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Secretion and anchoring of a cancer antigen in *Lactobacillus plantarum*

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Biotechnology

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Abstract

Melanoma is one of the most lethal forms of skin cancer, and immunotherapy continues to be a hot topic for the treatment of this disease. This study is part of a larger project with the long-term goal to develop mucosal vaccines utilizing lactic acid bacteria (LAB) as delivery vectors of antigens. LAB have been exploited in the preservation of food for centuries. In addition to their status as generally recognized as safe (GRAS), LAB meet many of the requirements of a potential vector for delivery of antigens to mucosal sites. Many of these bacteria have a tolerance for low pH, and are natural inhabitants of the gastrointestinal tract. Furthermore, studies of *Lactobacillus plantarum* have indicated that this bacterium has immunological adjuvant effects, making it a good candidate for delivery of antigens with the aim of inducing immunity against pathogens or even cancer.

MART-1/Melan-A is an antigen found on the surface of melanocytes and cells of melanocytic lineage. This antigen has been shown to have potential as a cancer vaccine candidate due to its potential to generate tumor specific immune responses. In the present study, we have constructed plasmids for secretion and surface display of MART-1 on *L. plantarum*. It has previously been shown that the amino acid substitution A27L in the MART-1 epitope recognized by T cells elicits a stronger immune response compared to the native MART-1 epitope. Therefore, the modified epitope was translationally fused to the C-terminal end of the open reading frame of the native MART-1 protein. In addition, adjuvants are often necessary to induce an adequate immune response, thus a dendritic cell (DC) binding peptide was also incorporated into the translated protein. The recombinant fusion protein was denoted modMART1. Six plasmids for the expression of this protein were constructed to facilitate either secretion or surface anchoring of the cancer antigen. These included two lipoprotein anchors directing the protein to the cell membrane, a covalent anchor bound to the cell wall via a LPxTG (sortase) anchor, and a non-covalent anchor bound to the cell wall by a LysM domain, in addition to two plasmids for the secretion of modMART1_DC.

The bacteria harboring the different modMART1_DC containing plasmids were characterized according to their ability to produce the recombinant protein in terms of viability and surface localization. Successful production was verified through Western blot analysis for all constructs. Surface localization of the antigen was investigated through flow cytometry and

immunofluorescent microscopy, and was confirmed for the lipoprotein anchors and LPxTG (sortase) anchor cell wall anchor after lysozyme treatment. Interestingly, no secreted antigen was observed in the culture supernatant from recombinant *L. plantarum* harboring the secretion plasmids. Subsequent lysozyme treatment, flow cytometry and immunofluorescent microscopy indicated that modMART1_DC was retained in the cell wall. modMART1_DC was also purified from an *E. coli* BL21 expression host to semi-quantify the amount of antigen produced by each construct, as knowledge of the amount of antigen administered is of great importance in a potential vaccine trial. The successful surface exposure of the antigens, alongside the continued viability of the *Lactobacillus* strains, indicate that the recombinant bacteria have potential as delivery vectors of MART-1 antigens to mucosal sites

Sammendrag

Dette studiet er del av et større prosjekt med det langsiktige målet å utvikle vaksiner med melkesyrebakterier som leveringsvektorer av terapeutiske proteiner og antigener til det slimhinne-assosierte immunforsvaret. Melkesyrebakterier har lenge blitt utnyttet i bevaringen av mat, og mange arter anses derfor som helt trygge for konsum. Det er også blitt vist at mange arter av melkesyrebakterier har probiotiske effekter på verten. Dette, i tillegg til at mange er en del av den naturlige tarmfloraen hos mennesker, gjør melkesyrebakterier til gode kandidater for leveringsvektorer av antigener. En av artene som det er forsket mye på er *Lactobacillus plantarum*, og studier har indikert at denne arten kan inneha immunomodulerende egenskaper som gjør den til en attraktiv kandidat som leveringsvektor av antigener med målet å indusere immunitet mot patogene mikroorganismer og til og med kreft.

MARTt-1/Melan-A er et protein uttrykt i melanocytter, og antigener fra dette proteinet har blitt vist å bli gjenkjent av cytotoxiske T celler som angriper kreftceller. Å indusere en videre immunrespons mot dette antigenet har derfor blitt ansett som en god strategi for å bekjempe føflekkreft. I dette studiet har vi konstruert plasmid for sekresjon og ankring av et modifisert MART-1 protein på overflaten av *L. plantarum*. Det har tidligere blitt vist at en aminosyresubstitusjon fra alanin til leucin (A27L) i epitopen gjenkjent av cytotoxiske T celler, øker immunresponsen. Derfor ble denne epitopen translasjonelt koblet sammen til den C-terminale delen av den åpne leserammen til det native MART-1 proteinet. I tillegg ble et dendrittisk cellebindende peptid inkorporert i proteinet for å forsterke en eventuell immunrespons ved å øke sjansen for å bli tatt opp av dendrittiske celler. Proteinets ble kalt modMART1_DC, og totalt seks plasmider ble konstruert: to lipoproteinankere som ville føre til ankring til cellemembranen, et kovalent anker bundet til celleveggen via et LPxTG (sortase) anker, et ikke-kovalent anker bundet til celleveggen via et LysM domene, og til slutt to sekresjonsplasmider.

De rekombinante bakterienes evne til å produsere proteinet og proteinets overflatelokalisering ble deretter undersøkt. Proteinuttrykk ble verifisert for alle konstruktene gjennom Western blot analyse. Proteinets overflatelokasjon ble undersøkt med strømningscytometri og immunofluorosensmikroskopi, og ble bekreftet for lipoproteinankerene og det kovalente LPxTG celleveggsankeret etter lysozymbehandling. Imidlertid ble ikke sekretert antigen

funnet i supernatantene til *L. plantarum* med sekresjonsplasmider. Påfølgende lysozymbehandling, strøminingscytometri og immunofluoresensmikroskopi indikerte at modMART1_DC var fanget i celleveggen til bakterien. I tillegg ble modMART1_DC uttrykt i en overekspresjonsstamme, *E. coli* BL2, for å isolere rent modMART1_DC protein som kunne brukes til å fastslå et semi-kvantitativ estimat av hvor mye antigen hver bakterie med ulike plasmider produserte. Dette er viktig å vite i et potensielt vaksineeksperiment, da det er nødvendig å vite hvor mye antigen som blir administrert. Den vellykkede produksjonen og ankringen av modMART1_DC på overflaten av *L. plantarum* indikerer at denne bakterien har potensiale til å brukes som leveringsvektor av kreftantigener til det slimhinneassosierte immunforsvaret for å indusere immunitet

Abbreviations

BSA	Bovine Serum Albumin
DC	Dendritic cell
dNTP	Deoxynucleotide phosphate
DTT	Dithiotreitol
FITC	Fluorescein isothiocyanate
GRAS	Generally recognized as safe
HK	Histidine protein kinase
HPV 16	Human Papillomavirus 16
HRP	Horseradish Peroxidase
Ig	Immunoglobulin
IL	Interleukin
LysM	Lysin Motif
MHC	Major Histocompatibility Complex
NICE	Nisin Controlled Expression
OFA	Oncofetal antigen
PCR	Polymerase Chain Reaction
RR	Response regulator
SRP	Signal recognition particle

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1. Introduction

The food preserving properties of Lactic acid bacteria (LAB) have been exploited for thousands of years. Many of these bacteria are part of the human gut-flora, and are thought of having a probiotic effect on the host. Due to their safe status, and continuous use in the food industry, the possibility of utilizing LAB as delivery vectors of therapeutic molecules and even vaccines have been explored for the past decades.

The delivery of therapeutic molecules or antigens is also a main strategy in inducing immune responses against cancer which still is a leading cause of death worldwide. However, to induce an adequate immune response that yield a positive and lasting effect, the antigens have to be associated with different adjuvants that will enhance the subsequent immune response. Due to their immunomodulatory properties and safe status, LAB is therefore also a promising candidate for delivery of cancer antigens. This study describes the generation of recombinant *Lactobacillus plantarum* for the delivery of the melanoma cancer antigen MART-1/Melan-A to mucosal sites.

1.1. Lactic Acid Bacteria

Lactic acid bacteria (LAB) comprise a group of Gram-positive bacteria with DNA with a low guanine and cytosine content. The bacteria have the ability to ferment carbohydrates through a homo- or heterofermentative pathway which both yield lactic acid as a by-product (Kandler 1983). The ability of LAB to produce lactic acid from hexose sugars has been exploited in the preservation of foods throughout history, however, in recent years, the possibility of utilizing these bacteria as both delivery vector of therapeutic molecules and live vaccine vectors has been explored (Daniel et al. 2011; Wszyńska et al. 2015).

Lactic acid bacteria inhabit primarily nutrient rich habitats as they are auxotrophic for a number of amino acids and essential nutrients (Christiansen et al. 2008; Deguchi & Morishita 1992) Many lactic acid bacteria are therefore also natural inhabitants of the human gastrointestinal tract (GIT) which makes them suitable for use as a vaccine delivery vector due to their resistance to low pH and ability to persist in the GIT for a longer period of time

A review of literature examining the potential health benefits of LAB found that the ingestion of these bacteria has the potential to prevent *Clostridium difficile*-associated diarrhea in adults and children (Goldenberg et al. 2013). Several LAB have also been linked to potential probiotic effects through imposing immunomodulatory effects on the host such as inducing tolerance against foreign non-lethal antigens (Ljungh & Wadstrom 2006; Perdigon et al. 2001). In a recent study, the ingestion of LAB was also shown to decrease stress-related sleep deprivation in students during their final exams (Takada et al. 2017).

The immunomodulatory potential and host interactions are of great importance, as the quality of a vaccine delivery vector is determined by the ability to interact with the host. Due to their continuous use in the preservation of food, many LAB have gained the Generally Regarded As Safe (GRAS) status from the American Food and Drug Administration, which implies the safe use of these bacteria for consumption. The use of GRAS LAB as an antigen delivery vector poses therefore significant less problems in contrast to live attenuated pathogen delivery vectors which may regain their inherent pathogenicity (Wells & Mercenier 2008).

1.1.1. *Lactobacillus plantarum*

Lactobacillus plantarum is a bacteria found in many environmental niches such as different food products and the human GIT (de Vries et al. 2006). The bacterium *L. plantarum* WCFS1 is one of the most studied bacteria belonging to the family Lactobacilli with one of the main reasons being that the *L. plantarum* WCFS1 genome was one of the first genomes to be sequenced by Kleerebezem et al. (2003). Since 2003, studies have been performed on the properties of different *L. plantarum* strains regarding their probiotic and immunomodulatory effects, in addition to their use as delivery vectors for therapeutic peptides/proteins and antigens (van den Nieuwboer et al. 2016). Especially the probiotic effects have been explored. In a randomized clinical trial, daily administration of *L. plantarum* strains showed a positive effect on the lipid composition of patients, and lowering the LDL cholesterol levels (Fuentes et al. 2016). *L. plantarum* has also been shown to activate toll-like receptor (TLR) 2 on intestinal epithelial cells, and this reaction may enhance the epithelial stability, thus limiting inflammation connected to increased permeability of the mucosal membrane (Karczewski et al. 2010).

Several strains of *L. plantarum* have been shown to be able to survive the transit of the human GIT tract, including *L. plantarum* WCFS1, which is a desirable trait for a potential oral delivery vector. However, *L. plantarum* WCFS1 has not been shown to colonize the GIT for a longer period of time (Douillard & de Vos 2014; van den Nieuwboer et al. 2016; Vesa et al. 2000). The presence of putative extracellular adherence proteins and mucus binding proteins further highlights the potential of this bacterium to interact with the host, and therefore induce an immune response (Boekhorst et al. 2006a; Boekhorst et al. 2006b; Pretzer et al. 2005). In a study by Smelt et al. (2012) *L. plantarum* WCFS1 was found to promote the generation of regulatory immune cells that could modulate undesired immune responses towards food antigens. In addition, *L. plantarum* also promoted the generation of cytotoxic T cells, that play a role in the defense and recognition of pathogen infected cells or cancerous cells. The latter, make *L. plantarum* a good prospective candidate for the delivery of cancer antigens.

1.2. The Mucosal Immune System

Mucous membranes consist of a layer of epithelial cells in tight conjunction and line various cavities in the human body such as the respiratory, digestive and genital tracts. These membranes serve as barriers between the body and the external environment and, as a consequence, immune cells are highly associated with these areas through mucosa-associated lymphoid tissue (MALT), since these membranes represent the main entry points for potential pathogens into the body. The tight connection with the immune system makes these areas good potential sites for vaccine delivery to elicit immune responses.

MALT may be subdivided according to the different mucosal areas it associates with. These include gut-associated lymphoid tissue (GALT), nose-associated lymphoid tissue (NALT), bronchial/tracheal-associated lymphoid tissue (BALT) and vulvovaginal-associated lymphoid tissue (VALT) (Montilla et al. 2004). The MALT is dispersed through the mucosal membranes and contains concentrated regions of immune cells such as T cells and B cells. In addition, MALT encompasses a sub-epithelial region with antigen-presenting cells (APCs) that may initiate specific immune responses (as described below) (McGhee & Fujihashi 2012). Together, the different types of MALT are responsible for inducing immune responses to foreign antigens, but also for inducing tolerance against antigens derived from non-pathogenic bacteria or food. Antigens are sampled continuously from the intestinal and nasal

lumen either through microfold cells (M cells) or directly by protruding dendrites of dendritic cells (DCs) (Fujimura et al. 2006; Wang et al. 2014a). Microfold cells (M cells) are specialized epithelial cells covered only by a thin layer of mucus. M cells are part of the follicle-associated epithelium (FAE) in MALT and of the Peyer's patches of GALT, and transport lumen-derived antigens over the epithelial barrier in a process called transcytosis (Mabbott et al. 2013). The translocated antigens are delivered to APCs such as DCs, B cells and macrophages that further initiate immune responses (Holmgren & Czerkinsky 2005).

