilizing the plasmid pSIP 411 could be an alternative (Karlskås et al., 2014).

The genes *sppK* and *sppR* associated with the inducible system was thought to not have a function in the plasmids constructed with constitutive promoters. They were removed to construct a smaller plasmid with the hypothesis that a smaller plasmid might be reduce the fitness cost in the host and therefor one could expect to see higher growth rates resulting in more overall AgE6 expressed. *sppK* and *sppR* were successfully removed using restriction enzymes.

5.2 Bacterial growth

Expressing heterologous proteins can hamper bacterial growth because vital metabolic systems can become limited for resources. When utilizing a constitutive promoter providing an uncontrolled overexpression of the heterologous protein, it may have greater consequences on the bacterial growth (Bienick et al., 2014). However, higher bacterial growth rates are preferred as there is a possibility to produce a higher antigen dosage and therefore possibly a more effective vaccine. Because of this, it was important to construct plasmids with a constitutive promoter that did not only produce high levels of AgE6 but was also fairly easy to cultivate in large quantities.

All plasmids producing heterologous protein used in this study had hampered growth compared to *L. plantarum* colonies harboring pEv (Figure 4.10). However, the growth rate of *L. plantarum* strains harboring plasmids with pSIP_PgM were adjacent to cultures harboring pEv after 15 hours. Moreover, the pSIP_PgM cultures had a significantly higher growth rate than strains harboring plasmids with pSIP_SlpA. (Figure 4.10). Similar results were also found by Nguyen et al., (2019) which indicated a higher protein production causing metabolic stress in *L. plantarum* cultures harboring plasmids with the SlpA promoter.

All *L. plantarum* cultures harboring plasmids constructed without *sppK* and *sppR*; pSlpA, pPgM and pSlpD, had the highest growth rate of all strains used in this study. The growth rates strongly indicated that plasmids without *sppK* and *sppR* did not promote the expression of AgE6. pSIP_SlpD had a similar growth rate to pSlpD thus consequently indicated that *L. plantarum* harboring pSIP_SlpD did not produce AgE6. The lack of SlpD promoter activity was likely due to the mutation in the -10 box indicating that the point mutation greatly affected promoter activity and thus the transcription of the associated gene (Peirotén & Landete, 2020).

5.3 Western blot analysis of antigen production

Cell free protein extracts from *L. plantarum* were applied to SDS-PAGE and analyzed by western blot to investigate the production of AgE6. The analysis included cultures harboring plasmids with promoters SlpA and PgM, without the genes *sppK* and *sppR,* and the inducible P_{sppA}. *L. plantarum* strains harboring pPgM is not present, however based on the lack of antigens found in pSlpA1261, the flow cytometry (Figure 4.11), the immunofluorescent microscopy (Figure 4.14) and the similar growth rates to pEv (Figure 4.7), cultures harboring pPgM was presumed to not produce AgE6.

In contrary to Nguyen et al., (2019), *L. plantarum* strains harboring pSIP_PgM appear to have produced slightly more antigens than pSIP_SlpA (Figure 4.9). Interestingly, the *L. plantarum* culture harboring pSlpD (without *sppK* and *sppR*) seems to have a weak band at the correct band size, while the culture harboring pSIP_SlpD does not (Figure 4.9). Both promoters have a point mutation and the difference in AgE6 production might be due to this point mutation together with the proximity to the erythromycin promoter in pSlpD. The SlpD promoter is smaller in size (Appendix 7.1.2) compared to SlpA and PgM. The proximity from the erythromycin promoter to AgE6 is greater in pSlpD, which might explain why this overlapping promoter phenomenon occurred in pSlpD and not in pSlpA or pPgM.

A western blot analysis on *L. plantarum* harboring pSIP_SlpA or pSIP_PgM harvested six hours after induction of the inducible plasmids would be interesting to perform. This would enable the analysis of any changes in AgE6 production compared to growth time. However, this was unmanageable due to the current situation with SARS-CoV-2.