APCs, such as dendritic cells, internalize and process the antigens which are then presented via major histocompatibility complexes (MHC) of class I or II (Siegrist 2013). The MHCs are located on the surface of APCs, and present antigens to naïve T cells either locally, or in the draining lymphnodes. Which class MHC that presents the peptide fragments is dependent on the sampling of the antigen. If the antigen is derived from the cytosol of a human cell (e.g. an infected cell), it is presented through MHC class I. If the antigen is derived from an exogenous source (e.g. from a bacteria of the intestinal lumen), however, it is presented through MHC class II. MHC class I is found on the surface of nearly every cell in the human body, and by displaying peptide fragments of antigens from the cytosol, enables cells to signal that an infection has occurred or that cellular processes are carried out incorrectly, such as in the case of cancer (Garrido et al. 2016).

The activation of T cells is dependent on the class of the MHC, and also upon the nature of the antigen. In order to elicit immune responses correctly, the immune system must be able to differentiate between normal microbiota and pathogens through a process called oral tolerance. This tolerance is mediated through the generation of antigen-specific T lymphocytes that suppress subsequent immune responses towards specific antigens (Kraal et al. 2006). A low antigen concentration is believed to be a main contributor to the development of oral tolerance, and in designing oral vaccines, this must be taken into account, since an oral tolerance for a specific pathogen is not desired. On the other hand, inducing oral tolerance has been considered a good strategy in various gut-associated autoimmune diseases such as irritable bowel disease (IBS) and Crohn's disease (Kraus et al. 2004).

Naïve CD4⁺ T cells are activated by antigens displayed via MHC class II on the APC surface. The activated CD4⁺ T cells may then differentiate into T helper cells (CD4⁺ Th1 or CD4⁺ Th2 cells), that will produce response molecules called cytokines (Siegrist 2013). The Th1 cells

produce the cytokines IFN- γ and TNF- α that mediate the elimination of intracellular pathogens and activate macrophages, in addition to promoting CD8⁺ T cell differentiation. In contrast, Th2 cells produce the interleukins IL-4, IL-5 and IL-13, which are involved in the defense against extracellular pathogens, as well as in stimulating B cell antibody production.

The generation of cytotoxic T cells is achieved through the activation of naïve CD8⁺ T cells (Siegrist 2013). These cells recognize the MHC class I molecules and are subsequently activated upon binding. As mentioned, MHC class I is found on the surface of nearly all cells in the human body, and cytotoxic T cells have the potential to destroy these cells if the antigen is displayed on the cell surface. The generation of cytotoxic T cells is therefore of great interest when designing cancer vaccines, as these have the potential to destroy cancer cells.

1.3. Cancer Vaccines and Immunotherapies

Cancer vaccines seek to promote tumor specific immune responses mainly through the generation of cytotoxic CD8⁺ T cells that target a specific tumor antigen (Wang et al. 2014b). The effectiveness of a cancer vaccine or immunotherapy is thus reliant on the identification of cancer antigens for the generation of antigen-specific T cells. Several tumor-associated antigens (TAAs) have been identified, and may be divided into antigens overexpressed in tumor cells, cancer-testis antigens, oncofetal antigens and mutated antigens (Butterfield 2015). The overexpressed antigens such as the melanoma lineage antigens tyrosinase, gp100 and MART-1/Melan-A, are antigens that are expressed in significant higher amounts in tumor cells in comparison to normal cells. Other TAAs are recognized on the basis of being expressed in cells that normally should not express them, examples are the oncofetal antigens and the cancer-testis antigens. Oncofetal antigens are normally only expressed during fetal development, but are re-expressed in some cancers (Wepsic 1983). The expression of antigens normally only expressed in testis cells is also seen in many tumor cells, these antigens are denoted as cancer-testis antigens and examples include the MAGE, GAGE and NY-ESO-1 antigens (Scanlan et al. 2002) TAAs belonging to the mutated antigens are specific tumor antigens resulting from mutation of genes such as the *RAS* oncogene and *P53* tumor repressor gene. These antigens are often highly specific both to patients and cells, and therefore vaccine

targeting these mutations have previously proved to be difficult, but in recent years patient-specific treatments have been investigated (Butterfield 2015).

Cancer vaccines and immunotherapies try to activate the generation of cytotoxic T cells that will kill cancer cells. The generation of such cells is largely dependent on the activation of the host immune system. The vaccine must be able to elicit an uptake by the antigen presenting cells (APC) such as dendritic cells, that will facilitate a further immune response. The APCs proteolyze the peptides into antigenic peptides and presents them through MHC class I. In humans the MHC is often referred to as human leukocyte antigen (HLA). T cells recognize HLA molecules through T cell receptors (TCRs), but in order to achieve optimal T cell activation, co-stimulatory factors such as B7 and CD28 must be present (Wang et al. 2014b). These are surface molecules that binds to the APC and T cell respectively, and in the absence of these molecules a tolerance of the presented antigen may be induced, thus yielding no cytotoxic T cells that could kill off tumor cells. The presence of other suppression factors such as CTLA-4 and PD-1 is also a limiting step in the generation of cytotoxic T cells, and drugs targeting the inhibition of these suppression factors have currently been approved by the FDA (Drake et al. 2014). In addition, the presence of other suppressor cells in the tumor microenvironment may further hamper the function of cytotoxic T cell. For a full review of evasion mechanisms see Drake et al. (2006).

Cancer vaccines may be administered as peptides, full proteins, DNA, *ex vivo* activated dendritic cells, killed tumor cells or tumor lysates that could elicit an immune response (Butterfield 2015). Due to the problems associated with an optimal activation, cancer vaccines often incorporate an adjuvant factor that will lead to an enhanced immune response such as immunizing with co-stimulatory factors that enhance the antigen uptake or suppress the generation of regulatory T cells (Drake et al. 2014; Khong & Overwijk 2016). As the dendritic cells are often the main contributor to the desired effect, several strategies attempts at enhancing the uptake of these cells, either by activating DCs *ex vivo* or by adding adjuvant factors. Curiel et al. (2004) found that the peptide FYPSYHSTQRP specifically binds to DCs, and that this could be used as an uptake-enhancing peptide when translationally fused to antigens. Although not a cancer antigen, a later study by Mohamadzadeh et al (2009) showed that mice immunized with *Lactobacillus acidophilus* carrying an antigen from *Bacillus anthracis* translationally fused to the DC-binding peptide were significantly more protected

against anthrax infection than mice immunized with bacteria harboring the antigen without the DC-binding peptide.

Strong immune responses that are able to elicit the generation of cytotoxic T cells may also be achieved by associating the cancer antigens with a foreign vector such as viral, bacterial, or yeast vectors. The vectors are able to elicit immune responses due to their inherent pathogenicity factors or ability to interact with the host (Heery et al. 2017).

1.4. Bacteria as Live Delivery Vectors of Antigens to Mucosal Sites

Traditional delivery vectors mimic mucosal pathogens to warrant the uptake of the antigen by the host either by adhering factors or other immune response inducing factors (Neutra & Kozlowski 2006). Live attenuated (reduced virulence) pathogens have successfully been used as delivery vectors of antigens to mucosal sites, and have the advantage of being able to induce a strong immune response due to pathogenicity factors recognized by the immune system (Toussaint et al. 2013). These pathogen-associated molecular patterns (PAMPs) include lipopolysaccharides (LPS), lipoproteins and flagellin, however, in addition to the possibility of reactivation of virulence, attenuated pathogens have been shown to pose a significant health risk, especially in immunocompromised patients where they have the potential to proliferate freely (Sartori 2004).

The possibility of using non-pathogenic bacteria has therefore also been explored. Bacteria with a GRAS status such as Lactic Acid Bacteria have been considered as a good candidate for delivery of both therapeutic molecules and antigens to mucosal sites (Bermúdez-Humarán et al. 2011; Medina & Guzmán 2001). A range of LAB have been utilized for the construction of recombinant strains expressing different antigens. The strains are genetically modified to produce heterologous proteins that are localized either in the cytosol or at the cell surface, or secreted into the environment (Daniel et al. 2011). In a study by Benbouziane et al. (2013) recombinant *Lactococcus lactis* expressing the human papillomavirus type-16 (HPV-16) E7 antigen was administered to mice orally and intranasally resulting in a tumor regression in comparison to the control mice, in addition to decreased mortality. Further investigations also indicated that the immunity was due to a generated cytotoxic T cell response (Benbouziane et al. 2013).

However, strict rules regarding the use of recombinant bacteria especially concerning the use of antibiotic resistance genes, limits their use as vaccines. Specifically, in heterologous protein production utilizing plasmids, an antibiotic resistance gene is normally used to maintain the plasmid within the bacteria. The creation of balanced lethal system is one approach to overcome the use of antibiotic resistance genes in plasmids (Toussaint et al. 2013). By deleting essential genes in the bacteria used in biosynthetic pathways such as genes essential for amino acid synthesis, and incorporating these genes in the expression plasmids, the plasmid will be kept by the bacteria. Nguyen et al. (2011) generated a food-grade system for *L. plantarum* by deleting the alanine racemase gene, *alr*, from the *L. plantarum* genome, and incorporating it in an expression vector based on the pSIP-system, which yielded a comparable expression level as compared to a plasmid containing an erythromycin resistance gene.

Bacteria may also be biologically contained by the complete elimination of essential genes. Steidler et al. (2003) used *L. lactis* where the thymidylate synthase gene *thyA* was replaced with a gene encoding interleukin-10, thus generating bacteria that was entirely reliant on the presence of thymidine in the environment. This strategy was employed by Braat et al. (2006) in a phase I trial with transgenic bacteria expressing interleukin-10 for the treatment of Crohn's disease, where it was shown that the transgenic bacteria were not able to survive outside the host.

Other approaches circumventing the use of antibiotics have also been developed. The previously mentioned human papillomavirus type-16 (HPV-16) E7 antigen was also used in another study where a non-GMO approach was used to immobilize the cancer antigen on the surface of *Lactobacillus casei* and *L.lactis*. Ribelles et al. (2013) utilized the cell-binding domain from the *L. casei* A2 phage lysin to immobilize the E7 on the bacterial surface. After immunization, 60 % of the vaccinated mice remained tumor free, while 100 % of the control mice developed tumors. The use of LAB for the delivery of antigens is thus a promising strategy both as producing heterologous proteins and carrier of antigens. The possibility of anchoring other cancer antigens have also been explored. The oncofetal antigen (OFA) has successfully been anchored to the *L.plantarum* cell wall through a LPxTG anchoring motif derived from the *L. plantarum* WCFS1 genome. Mice were immunized with the recombinant bacteria, and a subsequent immune response towards the antigen was detected (Fredriksen et

al. 2010). *L. plantarum* have also been utilized as a delivery vector of the cancer-testis antigen NY-ESO-1 with an observed immunogenic effect (Mobergslie et al. 2015).

1.5. Inducible Heterologous Gene Expression in Lactic Acid Bacteria

Expression systems have been developed to generate recombinant bacteria that are able to produce heterologous proteins that may be targeted to mucosal sites or used in the food industry (de Vos 1999; Peterbauer et al. 2011). Several of the expression systems take advantage of a cellular process called quorum sensing, where expression of specific proteins is induced by extracellular peptide pheromones secreted from other bacteria. As the cell density of the bacterial population increases, the pheromone concentration follows which lead to the activation of different cellular processes (Miller & Bassler 2001). The pheromones interact with a membrane-bound histidine kinase which is subsequently dephosphorylated. The phosphate-group is transferred to an intracellular response regulator that in turn binds to specific promoter segments which leads to explosive production of the cognate proteins/peptides.

One of the earliest examples utilizing the quorum sensing mechanism for the production of heterologous proteins in LAB, was the Nisin Controlled Expression (NICE) system developed by de Ruyter et al. (1996). The expression of a target protein is controlled by the bacteriocin nisin, and in a vector lacking the nisin encoding gene, the expression of a gene inserted after the nisin-activated promoter may be strictly controlled by the addition of nisin to the growth medium (Mierau & Kleerebezem 2005)

This principle has also been employed in the construction of the pSIP expression system (Sørvig et al. 2003; Sørvig et al. 2005). The expression vector (Fig 1.1) includes genes for the expression of histidine-kinase (SppK) and response regulator (SppR), which is under the control of an inducer peptide promoter, P_{sppIP} . However, the gene encoding for the inducer peptide SppIP is deleted and not present in the vector, thus, P_{sppIP} only regulate the transcription of the genes coding for the histidine-kinase and the response regulator. The target gene is translationally fused to the P_{sspA} , and after addition of the inducer peptide SppIP to the growth medium, the phosphorylated response regulator binds to segments of the P_{sspA} and P_{sppIP} , that induce an explosive production of target protein, histidine-kinase and response

regulator, respectively. All of the vector components are also easily exchangeable through digestion with restriction enzymes and subsequent ligation. The pSIP-system has been further modified for protein secretion by Mathiesen et al. (2008) and for anchoring of heterologous proteins to the bacterial cell surface by (Fredriksen et al. 2010; Fredriksen et al. 2012).

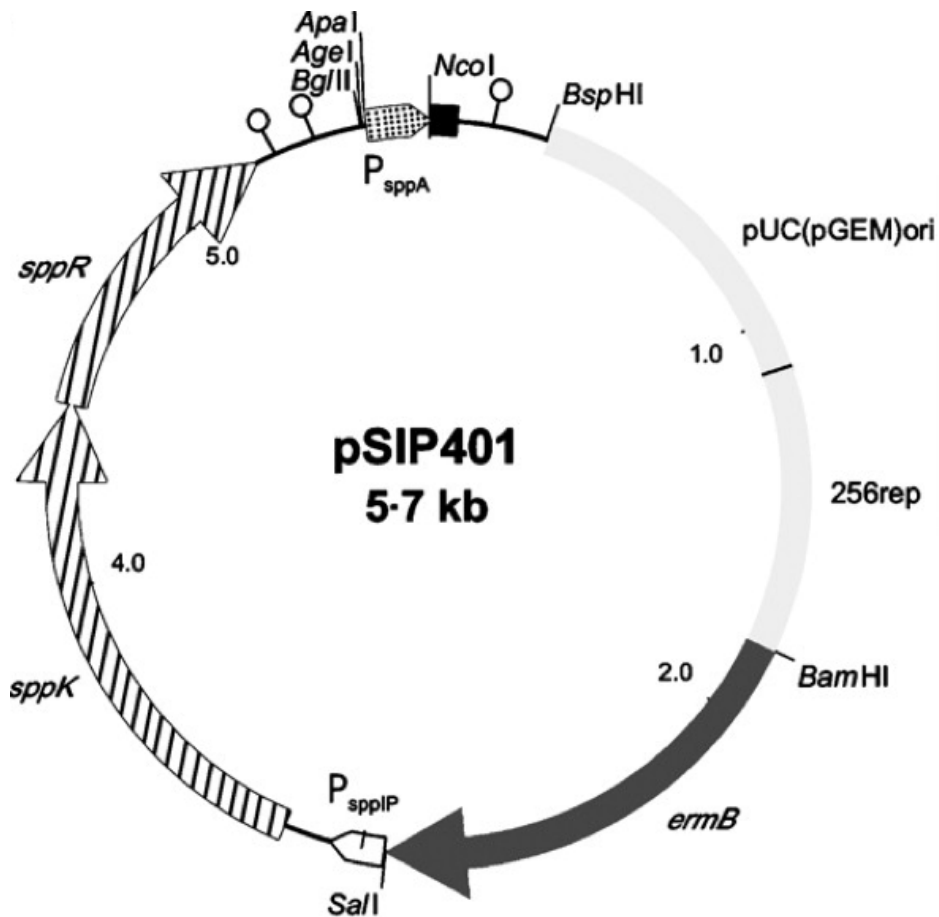


Figure 1.1 Schematic overview of the pSIP401 plasmid. Light grey areas, replicon pUC(pGEM)ori derived from *E. Coli* and replicon 256 from *L. plantarum*; Dark grey areas, erythromycin resistance gene; white box, P_{sppIP} promoter; Vertically hatched regions, histidine kinase and response regulator genes; dotted region, P_{sppA} promoter; black box, multiple cloning site; Lollipop structures, transcriptional terminators. Figure taken from Sørvig et al. (2005).

1.6. Secretion of Proteins in Gram-positive Bacteria

In order for proteins and peptides to be localized on the bacterial surface or secreted into the environment, the proteins must be translocated over the cell envelope. In Gram-positive bacteria, such as LAB, the cell envelope consists of only one membrane and a thick cell wall of peptidoglycan. Peptides destined for secretion, thus only have to cross one membrane, in contrast to two in Gram-negative bacteria. Seven main peptide and protein secretion mechanisms have been identified in Gram-positive bacteria: Secretion (Sec), twin-arginine translocation (Tat), flagella export apparatus (FEA), fimbriin-protein exporter (FPE), holin (pore-forming), peptide-efflux ABC and the WXG100 secretion system (Wss) (Desvaux et al. 2009). Only genes linked to the Sec, FEA, ABC-transporter and holin pathways were identified in lactobacilli in a study by Kleerebezem et al. (2010) on 13 lactobacilli genomes.

The holin (pore-forming) pathway of secretion allows for the translocation of proteins across the membrane without the need of added energy. The holins are membrane proteins originating from bacteriophages, and are often found to secrete bacterial toxins (Desvaux & Hebraud 2006). Of the active transport systems present in lactobacilli, the flagella export apparatus is mainly involved in the translocation of flagellar components through a transmembrane export gate driven by proton motive force, and a cytoplasmic ATPase complex (Minamino et al. 2014). ATP is also required for the transport of small molecules, such as bacteriocin peptides produced by the bacteria, via the peptide efflux ABC-transporter pathway (Kleerebezem et al. 2010).

The Sec pathway is conserved across archaea, bacteria and eukaryotes, and is the most widely used in Gram-positive bacteria for the translocation of proteins across the plasma membrane (Green & Mecsas 2016; Kleerebezem et al. 2010). Proteins are targeted to the Sec pathway through a N-terminal signal peptide consisting of three distinct parts; a N-domain with 1-3 positively charged amino acids, a hydrophobic domain of 10-15 amino acids, and finally a polar C-domain with a signal peptidase cleavage site (Driessen & Nouwen 2008). During translation, the N-terminal signal peptide is recognized and bound by a signal recognition particle (SRP) (Fig. 1.2). The FtsY protein is subsequently recruited to the SRP-peptide complex, and stabilizes the protein until it reaches the Sec translocase (SecYEG and SecA). SecA recognizes the polypeptide indirectly through and SecY hydrolyzes ATP consecutively

to drive the translocation of the protein across the membrane through the SecYEG channel (Schneewind & Missiakas 2012; Tjalsma et al. 2000)

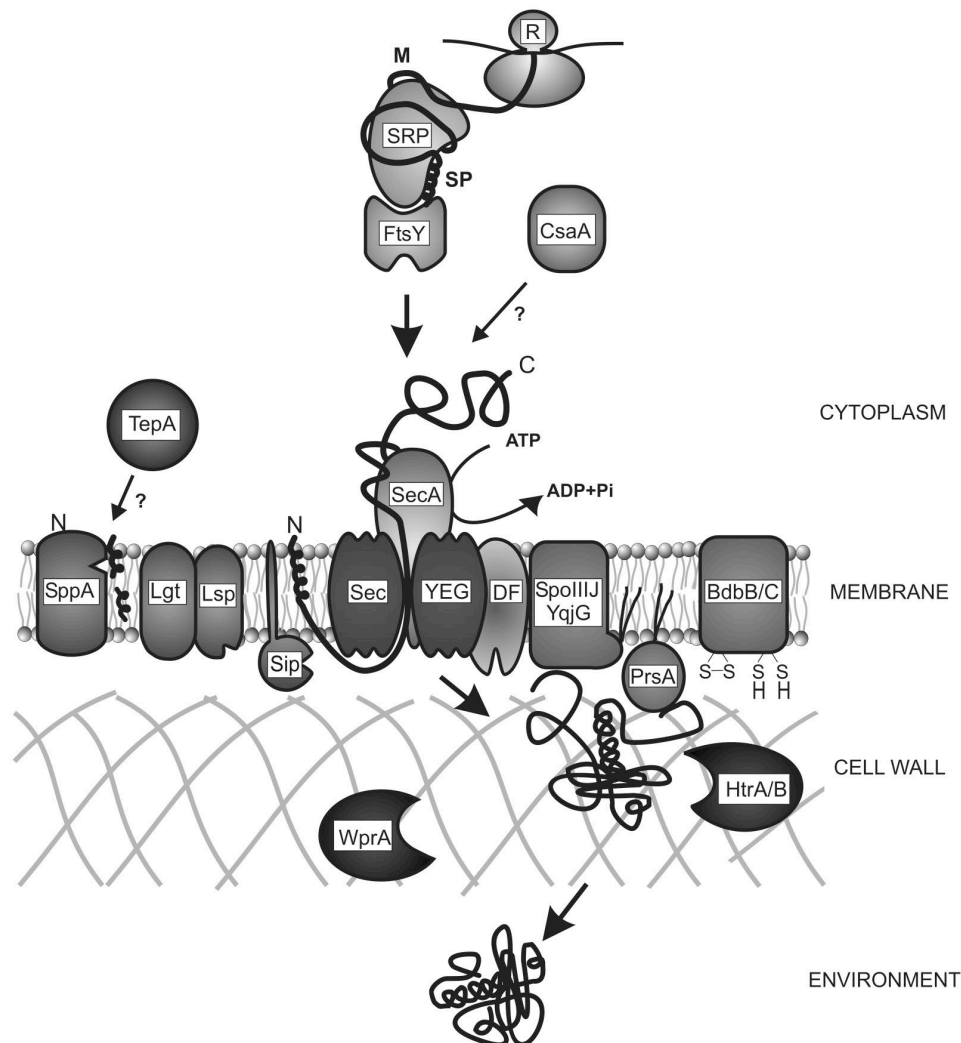


Figure 1.2 Schematic representation of the Sec-system and its components. Proteins destined for secretion are synthesized in the cytosol containing a N-terminal signal peptide. This motif is recognized by the signal recognition particle (SRP) which binds. FtsY binds the SRP and stabilizes the interaction during the transport to the Sec- translocase which mediates the translocation across the membrane. The Sec-translocase consists of a ATP-driven motor protein (SecA) and the protein channel SecYEG. During or immediately after the translocation, the signal peptide is cleaved off. For further details regarding the Sec-pathway see Tjalsma et al (2004) where this figure is taken from.

The protein structure and the presence or lack of cleavage sites determines the fate of the protein after translocation. Signal peptidases cleave the signal peptide and the protein during or straight after translocation if the protein sequence contain a signal peptidase cleavage site. Due to the hydrophobic nature of the N-terminal end of the signal peptide, the peptide is temporarily arrested in the cell membrane, thus keeping the cleavage site near to the signal

peptidase for cleavage (Tjalsma et al. 2000). If the cleavage site contains the peptide motif A-X-X-A, signal peptidase I cleaves, and the protein is subsequently secreted. However, the peptide motif L-X-X-C, is recognized by signal peptidase II, and after cleavage the protein is covalently attached to the lipid bilayer.

1.7. Anchoring of Proteins in Gram-positive Bacteria

Gram-positive bacteria employ several mechanisms to anchor proteins to the cell surface either in the membrane or the cell wall. It is therefore possible to exploit the anchoring process in the development of bacterial vectors for antigen delivery through translationally fusing antigens with anchoring motifs (Desvaux et al. 2006). Retention of proteins at the bacterial surface is achieved through either covalent interaction or non-covalent interaction between the protein and cell components (Boekhorst et al. 2006a). The anchoring mechanisms are usually classified as an example of either transmembrane anchoring, lipoprotein anchoring and LPXTG-anchoring or non-covalent binding to the cell wall.

Several recombinant bacteria with surface localize antigens have been constructed, as in the aforementioned study by Fredriksen et al. (2012) where the OFA antigen was anchored to the cell wall. The anchoring of proteins is not limited to antigens; enzymes have also been successfully anchored to the cell surface (Nguyen et al. 2016). Recently, *L. plantarum* was shown to be able to degrade cellulose via a cellulosome complex loaded onto a scaffolding protein. This protein was attached to the bacterial surface through a cell wall anchor (Morais et al. 2014). In order to achieve the desired effect, the choice of the anchoring mechanism must be carefully considered, as the nature of the anchor could affect the result. One aspect to consider is the degree of exposure to the extracellular environment, as enzymes must be able to reach its substrate, but bacteria used as oral delivery vectors could benefit from more embedded antigens to protect the protein from the harsh environment of the GIT. (Michon et al. 2016)

1.7.1. Transmembrane anchoring of Proteins

In the absence of a signal peptidase cleavage site or other recognition motifs, the 10-15 residue hydrophobic region of the signal peptide may insert itself into the membrane as a N-terminal transmembrane helix, thus resulting in the non-covalently anchoring of proteins within the membrane (Kleerebezem et al. 2010; Michon et al. 2016). The function of N-terminally anchored proteins is often related to transport and cell envelope metabolism (Kleerebezem et al. 2010). In heterologous protein production, the transmembrane PgsA protein from *Bacillus cereus* is often used to anchor proteins to the cell membrane (Michon et al. 2016) exemplified by the anchoring of a functional α -amylase on the surface of *L. casei* in a study by Narita et al. (2006).

1.7.2. Lipoprotein anchoring of Proteins

As mentioned, the presence of a L-X-X-C motif (lipobox) in the signal peptide leads to anchoring to the plasma membrane. After secretion through the Sec pathway, the enzyme diacylglycerol transferase couples the cysteine (C) of the lipobox motif to a phospholipid in the plasma membrane by the transfer of a diacylglycerol group to the SH-group of the cysteine (Desvaux et al. 2006). Signal peptidase II subsequently cleaves the signal peptide, N-terminally of the cysteine residue, and the protein is thus covalently anchored through its N-terminus to the cell membrane (Kleerebezem et al. 2010). Proteins coupled to the membrane through a lipoprotein anchor is involved in an array of processes such as adhesion, transport and receptors (Kleerebezem et al. 2010). The lipoproteins of *Lactobacillus* is also shown to interact with Toll-like receptor 2, and that this interactions leads to the generation of a host immune response (Sengupta et al. 2013).