5.4 Detection of surface displayed antigens

Flow cytometry was used to analyze and confirm the presence of antigen AgE6 anchored on the surface by lipo-protein anchor Lp_1261 in *L. plantarum* (Figure 4.11). Antigen detection on the cell surface using immunofluorescent microscopy confirmed the results found in western blot analysis and flow cytometry (Figure 4.14). *L. plantarum* cultures harboring the constitutive plasmids pSlpA and pPgM (without *sppK* and *sppR* genes) were adjacent to

cultures harboring pEv indicating no AgE6 on the cell surface. Thus, the flow cytometry analysis (Figure 4.11) together with the western blot results (Figure 4.9) strongly correlates *sppK* and *sppR* genes to the activity of constitutive promoters. There is likely a gene interaction or perhaps a co-transcription between the constitutive promoter and the *sppK* and *sppR* genes, resulting in no AgE6 production without *sppK* and *sppR*.

The western blot and flow cytometry analysis (Figure 4.9 and 4.11) showed some antigen production and anchoring in *L. plantarum* cultures harboring pSlpD (which contains the point mutation in the -10 box). These results further indicated the correlation between the small size of SlpD and a close proximity to the promoter for erythromycin resistance, resulted in an overlapping promoter function.

Surprisingly, *L. plantarum* cultures harboring pSIP_PgM or pSIP_SlpA (with *sppK* and *sppR* genes) produce approximately the same amount of AgE6 on the cell surface at two different times of cell growth, indicating a stable antigen production over time. As the promoter is constitutive it was thought that the AgE6 production would increase over time. However, this consistent antigen production could prove desirable in a live vaccine vector as the vaccine might be more effective at delivering antigens over time.

The similar amounts of AgE6 presented on the *L. plantarum* surface in strains harboring the plasmids pSIP_SlpA and pSIP_PgM (Figure 4.12 and 4.13 D), is not consistent with the results found by Nguyen et al., (2019). In the present study, by examining the growth curve pSIP_SlpA has a hampered growth compared to pSIP_PgM which indicates a higher heterologous protein production. (Figure 4.10). However, western blot (Figure 4.9) analysis and flow cytometry (Figure 4.12) indicated that *L. plantarum* cultures harboring pSIP_SlpA did not appear to surpass pSIP_PgM in production of AgE6, despite other studies finding SlpA to be a stronger promoter (Nguyen et al., 2019) (Figure 4.9, 4.12 and 4.13 D). A hypothesis for this phenomenon might be that the growth and fitness cost of *L. plantarum* is determined by an abundance of mRNA compared to available tRNA, amino acids and ribosomes (Baquero et al., 2019). When there a significant discrepancy of transcribed genes to ribosomes/tRNA/amino acids it could lead to ribosome pausing. Moreover, it could lead to protein mistranslation and protein misfolding subsequently affecting translation efficiency of the heterologous proteins. The effects of low translation efficiency commonly increases with the level of gene expression (Baquero et al., 2019). If SlpA over-promoted the transcription

of AgE6 to a level when a significant discrepancy occurred between mRNA and available ribosomes, it might have led to ribosome pausing, mistranslation and misfolded proteins. A high transcription rate promoted by SlpA could therefore explain the growth curve analysis and the lesser AgE6 production *L. plantarum*. Why these deleterious effects of high transcription rates of AgE6 did not appear to happen in *L. plantarum* cultures harboring the inducible plasmid is unclear. However, it is likely due to the difference in stress and fitness cost of having an uncontrolled constitutive promoter compared to a controlled inducible promoter. These effects might also have been avoided in *L. plantarum* harboring the inducible plasmids because they were allowed to grow before they were induced. When the inducible culture started the heterologous protein production, they might have been more suited to overcome the fitness cost.

A stable antigen production is favorable as a stable introduction of antigens to the immune system could elicit a stable immune response. However, *L. plantarum* cultures harboring the inducible pLp1261 still have a significantly higher overproduction of antigens and a better candidate for vaccine delivery than pSIP_SlpA and pSIP_PgM.

5.5 Tagging the promoter with mCherry

Tagging promoters with mCherry was done to measure promoter activity and to potentially be able to follow *L. plantarum* through the GIT. Using *L. plantarum* as a live vaccine vector to the mucosal surfaces, survival through and adherence to the GIT cells plays a role in the vaccine efficacy. The vaccine elicits an immune response through the mucosal surfaces in the intestinal area hence tracking the live vector is of interest. mCherry was utilized as it is a non-toxic, stable protein and matures into a folded fluorescent protein within 15 minutes (van Zyl et al., 2015). The mCherry protein has also shown the ability to be expressed at high levels without posing any significant physiological stress to the host, which was also observed in the present study (Figure 4.17). Furthermore, mCherry could possibly be fused to the AgE6 antigens for future studies as it has no significant disruptive qualities to the function of the protein to which they are fused (van Zyl et al., 2015).

The fluorophore mCherry is a promising candidate to study *L. plantarum* through the GIT as it did not lose its ability to fluoresce over time (Figure 4.18) and has previously been utilized

in a *L. plantarum* strain to study the intestinal tract of mice (van Zyl et al., 2015). Because mCherry does not hamper the growth of *L. plantarum* as much as AgE6 production does (Figure 4.17 and 4.10), the number of bacteria adhering to the GIT cells may not be true to the actual vaccine. This can be overcome by constructing a plasmid with the antigens and the mCherry gene or by fusing the mCherry gene to the antigens to follow the actual antigen production through the intestines.

Surprisingly, *E. coli* expressed the mCherry gene despite being controlled by the SIP system. The SIP system is thought not to be active in *E. coli* yet the mCherry protein was produced constitutively (Figure 4.16 A). pSIP_mCherry in *E. coli* did not seem to be affected by the presence of SppIP, or lack thereof (Figure 4.19). An attempt was made to transform pSIP_SlpA_mCherry into *E. coli* TOP10 which yielded no colonies, however pSIP_SlpA was able to be transformed directly into *L. plantarum*. This might be due to the excessive production of mCherry and having the constitutive promoter SlpA promoting mCherry could be toxic in *E. coli*. This strengthens the hypothesis that using *E. coli* TOP10 to initially transform a constructed plasmid is less productive than possibly other bacteria within the same genus as *L. plantarum*. Constructing a shuttle vector which includes more similar bacteria as *L. plantarum* or clone non-methylated plasmid directly into *L. plantarum* might yield more successful transformations.

5.6 Concluding remarks and future prospects

This thesis describes the challenges of constructing constitutive vectors to promote an overproduction of AgE6 in *Lactobacillus plantarum* WCFS1. Colonies from transformation attempts of constitutive plasmids were typically religated. *E. coli* colonies, for most of the constitutive promoter constructs survived only when selected for inactive mutants. Only three colonies contained the pSIP_SlpA8287 and they were all severely mutated, only one colony was obtained with the pSIP_SlpD promoter which had a base substitution in the -10 box. This indicated that the promoters were functional in *E. coli* and the production of AgE6 was toxic. An alternative would be to clone directly into *L. plantarum* (Spath et al., 2012) or via *L. lactis* (Karlskås et al., 2014).

One of the goals was also to construct a smaller plasmid which in return could benefit the growth of *L. plantarum* and therefore potentially produce more AgE6. The genes *sppK* (HK) and *sppR* (RR) were removed because they were thought to only have a function with the inducible system and not the constitutive system. However, constitutive plasmids constructed without the *sppK* and *sppR* genes resulted in inactivated promoters. In the pSIP system it is likely that an interaction between the *sppK*, *sppR* and promoter occurs and is vital for transcription of the associated gene.

The inducible P_{sppA} and the constitutive SlpA promoter were successfully tagged with mCherry. These promoters were measured for promoter activity and has the opportunity to follow *L. plantarum* through the GIT. mCherry did not hamper the growth of *L. plantarum* and fluorescence did not subside over 15 hours making it a good candidate for this analysis. Because the *L. plantarum* growth was not significantly hampered by the production of mCherry compared to AgE6, it could be beneficial to construct a plasmid with the anchor, antigens and mCherry, or fusing mCherry to AgE6. An important note is that *E. coli* TOP10 harboring pSIP_mCherry appeared to produce mCherry and promotes the hypothesis that the SIP system is, to some degree, active in *E. coli*.

Even though *L. plantarum* cultures harboring pSIP_PgM were the more suited constitutive plasmid alternative in this study considering bacterial growth and AgE6 production, it is not comparable to cultures harboring the inducible system. Several strategies should be further explored to successfully construct a constitutive plasmid that is comparable to the inducible plasmid in AgE6 production. Using subcloning species in the *Lactobacillus* genus where the promoter is likely to be less toxic could prove beneficial. Moreover, constitutive promoters are species dependent and can vary greatly in activity (Rud et al., 2006). It may prove advantageous to choose strong constitutive promoters native to *L. plantarum*, such as the elongation factor promoter Tuf33 (Peirotén & Landete, 2020), and clone it directly into *L. plantarum* or a *Lactobacillu*s subcloning vector.