1.7.3. LPXTG-anchoring of Proteins

Proteins may also be covalently attached to the peptidoglycan layer in the bacterial cell wall. This is achieved through the recognition of a C-terminal LPxTG motif by the enzyme sortase (SrtA) after secretion through the Sec pathway. SrtA cleaves the peptide bond between the threonine (T) and the glycine (G) in the LPxTG motif, and the protein is covalently attached to the cell wall through the threonine (Kleerebezem et al. 2010). This type of anchoring

mechanism has been found in nearly all Gram-positive bacteria (Kleerebezem et al. 2010). Therefore, this anchoring mechanism has been employed in studies with the aim of anchoring antigens to the cell wall. However, there are differences in sortase activity between different strains that may hamper the anchoring efficiency (Narita et al. 2006; Paterson & Mitchell 2004)

1.7.4. Non-covalent binding of Proteins to the Cell Wall

The non-covalent binding of proteins to the bacterial cell wall is mainly achieved through binding of different peptide domain motifs to peptidoglycan (Desvaux et al. 2006). One prevalent motif is the Lysin Motif (LysM) domain. This motif may be located both at the C- and N-terminal of proteins, and multiple copies may also be present in surface anchored proteins (Buist et al. 2008; Visweswaran et al. 2014). The ability of the LysM domain to bind non-covalently to the cell wall of bacteria makes it possible to circumvent the problem of utilizing antibiotic resistance for the production, as the recombinant protein may be expressed in and purified from an expression host. The purified protein may then be mixed with lactic acid bacteria, and bind non-covalently and strongly to the cell wall (Bosma et al. 2006; Michon et al. 2016).

1.8. Melanoma

Malignant melanoma is associated with the highest mortality rate of all skin cancers with a causative agent of melanoma development mainly being UV-radiation that damages the skin. (Bandarchi et al. 2010). Melanoma arises from cells called melanocytes that are mainly located in the bottom layer of the epidermis, in addition to in the eye and the inner ear. Melanocytes produce the pigment melanin which is synthesized, stored and transported in specialized organelles called melanosomes. Melanosomes undergo four distinct maturation stages before they are ready to transport the melanin to the keratinocytes which forms the barrier against UV-radiation and other environmental damage (Hoashi et al. 2005).

The transition from regular melanocytes to metastatic melanoma (Fig. 1.3), starts with the generation of a benign nevus of melanocytes. An abnormal growth of the melanocytes ensues, resulting in a pre-malignant lesion characterized by irregular borders and inconsistent

pigmentation. The melanocytes gain the ability to proliferate horizontally in the skin resulting in melanoma *in situ*. Some of the malignant cells could then proliferate vertically in the dermis and may spread to other parts of the body resulting in a metastatic melanoma (Arrangoiz et al. 2016).

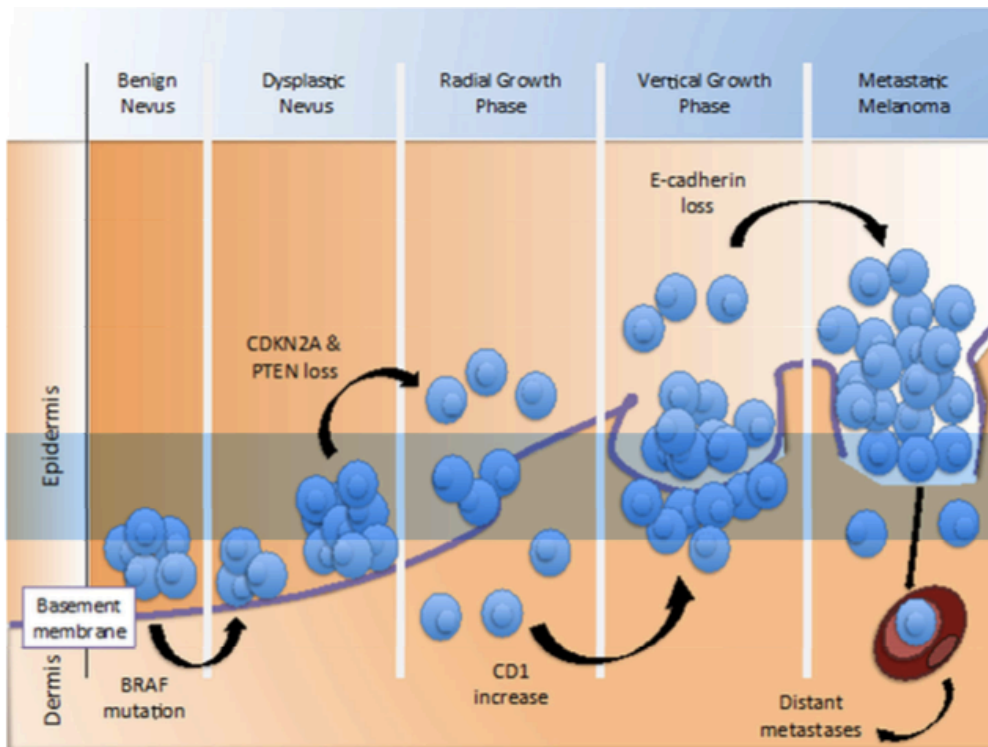


Figure 1.3 Transition from regular melanocytes to metastatic melanoma. A benign nevus starts to grow abnormally resulting in a pre-malignant melanoma. Acquiring the ability of horizontal proliferation results in a melanoma *in situ*. The acquisition of the ability to proliferate vertically is associated with the development of metastatic melanoma. The figure is taken from Arrangoiz et al. (2016).

The presence of an already existing immune response against melanoma tumor cells were discovered early on. This response consists largely of T cell responses that recognize the antigens on the cell surface and destroys the cells. In melanoma, several of these antigens is related to the melanocytic differentiation process such as tyrosinase, gp100 and MART-1. These antigens are also expressed in normal melanocytes, but are overexpressed in melanoma. Several strategies to enhance the immune response against cancer cells have targeted these antigens, and still remain an intriguing approach in treating cancer (Hodi 2006).

1.8.1. Melanoma Antigen used in this Study

The MART-1/Melan-A antigen was described by two independent research groups in 1994 after identification of a melanoma cytolytic T lymphocyte and a tumor-infiltrating lymphocyte, respectively (Coulie et al. 1994; Kawakami et al. 1994a). The antigen recognized by the lymphocyte cells was mapped to a gene corresponding to a putative 118 amino acid protein with a transmembrane region. The antigen epitope was found to correspond to the residues 27-35 (AAGIGILTV) and 26-35 (EAAGIGILTV) which both are part of the hydrophobic region of the protein (Fig. 1.4) Due to the presence of preexisting cytotoxic T cells that could destroy potential melanoma cells, the MART-1 protein has been utilized in numerous therapeutic and vaccination approaches after its initial characterization (Chodon et al. 2014; Lienard et al. 2009; Ribas et al. 2011; Valmori et al. 2000; Wang et al. 1999).

Recently, in a phase I/phase IIa clinical trial, it was found that by immunizing melanoma patients with the MART-1 peptide together Montanide ISA-51 (Incomplete Freund's Adjuvant) and LAG-3Ig (IMP321 a non-Toll like Receptor agonist showed to have adjuvant properties) yielded an antigen specific CD8⁺ and CD4⁺ response (Legat et al. 2016).

Kawakami et al. (1994b) showed that MART-1 was more readily recognized by cytolytic T cells in comparison to the other melanoma-associated antigens gp100, tyrosinase, and tyrosinase-related protein-1 (gp75). In a subsequent study the immunogenicity and ability of the MART-1 epitope to generate cytotoxic T cell was enhanced by an amino acid substitution A27L in the epitope (Valmori et al. 1998). The enhanced immunogenicity of the A27L substitution was also investigated by Abdel-Wahab et al. (2003) who observed a higher cytotoxic T cell response with dendritic cells transfected with mRNA of full-length MART-1 with the A27L substitution in comparison to native MART-1 mRNA.

The A27L substitution has thus also been incorporated into the gene used in the current study (Fig. 1.4). The native epitope was added at the C-terminal of the gene with the A27L substitution. In addition, the dendritic cell binding motif (FYPSYHSTQRP) described by (Curiel et al. 2004) was also fused C-terminally to the open reading frame to target the antigen to dendritic cells.

MART-1/Melan-A

MPREDAHF IYGYPKKGHGHSYTTAE**EAAGIGILTVILGVLL**LIGCWYCRRRNGYRALM
DKSLHVGTCALTRRC**PQEGFDHR**DSKVSLQEKNCEPVVPNAPPAYEKLSAEQSPPPY
SP

modMART1 DC

MPREDAHF IYGYPKKGHGHSYTTAE**EAAGIGILTVILGVLL**LIGCWYCRRRMGYRALM
DKSLHVGTCALTRRC**PQEGFDHR**DSKVSLQEKNCEPVVPNAPPAYEKLSAEQSPPPY
SPYTTAE**ELAGIGILTVILGVLFYPSYHSTPQRP**

Figure 1.4 Native MART-1/Melan-A and modMART1 protein sequence. Epitope region denoted in blue and A27L substitution marked in red. Putative transmembrane region is underlined, while the dendritic cell binding peptide is denoted in orange.

1.9. Aim of this Study

This study is part of a larger project with the aim of utilizing lactic acid bacteria as delivery vectors of antigens to mucosal sites. The construction of recombinant *L. plantarum* was based on the use of the pSIP-system developed for the inducible production of heterologous proteins by lactic acid bacteria (Sørvig et al. 2003; Sørvig et al. 2005). The aim of this study was (1) to successfully express the modMART1_DC fusion protein in *L. plantarum* WCFS1, (2) characterize the different constructs in regards to growth, surface localization of the antigen and secretion efficiency, and (3) identify the most promising candidate(s) for further immunological analyses which may lead to a cancer vaccine.

The work was carried out as follows. First, six constructs with different anchoring or secretion mechanisms were constructed and characterized according to their growth ability. Western blot analysis was used to verify the modMART1_DC production, and subsequent flow cytometry and immunofluorescent microscopy was used to confirm the successful surface localization. In addition, the bacteria harboring plasmids for secretion of modMART1_DC were investigated for their secretion ability. Furthermore, purified modMART1_DC protein was produced from an *E. coli* BL21 expression host to yield protein for a semi-quantitative Western blot that would give an estimate of protein produced by each strain as it is vital to know how much antigen is administered in a putative vaccine trial.

2. Materials

2.1. Laboratory Equipment

Table 2.1. Laboratory Equipment.

Laboratory equipment	Manufacturer
Automated pipettes	Thermo Scientific
Disposable cuvette, 1,5 mL	Brand
Centrifuge tubes, CELLSTAR®, 15 mL and 50 mL	Greiner Bio-One
Centrifuge tubes, Nalgene® Oak Ridge Style 3119	Sigma-Aldrich
Centrifuge tubes, Nalgene® Wide-Mouth with Sealing Caps, Style 3141, 500 mL	Sigma-Aldrich
Eppendorf tubes, 1.5 mL and 2 mL	Axygen
epT.I.P.S.®	Eppendorf
FastPrep® tubes and lids	Fisher Scientific
Gene Pulser® electroporation cuvette, 0.2 cm	Bio-Rad
Glass beads	Sigma-Aldrich
Glass equipment	
Mini-PROTEAN® TGX Stain-Free™	Bio-Rad
Precast Gels	
PCR tubes, 0.2 mL	Axygen
Petri dishes, 9 cm	Heger
Pipetboy comfort	Integra
Protein LoBind Tubes, 2.0 mL	Eppendorf
Scalpel, Stainless steel surgical blade 10	Swann-Morton
Serological pipette 10 mL	Sarstedt

Sparger, GL45 (1 L)	Epiphyte Three
Sterile filter, Filtropur 0.2 µm pore size	Sarstedt
Strata C18-E (55µm, 70A) Column, 1 mL	Phenomenex
Syringes, 10-50 mL	Plastipak
Toothpicks	Playbox AB
Tube, 13 mL, PP	Sarstedt
Vivaspin® 20, 10.000 MWCO PES	Sartorius

2.2. Instruments

Table 2.2: Instruments.

Instrument	Manufacturer
Azure c400	Azure Biosystems
Centrifuges	
Allegra X-30R Centrifuge	Beckman-Coulter
Avanti™ J-25	Beckman-Coulter
Eppendorf 5418R	Eppendorf
Microcentrifuge MiniStar Silverline	VWR
Vacuum Centrifuge	Maxi Dry Lyo, heto
CertoClav	OneMed
Electrophoresis electricity supplier	Bio-Rad
FastPrep® - 24 Tissue and Cell Homogenizer	MP Biomedicals
GelDoc EZ Imager	Bio-Rad
Gene Pulser II	Bio-Rad
Incubators	
New Brunswick™ Innova ® 44	Eppendorf
Incubator	Termaks
LEX-48 Bioreactor	Harbinger Biotech

MacsQuant[®] Analyzer

Miltenyi Biotec

PCR machines

Mastercycler gradient

Eppendorf

SimpliAmp Thermal cycler

Applied Biosystems

pH-meter

Metrohm

Qubit[®] Fluorometer

Invitrogen

SNAP i.d. Protein Detection System

Millipore

Ultrospec 10 Cell Density Meter

Biochrom

Vibra-cell[™] Ultrasonic Liquid Processor

Sonics

Vortex, MS2 Minishaker

IKA

Zeiss LSM 700 Laser Scanning Confocal
Microscope

Zeiss

Äkta Pure

GE Healthcare Life Sciences

2.3. Software

Table 2.3: Software.

Software	Supplier
CLC DNA Main Workbench 7	Qiagen
MacsQuantify [™] Software	Miltenyi Biotec
pDRAW32	www.acaclone.com
Scaffold 4	Proteome Software Inc.

2.4. Chemicals

Table 2.4: Chemicals

Chemical	Supplier
Acetone, C ₃ H ₆ O	Merck
Acetonitrile, C ₂ H ₃ N	Sigma-Aldrich
Ammonium Citrate Tribasic, C ₆ H ₁₇ N ₃ O ₇	VWR
Ampicillin, C ₁₆ H ₁₉ N ₃ O ₄ S	Sigma-Aldrich
Antifoam 204	Sigma-Aldrich
Bacto™ Tryptone Pancreatic Digest of Casein	Becton, Dickinson and Co
Bacto™ Yeast Extract	Becton, Dickinson and Co
Brain-Heart Infusion (BHI)	Oxoid
De Man, Rogosa, Sharpe (MRS)	Oxoid
Dithiothreitol (DTT), C ₄ H ₁₀ O ₂ S ₂	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA), C ₁₀ H ₁₆ N ₂ O ₈	Merck
Erythromycin, C ₃₇ H ₆₇ NO ₁₃	Merck
Ethanol, C ₂ H ₅ OH	Sigma-Aldrich
D-(+)- Glucose, C ₆ H ₁₂ O ₆	VWR
Glycerol, C ₃ H ₈ O ₃	Merck
Glycine, C ₂ H ₅ NO ₂	Duchefa Biochemie
Imidazole	Sigma-Aldrich
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich
Kanamycin, C ₁₈ H ₃₆ N ₄ O ₁₁	Sigma-Aldrich
Magnesium Chloride, MgCl ₂	Merck
Magnesium Phosphate, MgSO ₄	Sigma
Manganese(II) sulfate, MnSO ₄ ·H ₂ O	VWR
MES hydrate, C ₆ H ₁₃ NO ₄ S · xH ₂ O	Sigma-Aldrich
Methanol LC-MS gradel, CH ₃ OH	Sigma-Aldrich

NuPAGE® LDS Sample Buffer (4X)	Invitrogen
NuPAGE® Sample Reducing Agent (10X)	Invitrogen
PeqGreen	Peqlab
Polyethylene glycol, PEG ₁₄₅₀	Aldrich
Potassium Phosphate Dibasic, K ₂ HPO ₄	Sigma-Aldrich
Potassium Phosphate Monobasic, KH ₂ PO ₄	Sigma-Aldrich
SeaKem® LE Agarose	Lonza
Sodium Acetate, C ₂ H ₃ NaO ₂	Sigma-Aldrich
Sodium Chloride, NaCl	Merck
Sodium hydroxide, NaOH	Sigma
Sucrose, C ₁₂ H ₂₂ O ₁₁	VWR Chemicals
Super Optimal broth with Catabolite repression (S. O. C.)	Invitrogen
Trichloroacetic acid (TCA), C ₂ HCl ₃ O ₂	Sigma
Trifluoroacetic acid, C ₂ HF ₃ O ₂	Sigma-Aldrich
Tris-base, C ₄ H ₁₁ NO ₃	Sigma
Tris-Glycine-SDS (TGS) 10X	Bio-Rad
Tween-20	Sigma-Aldrich

2.5. Proteins and enzymes

Table 2.5: Proteins and enzymes. List of proteins and enzymes used in this study.

Protein/Enzyme	Supplier
Antibodies	
Polyclonal Rabbit Anti-Mouse Immunoglobulin HRP FITC	Agilent
Melan-A(A103): sc-200032	Santa Cruz Biotechnology Inc.
The BenchMark™ Protein Ladder	Invitrogen
Bovine Serum Albumin (BSA)	Sigma
FastDigest® Green Buffer	Thermo Scientific
FastDigest® Restriction enzymes	Thermo Scientific
EcoRI	
Sall	
HindIII	
MLuI	
BamHI	
Inducer peptide SppIP	CASLO
Lysozyme	Sigma
MagicMark™ XP Western Protein Standard	Invitrogen
RED Taq DNA Polymerase Master Mix	VWR
Trypsin/Lys-C Mix, Mass Spec Grade	Promega
T4 DNA ligase	NEB
T4 DNA ligase Buffer	NEB
Quick T4 DNA ligase	NEB
Quick T4 DNA ligase Reaction Buffer	NEB

2.6. DNA and Nucleoside triphosphates

Table 2.6: DNA. List of DNA and nucleoside triphosphates used in this study.

DNA	Supplier
dGTP-mix, 10 mM	New England Biolabs
DNA-standard	
GeneRuler™ 1kb DNA Ladder	Fermentas

2.7. Primers

The sequence of the primers used in this study is listed in Table 2.7. Table 2.8 gives an overview of the purpose of each primer.

Table 2.7: Primers. Name of primers and their sequences used in this study

Name	Sequence	Restriction site*
MartDC_F1	CGCAACGCCCGCCACGGGAAGAC GCAC	
MartDC_F2	TCAACCCCGCAACGCCCGCCA	
MartDC_F3	ACCCAAGTTATCACTCAACCCCG CAACGC	
MartDC_F4	GTCGACTTCTACCCAAGTTATCAC TCAAC	
MartDC_F5	GGCCTCCAAGGTCGACTTCTACCC AAGT	SalI
MartMLuR	G TTCAGTGACACGCGTTAAGACT CCCAGGATCAC	MluI
InFusion1452_F	ATTGCGGCGGTCGACCCACGGGA AGACGCACAT	SalI
InFusion1452_R	CGGGGTACCGAATTCTTACGGGC GTTGCGGGGT	EcoRI
InFusion1261_F	GATTGCGGCGGTCGACCCACGGG AAGACGC	SalI

InFusion1261_R	CTGTAATTTGA <u>AAGCTTTT</u> ACGGGC	HindIII
	GTTGC	
pNIC_LIC_F	TTAAGAAGGAGATATACTATGCC	
	ACGGGAAGACGCA	
pNIC_LIC_R	AATGGTGGT <u>GATGATGGT</u> GCGCC	
	GGGCGTTGCGGGGTT	
SekF	GGCTTTTATAATATGAGATAATGC	
	CGAC	
SekR	CCTTATGGGATTTATCTTCCTTAT	
	TCTC	

*Restriction sites are underlined.

Table 2.8: Description of primers used in this study.

Name	Description
MartDC_F1	First of five forward primers used to move the dendritic cell binding motif from the C-terminal of the modMART1-sequence N-terminally.
MartDC_F2	The second forward primer used to move the dendritic cell binding motif from C-terminal of the modMART1-sequence N-terminally.
MartDC_F3	The third forward primer used to move the dendritic cell binding motif from C-terminal of the modMART1-sequence N-terminally.
MartDC_F4	The fourth forward primer used to move the dendritic cell binding motif from C-terminal of the modMART1-sequence N-terminally.
MartDC_F5	The fifth forward primer used to move the dendritic cell binding motif from C-terminal of the modMART1-sequence N-terminally.
MartMLuR	Reverse primer used in all the reactions with the above mentioned forward primers (MartDC_F1 – MartDC_F5).

InFusion1452_F	Forward primer used to amplify modMART1_DC with a tail complementary to Lp_1452
InFusion1452_R	Reverse primer used to amplify modMART1_DC with a tail complementary to Lp_1452
InFusion1261_F	Forward primer used to amplify modMART1_DC with a tail complementary to Lp_1261.
InFusion1261_R	Reverse primer used to amplify modMART1_DC with a tail complementary to Lp_1261.
pNIC_LIC_F	Forward primer used to generate a gene fragment containing the target gene modMART1_DC suitable for LIC-cloning.
pNIC_LIC_R	Reverse primer used to generate a gene fragment containing the target gene modMART1_DC suitable for LIC-cloning.
SekF	Forward primer for sequencing of target gene in plasmids.
SekR	Reverse primer for sequencing of target gene in plasmids.

2.8. Bacterial strains and plasmids

The three different bacterial strains used in this study is listed in Table 2.9.

Table 2.9 Bacterial strains used in this study.

Strain	Source
<i>Escherichia coli</i> TOP10	Invitrogen
<i>Escherichia coli</i> BL21	NEB
<i>Lactobacillus plantarum</i> WCFS1	Kleerebezem et al. (2003)

Table 2.10 gives an overview of the plasmids used and constructed in this study.

Table 2.10: Plasmids used and constructed in this study.

Plasmid	Description	Source
pUC57_modMART1_DC	Vector containing the synthetic gene modMART1 fused with a dendritic cell binding peptide (DC-pep). The gene is codon optimized for <i>L. plantarum</i> .	GeneScript
pLp_1261-Ag85B-ESAT6	A pSIP401 derivate for membrane-anchoring of the antigen, where a lipobox motif from the protein Lp_1261 is fused to AG85B-ESAT6.	R. Tjåland (2011)
pLp_1452-Invasin	A pSIP401-derivate for the expression of Invasin with a Lp1452 lipoprotein anchor.	L. Fredriksen
pLp_3014-Ag85_ESAT6-DC	A pSIP401-derivate for the expression of Ag85E6 with a LysM domain.	N. Målbakken (2014)
pLp_0373-Ag85E6-cwa2	A pSIP401-derivate for the expression of Ag85E6 with a Lp0373 signal sequence and a cell wall anchor (cwa2).	R. Tjåland (2011)

pLp_3050-DC-Ag-E6-cwa2	A pSIP401-derivate for the expression of DC_AgE6_cwa2 with a Lp3050 signal sequence and a cell wall anchor.	L. Øverland (2013)
pNIC-CH	A cloning vector used for protein production in <i>E. coli</i> . Contains a C-terminal 6xHistidine-tag which allows for purification. Cloned gene is under an IPTG-inducible promotor. Vector also harbors a Kanamycin resistance gene and a SacB gene which allows for negative selection on 5% sucrose.	Opher Gileadi
pEV	Vector lacking an expressed target gene. Used for negative control.	L. Fredriksen (2010)
pLp_1261_modMART1_DC	A pLp_1261-Ag85B-ESAT6 for membrane anchoring of modMART1_DC.	This study
pLp_3050_DC_modMART1_cwa2	A pLp_3050-DC-Ag-E6-cwa2 derivate for cell wall anchoring of DC_modMART1	This study
pLp_3050_modMART1_DC	A pLp_3050-DC-Ag-E6-cwa2 derivate for secretion of modMART1_DC. The cell wall anchor (cwa2) is removed through restriction digestion, and the antigen is only fused to the Lp3050 signal sequence.	This study
pLp_1452_modMART1_DC	A pLp_1452-Invasin derivate for mebrane anchoring of modMART1_DC through the lipobox motif derived from the Lp1452 protein.	This study
pLp_3014_modMART1_DC	A pLp_3014-Ag85_ESAT6-DC derivate for cell wall anchoring of modMART1_DC through a LysM Domain.	This study

pLp_0373_modMART1_DC	A pLp_0373-Ag85E6-cwa2 derivate for the secretion of modMART1_DC. The cell wall anchor (cwa2) is removed through restriction digestion, and the antigen is only fused to the Lp0373 signal sequence.	This study
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2.9. Kits

GenElute™ HP Plasmid Midiprep Kit

Sigma-Aldrich

GenElute™ HP Midiprep Filter Syringes

GenElute™ HP Midiprep Binding Columns

Collection tubes, 15 mL conical

Column Preparation solution

RNase A Solution

Resuspension solution

Lysis Solution

Neutralization Solution

Binding Solution

Wash Solution 1

Wash Solution 2

Elution Buffer (10 mM Tris-HCL, pH 8,5)

iBlot® Dry Blotting System

Invitrogen

iBlot® Gel Transfer Device

Blotting roller

iBlot® Gel Transfer Stack, Regular

iBlot® Cathode stack, Top

iBlot® Anode stack, bottom

iBlot® Disposable sponge

iBlot® Filter Paper

In-Fusion [®] HD Cloning kit	Clontech
5X In-Fusion [®] HD Enzyme Premix	
Novex [®] NuPAGE [®] SDS-PAGE Gel system	Invitrogen
NuPAGE [®] Novex Bis-Tris gels 8 cm x 8 cm x 1 mm, 10 and 15 wells	
NuPAGE [®] LDS Sample Buffer (4X)	
NuPAGE [®] Reducing agent (10X)	
The NucleoSpin [®] Gel and PCR Clean-up	Macherey-Nagel
The NucleoSpin [®] Gel and PCR Clean-up columns	
Collections Tubes, 2 mL	
Binding Buffer NTI	
Wash Buffer NT3	
Elution Buffer NE	
NucleoSpin [®] Plasmid	Macherey-Nagel
Buffer A1	
Buffer A2	
Buffer A3	
Buffer A4	
Elution Buffer AE	
NucleoSpin [®] Plasmid/Plasmid (NoLid) column	
Collection Tubes, 2 mL	
Qubit [®] dsDNA BR Assay Kit	Invitrogen
Qubit [®] Assay Tubes	
Qubit [®] dsDNA BR buffer	
Qubit [®] dsDNA BR reagent	
Qubit [®] dsDNA BR standard 1 and 2	
Quick Ligation [™] kit	NEB
Quick T4 DNA Ligase	

2X Quick Ligation Reaction Buffer

SNAP i.d.[®] Protein Detection System

Millipore

SNAP i.d.[®] Single Well Blot Holder

SNAP i.d.[®] Spacer

SNAP i.d.[®] Blot roller

Filter paper

SuperSignal[®] West Pico Chemiluminescent Substrate

Thermo Scientific

Luminol/Enhancer

Stable Peroxide Buffer

2.10. Agar and media

All components and suppliers are listed in Table 2.4.

Brain-Heart-Infusion (BHI)

Medium:

37 g powdered BHI dissolved in 1 L dH₂O. Sterilized in a CertoClav at 115 °C for 15 minutes.

Agar:

BHI broth supplemented with 1,5 % (w/v) agar.