6.0 References

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7.0 Appendix

7.1 Constitutive promoters

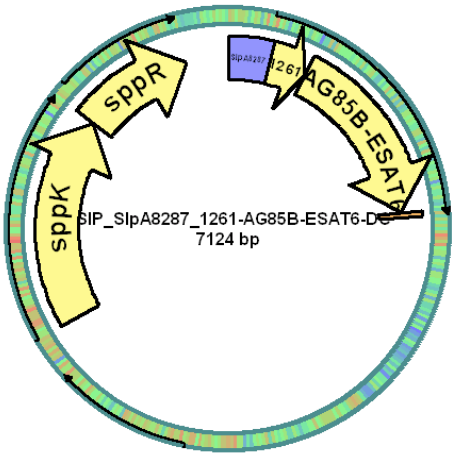
7.1.1 SlpA8287

The SlpA8287 gene is an S-layer protein gene (Vidgrén et al., 1992) from *Lactobacillus brevis* ATCC 8287

Sequence:

```
   1 AGATCT GATT ACAAAGGCTT TAAGCAGGTT AGTGACGTTT TAGTTATGTA
  51 ACAATAACAT TACAGGACAC CCATAATTGT TTCAATCCAA CGACTCAGAG
 101 CGTAATCCTT GTATCTCCTT AAGGAAATCG CTATACTTAT CTTCGTAGTT
 151 AGGGGATAGC TGATCGGGTC CGCTAATGTT ATGAAATAAA ATTCTTAACA
 201 AAAGCGCTAA CTTCGGTTAT ACTATTCTTG CTTGATAAAT TACATATTTT
 251 ATGTTTGGAG GAAGAAAG CA TATG
```

The sequence has two promoters P1 and P2. The regulatory -10 box is marked in red and the -35 box is marked in yellow. The RBS is highlighted in bold letters. The bases written in cursive letters marks the translation start signals. This information was found by using BLAST by NCBI.



Visualization of pSIP_SlpA8287

7.1.1.1 SlpA8287 after sequencing

pSIP_SlpA8287 sent to sequencing after correct band size from *E. coli* TOP10 colony PCR

Sequence:

```
    1 AGATCTGATT ACAAAGGCTT TAAGCAGGTT AGTGACGTTT TAGTTATGTA
   51 ACAATAACAT TACAGGACAC CCATAATTGT TTCAATCCAA CGACTCAGAG
  101 CGTAATCCTT GTATCTCCTT AAGGAAATCG CTATACTTAT CTTCGTAGTT
  151 AGGGGATAGC TGATCGGGTC CGCTAATGTT ATGAAATAAA ATTCTTAACA
  201 AAAGCGCTAA CTTCGGTTAT ACTATTCTTG CTTGATAAAT TACATATTTT
  251 ATGTTTGGAG GAAGAAAG CATATG
```

The bases marked in gray was deleted from the SlpA8287 promoter in clone I from *E. coli* TOP10 colony. The bases marked in gray and turquois was deleted from clone II from *E. coli* TOP10. The bases marked in gray, turquois and green was deleted from clone III from *E. coli* TOP10. RBS (highlighted in bold letters) was deleted in all clones. This information was found by using BLAST by NCBI.

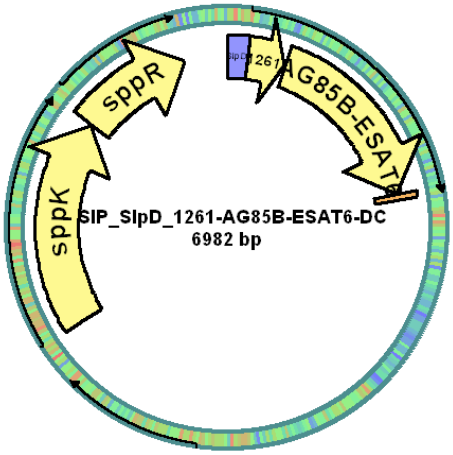
7.1.2 SlpD

The SlpD gene is a surface layer protein gene from *Lactobacillus brevis* ATCC14869 (Jakava-Viljanen et al., 2002)

Sequence:

```
   1  AGATCTTTTC CATTGTAAAA AAATAAATCA TTTTTTGGTT GTAGGTGTTT
  51 GCAATTTAAA TTAATTGTGA TAAGATATCG TTGTAGCATA AATGTTACGT
 101 AAATAAAACA ATATTTAGGG GGATTT CATA TG
```

The promoter sequence has two regulatory – 10 box and is marked in red and RBS is highlighted in bold letters. The bases written in cursive letters marks the translation start signals. The promoter sequence has two regulatory – 10 box and is marked in red and RBS is highlighted in bold letters. The bases written in cursive letters marks the translation start signals. The base written in white represents the bp substitution from the *E. coli* TOP10 clone, which was substituted with a G. This information was found by using BLAST by NCBI.