After sterilization in a CertoClav, the medium was allowed to cool down to ~60 °C, before the addition of the appropriate antibiotic. The medium was poured into petri dishes, and allowed to solidify, before storage at 4°C.

De Man, Rogosa, Sharpe (MRS)

Medium:

52 g powdered MRS broth in 1 L dH₂O. Sterilized in a CertoClav at 115 °C for 15 minutes.

Agar:

MRS broth supplemented with 1,5 % (w/v) agar.

After sterilization in a CertoClav, the medium was allowed to cool down to ~60 °C, before the addition of appropriate antibiotic. The medium was poured into petri dishes, and allowed to solidify, before storing them at 4°C

MRS medium without Tween

5 g Bacto™ Tryptone Pancreatic Digest of Casein

5 g Meat extract

10 g Glucose

1 g Potassium Phosphate Dibasic

2.5 g Sodium Acetate

1 g Ammonium Citrate Tribasic

0.1 g Magnesium Sulfate

0.025 g Manganese (II) Phosphate

in 500 mL dH₂O. pH adjusted to 6,25.

Autoclaved in a CertoClav at 115 °C for 15 minutes.

MRSSM medium

5,2 g MRS

17.1 g Sucrose (0,5 M)

2.0 g MgCl₂ (0,1 M)

in 100 mL dH₂O

Sterile filtrated using a 0,2 µm pore size filter.

Lysogenic Broth (LB)

Medium:

10 g Bacto™ Tryptone Pancreatic Digest of Casein

5 g Bacto™ Yeast Extract

10 g Sodium Chloride

in 1 L dH₂O. pH adjusted to 7.

Autoclaved in a Certoclav at 115 °C for 15 minutes

Agar:

LB supplemented with 1,5 % (w/v) agar.

After sterilization in a Certoclav, the medium was allowed to cool down to ~60 °C, before the addition of appropriate antibiotic. The medium was poured into petri dishes, and allowed to solidify, before storing them at 4°C

Super Optimal broth with Catabolite repression (S. O. C.)

Pre-made by the manufacturer of competent cells.

Terrific Broth (TB) for protein production in *E. coli* BL21

6 g Bacto™ Tryptone Pancreatic Digest of Casein

12 g Bacto™ Yeast Extract

2 mL Glycerol (85%)

in 450 mL dH₂O. A 1 L GL45 Sparger was attached to the flask, before autoclaving. The solution was allowed to cool down to ~60 °C, before the addition of 50 mL Phosphate solution (Section 2.11) and 150 μL Antifoam 204.

2.11. Buffers and solutions

All components and suppliers are listed in Table 2.4.

MES Buffer A

50 mM MES

MES Buffer B

50 mM MES

1 M NaCl

NiNTA Buffer A

5 mM Imidazole

50 mM Tris Buffer, pH 8

500 mM Sodium Chloride

NiNTA Buffer B

250 mM Imidazole

50 mM Tris Buffer, pH 8

250 mM Sodium Chloride

Phosphate Buffered Saline (PBS)

8 g/l NaCl

0.2 g/l KCl

1.44 g/l Na₂HPO₄

0.24 g/l KH₂PO₄

Phosphate Solution

23.14 g KH₂PO₄

125.4 g K₂HPO₄

dissolved in 1L dH₂O, before autoclaving.

Protein Storage Buffer

50 mM Tris-HCL, pH 8

200 mM Sodium Chloride

Tris Buffered Saline (TBS)

150 mM NaCl

10 mM Tris-HCl, pH 8

TTBS

TBS

0.1 % (v/v) Tween-20

Tris-acetate/EDTA (TAE) 50X

242 g Tris base

57.1 mL Acetic acid

100 mL 0,5 M EDTA, pH 8

dH₂O to 1 L

Tris-Glycine-SDS (TGS) 10X

From manufacturer

3. Methods

3.1. Bacterial Cultivation

Bacteria harboring different plasmids were either grown in liquid medium or on agar plates. The plasmids used in this study contain different antibiotic resistance genes which allowed for selective growth. The appropriate antibiotic and the concentration used for cultivation of bacteria carrying different plasmids are given in Table 3.1.

Overnight cultures of *E. coli* containing the pUC57- and pSIP401-derivates were cultivated in liquid BHI medium at 37 °C overnight, while subjected to shaking to ensure aeration. Bacteria carrying these plasmids were also grown on BHI agar plates at 37 °C.

E. coli carrying the pNIC-CH plasmid were cultivated in liquid TB medium, and on LB agar plates containing 5% sucrose to ensure selection.

Lactobacillus plantarum was cultivated at 30 or 37°C in either liquid MRS media or on MRS agar plates. Due to its facultative anaerobe nature, *L. plantarum* was not subjected to shaking during liquid cultivation.

Table 3.1: Antibiotics and concentrations used for the cultivation of bacteria harboring different plasmids.

Species	Plasmid	Antibiotic	Antibiotic concentration in liquid medium ($\mu\text{g}/\text{mL}$)	Antibiotic concentration on agar plates ($\mu\text{g}/\text{mL}$)
<i>E. coli</i>	pUC57-derivate	Ampicillin	200	100
<i>E. coli</i>	pSIP401-derivate	Erythromycin	200	200
<i>E. coli</i>	pNIC-CH-derivate	Kanamycin	50	50
<i>L. plantarum</i> WCFS1	pSIP401-derivate	Erythromycin	10	10

3.2. Long-term storage of bacteria

For long-term storage of bacteria, 300 μL of 85% glycerol was added to 1 mL of a bacterial culture cultivated overnight. The solution was stored at -80°C . The glycerol protects the bacterial cells from damage to the cell membrane, and thus allows for long term storage. These glycerol stocks were used to cultivate the bacteria by scraping the frozen culture with a sterile toothpick, which was subsequently added to a liquid growth medium containing the appropriate antibiotic.

3.3. Plasmid isolation from *Escherichia coli*

The NucleoSpin® Plasmid kit (Section 2.9) from Macherey-Nagel was used to isolate plasmids from *E. coli*. To achieve higher yields of plasmid, the GenElute™ HP Plasmid Midiprep Kit was used.

3.3.1. Isolation of plasmid with the NucleoSpin® Plasmid Kit.

The procedure was performed according to the protocol provided by the manufacturer.

Materials:

NucleoSpin® Plasmid kit (Section 2.9)

Procedure:

1. 2-3 mL of an overnight culture was centrifuged at 11,000 x g for 30 seconds, and the supernatant was discarded.
2. The pellet was resuspended in 250 μL Buffer A1.
3. 250 μL Buffer A2 was added, and the suspension was mixed gently to avoid shearing of DNA, and incubated at room temperature for 5 minutes.
4. 300 μL of Buffer A3 was added, and the Eppendorf tube was inverted 6-8 times to ensure proper mixing.
5. The tube was centrifuged at 11,000 x g for 5 minutes.

6. A NucleoSpin® Plasmid/Plasmid (NoLid) column was placed into a 2 mL Collection tube, and a maximum of 750 μL of the sample was transferred to the column.
7. The column was centrifuged at 11,000 x g for 1 minute, and the flow-through was discarded.
8. Step 7 was repeated with the remainder of the sample.
9. 600 μL of Buffer A4 was added, and the column was centrifuged for 1 minute at 11,000 x g. The flow-through was discarded, and the column placed back into the collection tube, before the tube was centrifuged for 2 minutes at 11,000 x g.
10. The column was transferred to a sterile Eppendorf tube. To elute the plasmid, 50 μL Buffer AE was added, and the column was incubated at room temperature for 1 minute. The column was centrifuged for 1 minute at 11,000 x g, and the eluate was stored at -20 °C.

3.3.2. Isolation of plasmid with the GenElute™ HP Plasmid Midiprep Kit

The procedure was performed according to the protocol provided by the manufacturer. A swinging bucket rotor was used from step 6 and onwards.

Materials:

GenElute™ HP Plasmid Midiprep Kit (see Section 2.9)

Procedure:

1. 50 mL of an overnight culture was harvested by centrifugation at 5,000 x g for 10 minutes. The supernatant was discarded.
2. The pellet was resuspended in 4 mL of Resuspension/RNase A solution.
3. The cells were lysed by the addition of 4 mL Lysis solution, and gently mixing by inverting the tube 6-8 times. The mixture was incubated for 5 minutes at room temperature.
4. 4 mL of chilled Neutralization Solution was added to the lysate and the tube inverted gently 4-6 times.
5. 3 mL of Binding solution was added to the neutralized lysate, and the solution was mixed gently. The solution was transferred to the barrel of a filter syringe, and

incubated for approximately 5 minutes until the white aggregate, consisting of cell debris, proteins, lipids SDS and chromosomal DNA, had floated to the top.

6. A GenElute HP Midiprep Binding Column was placed into a collection tube. 4 mL of Column Preparation Solution was added, and the column was spun in a swinging bucket rotor at 3,000 x g for 2 minutes. The flow-through was discarded.
7. Half of the lysate was added to the GenElute HP Midiprep Binding Column with the filter syringe. The column was spun at 3,000 x g for 2 minutes, and the flow-through discarded.
8. Step 7 was repeated with the remaining lysate.
9. 4 mL of Wash Solution 1 was added to the column, and the sample was spun at 3,000 x g for 2 minutes.
10. Step 9 was repeated with Wash solution 2.
11. The column was transferred to a new collection tube, and 1 mL of Elution Solution was added. The column was spun at 3,000 x g for 5 minutes and the eluate was stored at -20 °C.

3.4. Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction is a method used to amplify specific DNA fragments. The technique requires the presence of a DNA template, primers, the four different deoxynucleotide triphosphates (dNTPs), and a thermostable DNA polymerase. The reaction proceeds in three steps: denaturation, annealing and elongation. The denaturation of the double-stranded DNA is accomplished by subjecting the DNA to elevated temperatures (95-98 °C). In the subsequent annealing step, the temperature is lowered, and the primers anneal to the single-stranded DNA at complementary regions. In the final elongation step, the temperature is raised, and the DNA polymerase incorporates dNTPs from the 3'-end of the primers, thus generating a new copy of the strands. By repeating the cycle of denaturation, annealing, and elongation, DNA fragments can be amplified exponentially.

3.4.1. PCR using Q5[®] High-Fidelity 2 x Master Mix

The procedure was performed according to the protocol provided by the manufacturer. The Q5[®] High-Fidelity 2 x Master Mix contains a high-fidelity DNA-polymerase fused to a processivity-enhancing domain that reduce the error-rate compared to the *Taq* DNA polymerase. PCR with Q5 DNA Polymerase was used to move the dendritic cell-binding motif from the C-terminus to the N-terminus through overlap-extension PCR and to generate DNA-fragments for In-Fusion cloning (see Section 4.1 and 4.2).

Materials:

Q5[®] High-Fidelity 2X Master Mix

Primers (Section 2.7)

DNA-template

dH₂O

Procedure:

1. The reactants listed in Table 3.2 were added to a sterile 0,2 mL PCR tube and gently mixed. The reactants and PCR tubes were kept on ice.

Table 3.2 Reactants for Q5[®] High-Fidelity PCR.

Reactant	Volume (µl)	Final concentration
Q5 [®] High-Fidelity 2X Master Mix	25	1 x
Forward primer	1	1 µM
Reverse primer	1	1 µM
DNA-template	Variable	< 1000 ng
dH ₂ O	To 50 µl	N/A

2. The PCR tubes were placed in a thermal cycler and the program presented in Table 3.3 was used to amplify the DNA.

Table 3.3 Thermal cycler settings for Q5[®] High-Fidelity PCR

Process	Temperature (°C)	Time	No. Cycles
Initial denaturation	98	30 sec	1
Denaturation	98	5 sec	30
Annealing	*	10 sec	
Elongation	72	20 sec	
Final elongation	72	2 min	1

* Annealing temperatures were dependent on the T_m of the primers used.

3. After 10 cycles, 15 μ L of the PCR reaction was removed and purified. 1 μ L was used as template in the next PCR reaction. The remaining reaction mix was allowed to run the entire program.
4. Correct PCR-amplification was verified through gel electrophoresis.

3.4.2. PCR using VWR Red Taq DNA Polymerase Master Mix

The procedure was performed according to the protocol provided by the manufacturer, mainly to confirm the presence of the desired plasmid in bacterial colonies (Colony-PCR).

Materials:

Taq Master Mix RED

Primers (Section 2.7)

Sterile dH₂O

DNA-template

Procedure:

1. A sterile toothpick was used to pick a bacterial colony from an agar plate, and to transfer the colony to a sterile 0.2 mL PCR tube.
2. The PCR tubes and the reactants were kept on ice. The reactants listed in Table 3.4 were added to the PCR tubes and gently mixed by pipetting.

Table 3.4 Reactants for Red Taq PCR.

Reactant	Volume (μl)	Final concentration
Taq Master Mix RED	25	1 x
Forward Primer	1	1 μM
Reverse Primer	1	1 μM
DNA-template	n/a	
dH ₂ O	To 50 μl	

- The PCR tubes were placed in a thermal cycler with the program showed in Table 3.5.

Table 3.5 Thermal cycler settings for Red Taq PCR

Process	Temperature ($^{\circ}\text{C}$)	Time	No. Cycles
Initial denaturation	95	2 min	1
Denaturation	95	20 sec	30
Annealing	50-79*	25 sec	
Elongation	72	20 sec	
Final elongation	72	2 min	1

* Annealing temperatures were dependent on the T_m of the primers used.

- The PCR reaction mixture was applied to an agarose gel for plasmid verification.

3.5. PCR Clean-up

The NucleoSpin® Gel and PCR Clean-up kit from Macherey-Nagel (Section 2.9) was used to purify PCR products which were to be used in a consecutive PCR.

Materials:

NucleoSpin® Gel and PCR Clean-up (see Section 2.9)

Procedure:

1. To 1 volume of sample, 2 volumes of Buffer NTI was added, before it was loaded onto a NucleoSpin® Gel and PCR Clean-up column placed in a collection tube.
2. The sample was centrifuged for 30 seconds at 11,000 x g, and the flow-through was discarded.
3. 700 μL of Buffer NT3 was added to the column, and the sample was centrifuged as before.
4. The flow-through was discarded, and the column was centrifuged for 1 minute at 11,000 x g, before the column was transferred to a clean Eppendorf tube.
5. 15 μL Buffer NE was added to the column, and the sample was allowed to incubate at room temperature for 1 minute.
6. The sample was centrifuged for 1 minute, and the eluate was either used directly or stored at -20°C .

3.6. Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA-fragments according to their size. When an electric current is applied to an agarose gel, the negatively charged DNA will travel towards the anode. The nature of the agarose gel determines the movement of the DNA-fragments. The agarose gel consists of a network of pores which cause smaller DNA-fragments to travel more rapidly through the gel than larger fragments. This allows for the effective separation of DNA-fragments according to their size.

Materials

Seakem® LE Agarose

1X TAE Buffer (Section 2.9)

peqGREEN DNA/RNA Dye

10X FastDigest® Green Buffer

GeneRuler™ 1 kb DNA ladder

Procedure:

1. A 0.5 L batch of ready-to-use 1,2 % agarose was prepared by dissolving 6 g of Seakem® LE Agarose in 0.5 L 1X TAE buffer. The solution was autoclaved and stored at 60 °C.
2. To prepare one agarose gel, 2,5 μ L peqGREEN was added to 60 mL of the 1,2 % agarose batch and poured into a gel tray. A comb was inserted, and the gel was allowed to solidify for approximately 20 minutes.
3. The comb was carefully removed, and the gel tray was transferred to an electrophoresis chamber. The gel was subsequently submerged in TAE-buffer.
4. The samples were mixed with loading buffer, and applied into the wells. A DNA ladder was also added to the gel.
5. The DNA-fragments were visualized with a Gel Doc EZ System.

3.7. Extraction of DNA from agarose gels

The NucleoSpin® Gel and PCR Clean-up kit from Macherey-Nagel (Section 2.9) was used to extract DNA from agarose gels. The procedure was performed according to the protocol provided by the manufacturer.

Materials:

NucleoSpin® Gel and PCR Clean-up (see Section 2.9)

Procedure:

1. The pre-run agarose gel was placed on a UV-plate, and a sterile scalpel was used to excise the correct sized DNA-fragment from the gel. The gel-fragment was transferred to an Eppendorf tube.
2. 200 μ L NTI buffer was added per 100 mg of gel. The gel fragment was incubated at 50 °C for 5-10 minutes and vortexed every 2 minutes, until it was completely dissolved.
3. Up to 700 μ L sample was transferred to a NucleoSpin® Gel and PCR Clean-up column which was inserted into a collection tube. The sample was centrifuged at 11,000 x g for 30 seconds, and the flow-through was discarded.
4. Step 3 was repeated with the remainder of the sample.

5. After all of the sample had been applied to the column, 700 μL NT3 buffer was added to wash the column. The tube was centrifuged at 11,000 x g for 30 seconds and the flow-through discarded.
6. Step 5 was repeated.
7. The column was centrifuged at 11,000 x g for 1 minute to remove any residual NT3 buffer.
8. The column was transferred to an Eppendorf tube, and 20 μL of NE buffer was added. The sample was incubated for 5 minutes at room temperature, and centrifuged at 11,000 x g for 1 minute.
9. The eluate was stored at -20°C .

3.8. Restriction enzyme digestion and ligation of DNA fragments

Restriction enzymes are endonucleases which cleaves DNA at specific nucleotide sites called restriction sites. The cleavage site is specific to each restriction enzyme which cleaves the phosphodiester bond between the nucleotides on both strands. A blunt end is generated if the restriction enzyme cleaves both strands at the same nucleotide position, while sticky ends are generated when the enzymes cleave the two strands at different positions. The overhang generated in sticky ends may be ligated with another molecule cleaved with the same restriction enzyme. This allows for the generation of recombinant DNA.

3.8.1. Restriction enzyme digestion with FastDigest restriction enzymes

Digestion of DNA was performed with the use of FastDigest restriction enzymes. These enzymes are 100 % active in FastDigest Green buffers which allows for simultaneous digestion of DNA with several enzymes. The inert dyes found FastDigest Green buffer also allows for direct application on an agarose gel.

Materials:

Plasmid-DNA

FastDigest restriction enzymes (Section 2.5)

FastDigest Green Buffer 10 x

dH₂O

Procedure:

1. The reactants listed in Table 3.6 were mixed in an Eppendorf tube.

Table 3.6 Reactants for FastDigest restriction enzyme digestion

Reactant	Volume (µl)
DNA	15-50
FastDigest Green Buffer 10 x	5
FastDigest restriction enzyme(s)	5*
dH ₂ O	To 50 µl

* When using two restriction enzymes, the total enzyme volume was kept constant at 5 µl (2,5 and 2,5 µl).

2. The reaction was incubated at 37 °C for 15-45 minutes.
3. The DNA-fragments were separated through gel electrophoresis, isolated and stored at -20 °C.

3.9. Quantification of DNA

The DNA concentration of different solutions was determined with the Qubit[®] dsDNA BR assay kit. The procedure was performed according to the protocol provided by the manufacturer.

Materials:

Qubit[®] dsDNA BR assay kit (see Section 2.9)

Qubit[™] fluorometer

Procedure:

1. A Qubit[®] working solution was prepared by diluting the Qubit[®] dsDNA BR 1:200 in Qubit[®] dsDNA BR Buffer.
2. Calibration standards were made by mixing 10 µl of Qubit[®] dsDNA BR Standard 1 and 2 in 190 µl of the working solution. The fluorometer was calibrated.

3. To determine the DNA concentration, 1-5 µl of a sample with an unknown concentration was mixed with 195-99 µl working solution, and measured with the fluorometer.

3.10. Ligation of DNA fragments

Ligation of DNA-fragments generated from restriction enzyme digestion may be achieved through the use of a DNA ligase which catalyzes the formation of phosphodiester bonds between nucleotides.

3.10.1. Ligation using the Quick Ligation™ kit

The Quick T4 DNA Ligase is an enzyme used to ligate both blunt and sticky ends. Only sticky ends were used in this study.

Materials:

DNA-fragments

Quick Ligation™ kit (see Section 2.9)

dH₂O

Procedure:

1. Linearized vector and target gene was mixed with a final molar ratio of 1:3. dH₂O was added to a final volume of 10 µl.
2. 10 µl of 2X Quick Ligation Reaction Buffer was added.
3. 1 µl Quick T4 DNA Ligase was added, and the solution was vortexed. The sample was briefly centrifuged in a microcentrifuge.
4. The sample was incubated at room temperature for 5 minutes, and subsequently chilled on ice before transformation, or stored at -20 °C.

3.10.2. Ligation using T4 Ligase

Materials:

T4 DNA Ligase Buffer (10 X)

T4 DNA ligase

DNA-fragments

dH₂O

Procedure:

1. A molar ratio of 1:3, vector to insert, was used in the reaction.
2. Vector and insert were mixed with 2 µl 10 x T4 Ligation Buffer and 1 µl T4 DNA Ligase. The volume was adjusted to 20 µl with dH₂O.
3. The reaction was incubated overnight at 16 °C, and then transformed into competent cells (see Section 3.14 and 3.15) or stored at -20 °C.

3.11. In-Fusion cloning

In-Fusion cloning is a cloning technique which makes use of PCR to generate an insert to be cloned into a linearized vector. A forward and reverse primer is designed with a 15 base pair overhang complementary to the vector, and a part complementary to the gene to be inserted into the vector. A PCR is run with these primers together with a template containing the target gene. The amplified PCR fragments contain both the target gene and vector complementary overhangs at both ends. Linearized vector, purified PCR product and an In-Fusion HD Enzyme Premix are incubated together to allow for cloning of the PCR product into the vector.

Materials:

5 x In-Fusion HD Enzyme Premix (see Section 2.9)

Purified PCR product

Vector-DNA

dH₂O

Procedure:

1. In-Fusion[®] Molar Ratio Calculator
(<http://bioinfo.clontech.com/infusion/molarRatio.do>) was used to determine the amount of insert and vector needed for each reaction.
2. The reactants listed in Table 3.7 were added to Eppendorf tubes.

Table 3.7 Reactants for In-Fusion Cloning.

Reactant	Volume (μl) Cloning reaction	Volume (μl) Negative control
5 x In-Fusion HD Enzyme Premix	2	2
Insert	*	N/A
Linearized Vector	*	Same as cloning reaction
dH ₂ O	To 10 μl	To 10 μl

* Calculated with In-Fusion[®] Molar Ratio Calculator.

3. The reaction mixtures were vortexed and centrifuged in a microcentrifuge.
4. The tubes were incubated at 50 °C for 15 minutes, and then placed on ice.
5. The mixture was either transformed into competent cells (see Section 3.14 and 3.15) or stored at -20 °C.

3.12. Ligation-Independent Cloning

Ligation-independent cloning (LIC) is a molecular technique that circumvents the need for restriction enzymes. LIC exploits the dual activity of the T4 DNA Polymerase, which can act both as an exonuclease and a polymerase. The enzyme is used to digest both the vector and the insert prior to cloning, generating complementary overhangs.

While the vector is normally digested as is, the insert is generated through PCR with primers containing one part complementary to the vector and one part complementary to the target gene. Under standard PCR conditions, the polymerase activity is favored. However, by adding only one variety of dNTPs to the reaction, the exonuclease activity can be selected for. The enzyme will degrade the 3' → 5' template DNA-strand until it encounters a dNTP equal to the one added to the reaction. Since the polymerase activity is favored, the dNTP will be incorporated, and the enzyme will become stalled. To generate complementary overhangs between the vector and the insert, two complementary dNTPs are used to generate overhangs that will spontaneously anneal upon mixing.

The vector used in this study for protein production and purification was the pNIC-CH vector. This vector contains both a kanamycin resistance gene and a SacB selection gene, in addition to a 6xHistidine-tag for protein purification (see Section 3.23 for details regarding pNIC-CH as a protein production vector). The vector had previously been digested and linearized by Dr. Lasse Fredriksen.

Materials:

BSA, 10 mg/mL

dGTP

DTT

EDTA

NEB Buffer 2

T4 DNA Polymerase

Purified PCR-product

Predigested pNIC-CH vector

Procedure:

1. The reactants and volumes used in the digestion reaction are listed in Table 3.8.

Table 3.8 Reactants for digestion of insert.

Reactant	Volume
NEB Buffer 2	2 μ L
BSA	1 μ L
dGTP	2 μ L
DTT	1 μ L
T4 DNA Polymerase	1 μ L
Purified PCR-product	*
dH ₂ O	To 20 μ L

* Volume equal to 0,2 pmol DNA.

2. The reaction mixture was incubated in a thermocycler at 22 °C for 1 hour, followed by a 21-minute incubation at 75°C.
3. 2 μ L of the digested PCR-product and 1 μ L digested pNIC-CH vector was mixed and incubated at room temperature for 5 minutes.
4. 2 μ L 25 mM EDTA was added, and the mixture was incubated for 10 minutes at room temperature.
5. 3 μ L of the reaction mixture was transformed into TOP10 *E. coli* cells, which were spread on LB agar plates containing 2% sucrose and 50 μ g/mL Kanamycin.

3.13. Preparation of electrocompetent *Lactobacillus plantarum* WCFS1

To generate electrocompetent *Lactobacillus plantarum* WCFS1, the bacteria was grown in MRS medium containing 1 % glycine. The glycine replaces the L-alanine in the cell wall, thus making it more permeable to external DNA.

Materials:

L. plantarum

MRS medium (Section 2.10)

MRS medium with 1 % glycine (Section 2.10)

30 % PEG₁₄₅₀

Procedure:

1. 10 mL of MRS medium was inoculated with *L. plantarum* from a glycerol stock and incubated overnight at 37 °C.
2. A 10-fold serial dilution (10^{-1} - 10^{-10}) of the overnight culture was prepared in MRS containing 1 % glycine. The dilutions were incubated at 37 °C overnight.
3. 2 mL of one of the cultures with a measured OD₆₀₀ of $2,5 \pm 0,5$ was used to inoculate 40 mL of MRS containing 1 % glycine. When the OD₆₀₀ reached $0,7 \pm 0,07$ the culture was placed on ice for 10 minutes to stop the growth.
4. The culture was centrifuged at 4500 rpm at 4°C for 10 minutes, and the supernatant was discarded. The cell pellet was resuspended in 5 mL ice cold 30 % PEG₁₄₅₀, and additional 15 mL chilled 30 % PEG₁₄₅₀ was added. The suspension was left on ice for 10 minutes.
5. The cells were harvested by centrifugation at 4500 rpm at 4°C for 10 minutes. The supernatant was discarded, and the cell pellet was resuspended in 400 μ L ice cold 30% PEG₁₄₅₀.
6. 40 μ L of the suspension was aliquoted into sterile Eppendorf tubes which were immediately frozen in liquid nitrogen. The electrocompetent cells were then stored at -80 °C.

3.14. Electroporation of *Lactobacillus plantarum* WCFS1

Electroporation is a bacterial transformation method where bacterial cells are subjected to an electric pulse. Cells subjected to this pulse are more likely to take up external DNA, as the membrane is temporarily disrupted which allows for uptake of extracellular components.