Visualization of pSIP_SlpD

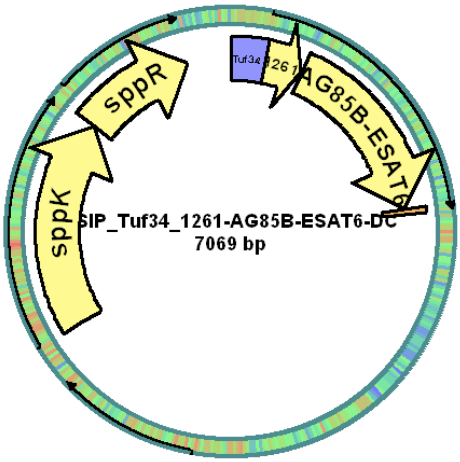
7.1.3 Tuf34

The Tuf34 gene is found upstream of a gene coding for translation elongation factor from *Lactobacillus buchneri* (Tauer et al., 2014).

Sequence:

```
   1 AGATCTGATC AGGAAATTAA AATTGGTCTC ATATAACTGA ATTATTTTCG
  51 GAAAATAAAG GGAATCTGTT TACAAACATT ACCAGTATCG ATATAATACT
 101 TAAGGATTCT TCGAAAATTG ACTACTTTGT CTTTTCCAGA AGATGTAGTA
 151 TAATAACACT TAGAAATGCA TTGATGCGAA ATTGATGTAA TTCTTGAAAA
 201 CAAGGAGATT TTA CATATG
```

The sequence has two promoters P1 and P2. The regulatory -10 box is marked in red and the -35 box is marked in yellow. The RBS is highlighted in bold letters. The bases written in cursive letters marks the translation start signals. This information was found by using BLAST by NCBI.



Visualization of pSIP_Tuf34

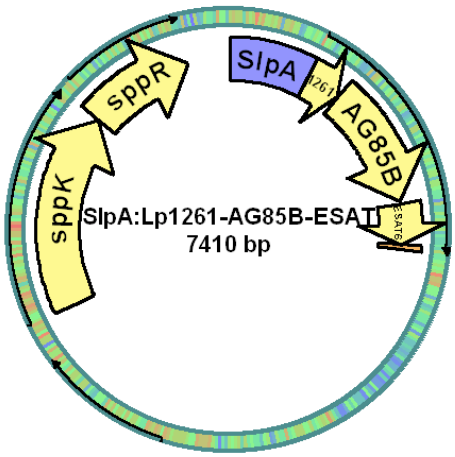
7.1.4 SlpA (not constructed in this study)

The SlpA gene was amplified from a plasmid pSlpA1261Man from *Lactobacillus acidophilus* ATCC4356.

Sequence:

```
   1 AGATCT ATAA AGTTGTTTGA TAAATGCTCA ACTTTAAGTA ATTTTAAGGA
  51 GCTAACTAAC TGTGGGGGAT GAAATAAAGC CAATAGAAAA AGCGAACCTA
 101 ATAAGATTAA TCTTTAGGAA AATCGAATAA AAATATTACT TTTTTGATAT
 151 GTTTTGTCAT AGTTTCGTAA AATTTAGTAA AGATTACGAG CGATAAATAG
 201 AGAACTTAAT CTTGTCTTTT TCTTGCTATA GCTAGGTTTA GCACATTTTA
 251 CAATTTTAAA GTGCTTGTAA TGCTTGTGGG GGTAAGCGGT AGGTGAAATA
 301 TTACAAATAG TATTTTTCGG TCATTTTAAC TTGCTATTTC TTGAAGAGGT
 351 TAGTACAATA TGAATCGTGG TAAGTAATAG GACGTGCTTC AGGCGTGTTG
 401 CCTGTACGCA TGCTGATTCT TCAGCAAGAC TACTACCTCA TGAGAGTTAT
 451 AGACTCATGG ATCTTGCTTT GAAGGGTTTT GTACATTATA GGCTCCTATC
 501 ACATGCTGAA CCTATGGCCT ATTACATTTT TTTATATTTC AAGGAGGAAA
 551 AGAC CATATG
```

The RBS is highlighted in bold letters. The bases written in cursive letters marks the translation start signals.



Visualization of pSIP_SlpA

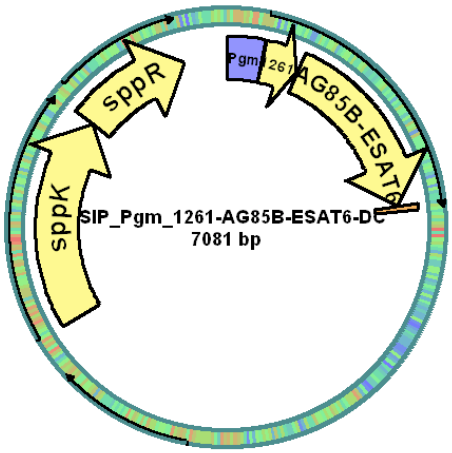
7.1.5 PgM (not constructed in this study)

The PgM gene is a glycogen metabolic gene from *Lactobacillus acidophilus* NCFM.

Sequence:

```
   1 AGATCT TGCG ACAAGTAATA AACTAAACAA AACAACTACA AAATATTTCT
  51 TTTTGTTTTT CATGATTTTT ACACTTCTCT TAGTATGCTT TTGTTATAAG
 101 TTAGCACAAA AAAGCAGAAA ATAAAAAGTA GAAATAAAAA AAGATGTTTT
 151 TTTGCCCATA TCTCTATGAA AAAAACTGTG AAATGTGTAA AATATGGATG
 201 AAACATTGAA TTTAAAAGGA GATAT CATAT G
```

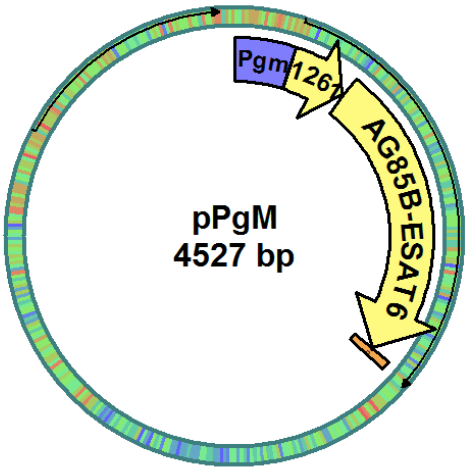
The RBS is highlighted in bold letters. The bases written in cursive letters marks the translation start signals.



Visualization of pSIP_PgM

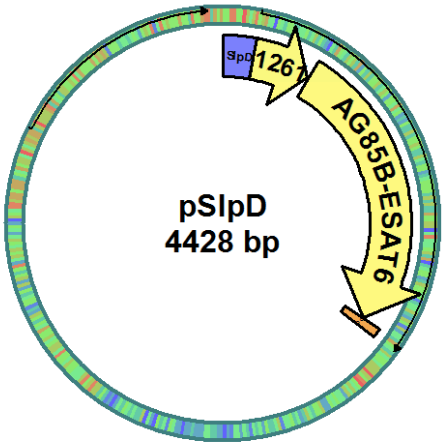
7.2 Plasmids with deleted sppK and sppR genes.

7.2.1 pPgM



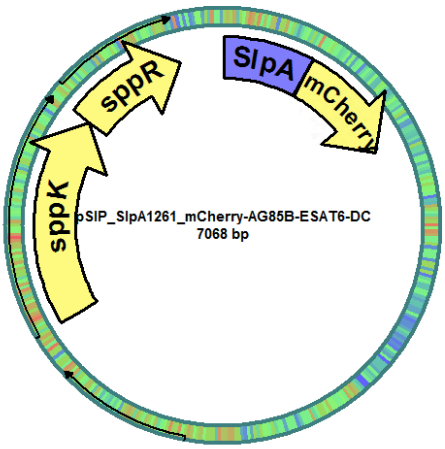
Visualization of pPgM

7.2.2 pSlpD



Visualization of pSlpD

7.3 pSIP_SlpA_mCherry



Visualization of pSIP_SlpA_mCherry

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