Materials:

Electrocompetent *L. plantarum* WCFS1

Gene Pulser[®] electroporation cuvette 0,2 cm

MRSSM medium (Section 2.10)

Plasmid

MRS agar plates with 10 $\mu\text{g}/\text{mL}$ erythromycin

Procedure:

1. A vial of electrocompetent *L. plantarum* was thawed on ice. 1 μL plasmid was added to the vial, and the mixture was transferred to a pre-chilled electroporation cuvette.
2. An electroporator was set to a voltage of 1,5 kV, a capacitance of 25 μF and a resistance of 400 ω . The cuvette was placed in a electroporator and subjected to the current.
3. 450 μL of MRSSM was added, and the cells were transferred to a sterile Eppendorf tube.
4. The cells were incubated at 37 °C for 2-4 hours, before 50-100 μL were spread out on MRS agar plates with 10 $\mu\text{g}/\text{mL}$ erythromycin. The plates were incubated at 37 °C over night.

3.15. Transformation of chemically competent *E. coli*

Materials:

Chemically competent *E. coli* TOP10 or BL21 cells

Plasmid or ligation reaction mixture

S. O. C. medium (Section 2.10)

LB or BHI agar plates with appropriate antibiotic

Tubes, PP (13 mL)

Procedure:

1. A vial of chemically competent *E. coli* TOP10 cells (50 μ L) or BL21 (50 μ L) was thawed on ice, and the entire volume was transferred to a pre-chilled tube.
2. 1-5 μ L of plasmid or ligation reaction mixture was added, and the cells were incubated on ice for 30 minutes.
3. The cells were then subjected to a heat shock at 42 °C for 30 seconds, and then placed on ice.
4. After 2 minutes 75-250 μ L S.O.C medium was added, and the cells were incubated at 37°C for approximately 1 hour.
5. 25-100 μ L of the cell suspension was spread out on either LB or BHI agar plates with appropriate antibiotic, and incubated at 37°C overnight.

3.16. DNA sequencing

Transformant colonies were sequenced to verify the correct insertion of the gene into the vector. Approximately 400 ng of plasmid-DNA was used for the sequencing, together with 25 pmol sequencing primers, SekF and SekR (see Section 2.7). Two tubes were prepared for each plasmid, one containing the forward primer and the other the reverse primer. The volume was adjusted to a total of 11 μ L, and sent to GATC Biotech for sequencing. The sequences were then analyzed with CLC DNA Main Workbench 7 (Qiagen).

3.17. Preparing samples for analysis of gene products in *L. plantarum*

The target genes are under the control of the SppIP promoter which requires the presence of an inducer peptide, SppIP, to initiate expression. To analyze the production of the target genes in *L. plantarum* harboring the different plasmids, the expression was induced by addition of the inducer peptide. After induction the bacteria were harvested and production was investigated through Western blot analysis. Flow cytometry and confocal laser scanning microscopy was used to determine the localization of the target proteins.

3.17.1. Cultivation and harvesting

Materials:

MRS (Section 2.10)

Erythromycin 10 mg/mL

Inducer peptide SppIP 0,1 mg/mL

PBS Buffer (Section 2.11)

Procedure:

1. *L. plantarum* was cultured in MRS-medium with 10 µg/mL erythromycin at 37°C overnight.
2. The overnight cultures were diluted in 50 mL fresh MRS-medium containing 10 µg/mL erythromycin the next day to an OD₆₀₀ of 0.1-0,15.
3. The dilutions were incubated at 37°C until the OD₆₀₀ reached 0,28-0,33, at which point the inducer peptide SppIP was added to a final concentration of 25 ng/mL.
4. After induction, the cultures were incubated at 37°C for 3 hours, and then put on ice to stop the bacterial growth.
5. The cultures were harvested by centrifugation at 5,000 x g and 4 °C for 5 minutes, and the supernatant was decanted into sterile 50 mL CELLSTAR® tubes for protein precipitation (see Section 3.17.3)
6. The pellet was washed 1-3 times with 10 mL cold PBS, and centrifuged at 5,000 x g and 4 °C for 5 minutes.
7. After the washing steps, the pellet was either resuspended in 1 mL PBS or stored at 4 °C for further use.

3.17.2. Cell disruption by glass beads

The harvested bacterial cells were disrupted using glass beads to investigate whether the target gene had been expressed after induction with SppIP. By adding glass beads and subjecting the cells to vigorous shaking, the cells are lysed generating a cell free extract which may be used to investigate the production of the target genes.

Materials:

PBS Buffer (Section 2.11)

FastPrep tubes

Glass beads, 100 micron

Procedure:

1. Harvested bacterial cells were resuspended in 1 mL PBS, and the suspension was transferred to FastPrep tubes containing approximately 1,5 g glass beads.
2. The FastPrep tubes were placed in a FastPrep[®] - 24 Tissue and Cell Homogenizer set to 6,5 m/s and 45 seconds.
3. After one run, the tubes were kept on ice for 5 minutes before the run was repeated.
4. The tubes were centrifuged at 16,100 x g and 4 °C for 1 minute to separate the supernatant from the cell debris and glass beads. The supernatants were subsequently transferred to new Eppendorf tubes.
5. The centrifugation step was repeated to remove any residual glass beads.
6. The supernatant was transferred to new Eppendorf tubes, and stored at -20°C.

3.17.3. Trichloroacetic acid/Sodium Deoxycholate precipitation of proteins in culture supernatant

Trichloroacetic acid (TCA) is used to precipitate proteins through dehydration of the hydration shell around the proteins. This process leads to the exposure of hydrophobic part on the protein surface, and proteins start to aggregate to re-shield these patches. Sodium deoxycholate acts as a co-precipitant which leads to a higher yield of precipitated proteins. This method was used to investigate whether the target protein was located in the supernatant after induction.

Materials:

BSA, 10 mg/mL

pH-strips

2 % (w/v) Sodium Deoxycholate

100 % (w/v) Trichloroacetic acid (TCA)

Acetone

6 M NaOH

Procedure:

1. 15 μL BSA was added to the sample supernatants as a control.
2. The pH was measured with pH-strips and adjusted to pH 7.
3. 2% DOC was added to a final concentration of 0,2 % v/v, and the samples were kept on ice for 30 minutes.
4. TCA was added to a final concentration of 16% v/v, and the samples were vortexed and kept on ice for additional 30 minutes.
5. The samples were centrifuged at 16,100 x g and 4 °C for 15 minutes, and the supernatant discarded.
6. The protein pellet was washed with 200 μL ice cold acetone, and the samples were centrifuged as before. The supernatant was discarded, and the pellet was dried in a vacuum centrifuge for 3 minutes to remove residual acetone.
7. The dried pellet was stored at -20°C, or used directly by dissolving it in 20 μL 50 mM NaOH before SDS-PAGE analysis.

3.18. Gel Electrophoresis of Proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a well-known technique used to separate denatured proteins according to mass. Precast gels and automated gel imaging is readily available, providing a rapid and safe method for visualization and analysis of protein samples.

The addition of the anionic detergent lithium dodecyl sulfate (LDS) and the reducing agent dithiothreitol (DTT) to protein samples denatures the proteins by breaking non-covalent bonds and disulfide bridges respectively. This gives the proteins a uniform negative charge which is then used to separate the proteins in a stain-free polyacrylamide gel according to molecular weight.

By applying an electric current to the gel, the negatively charged proteins will migrate towards the anode. Due to the pore structure of the gel, proteins of lower molecular weight (kDa) will migrate more rapidly through the gel than heavier proteins, which allows for the effective separation of proteins. A protein standard is used to determine the approximate molecular weight of the proteins.

Materials:

Mini-PROTEAN[®] TGX Stain-Free[™] Precast Gels

NuPAGE[®] LDS Sample Buffer (4X)

NuPAGE[®] Reducing agent (10X)

TGS Buffer (see Section 2.11)

Benchmark[™] Protein Ladder

MagicMark[™] XP Western Protein Standard

Procedure:

1. A 2 X working solution was prepared from mixing NuPAGE[®] LDS Sample Buffer (4X) and NuPAGE[®] Reducing agent (10X).
2. Protein solution and working solution were mixed 1:1, and boiled for 10 minutes.
3. The Mini-PROTEAN[®] TGX Stain-Free[™] Precast Gel was assembled in the electrophoresis chamber, and TGS Buffer was added.
4. The boiled samples and ladders were applied to the gel, and the gel was run at 280 V for 18 minutes.
5. After the electrophoresis, the gel was placed briefly in dH₂O before further analysis.

3.19. Western Blot

Western blot analysis is a widely-used antibody hybridization technique for detection of specific proteins. Proteins separated by SDS-PAGE are transferred from the polyacrylamide gel to a membrane using an electric current through a process called electroblotting. When transferred to the membrane, proteins are free to hybridize with antibodies. To prevent non-specific binding between proteins and antibodies, the membrane is treated with a blocking solution usually containing Bovine Serum Albumin (BSA). The membrane is subsequently incubated with a primary antibody which bind specific epitopes of the target protein. A wash step is performed to remove any unbound primary antibody before the addition of a secondary antibody. The secondary antibody binds to the primary antibody which is linked to a reporter enzyme that produces a detectable signal in the presence of a specific substrate. The secondary antibody binds species-specific immunoglobulins. In this study the primary antibody is produced in mouse, and the secondary antibody is produced in goat targeted against mouse immunoglobulins. The secondary antibody is conjugated to a reporter enzyme

called horse radish peroxidase (HRP) that oxidizes luminol. This oxidation causes a detectable emittance of light that allow for visualization of target proteins.

3.19.1. Blotting with iBlot[®] Dry Blotting System

The iBlot[®] Dry Blotting System was used to transfer proteins from a polyacrylamide gel to a nitrocellulose membrane. The schematic representation of the system is shown in figure 3.1

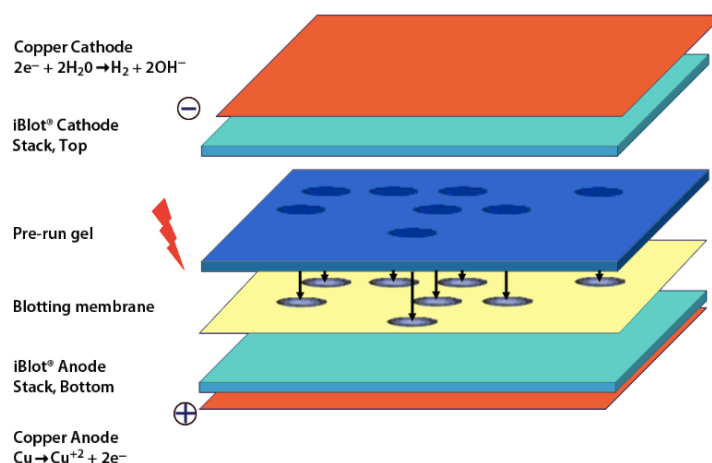


Figure 3.1 Assembly of the components of the iBlot[®] Dry Blotting System. The bottom part consists of a copper anode, anode buffer gel and the nitrocellulose membrane. The pre-run polyacrylamide gel is placed on top of the nitrocellulose membrane, and covered with a filter paper soaked in dH₂O. The top part consists of a layer of cathode buffer gel and copper cathode. Figure reprinted from the iBlot[®] Dry Blotting System Manual (MAN0000560).

Materials:

iBlot[®] Dry Blotting System (Section 2.9)

TBS (see Section 2.11)

Procedure:

1. The polyacrylamide gel was washed with dH₂O for 5 minutes.
2. The anode stack was placed in the iBlot[®] Gel transfer Device, and the gel was transferred to the nitrocellulose membrane.
3. A filter paper was soaked in dH₂O and placed on top the gel, and a blotting roller was used to remove any air bubbles.
4. The cathode stack was placed on top of the filter paper with the copper-electrode facing upwards.

5. A disposable sponge was placed in the lid of the iBlot[®] Gel transfer Device, and the lid was closed.
6. Program P3 (20 V for 7 minutes) was used to blot the proteins on the nitrocellulose membrane.
7. After the program had finished, the nitrocellulose membrane was kept in TBS before antibody hybridization.

3.19.2. SNAP i.d.[®] immunodetection

The SNAP i.d. immunoblotting system is a fast and easy method for antibody hybridization. The membrane is placed in a blot holder which is mounted on the SNAP i.d.[®] immunodetection device before a vacuum is applied to pull the antibody and wash solutions through the membrane.

Materials:

SNAP i.d.[®] immunodetection system (Section 2.9)

TTBS (see Section 2.11)

TTBS/0,5 % BSA

Blocking solution TTBS/ 1% BSA

Primary antibody, Melan-A(A103): sc-200032 (Santa Cruz Biotechnology Inc)

Secondary antibody, Polyclonal Rabbit Anti-Mouse Immunoglobulin HRP FITC (Agilent)

Procedure:

1. The blot holder was drenched in water. The nitrocellulose membrane was placed with the protein side down in the middle of the holder.
2. A filter paper was placed between the membrane and the back of the blot holder, and the blot holder closed. A blotting roller was used to remove air bubbles.
3. The blot holder was mounted in the SNAP i.d.[®] immunodetection device.
4. 30 mL of blocking solution was added, and the vacuum was turned on. When all of the blocking solution had passed through the system, the vacuum was turned off.
5. 7 μ L primary antibody was added to 3 mL TTBS/0,5% BSA, and the solution was poured over the membrane.

6. After 10 minutes, the vacuum was turned back on, and the membrane was washed with 3x10 mL TTBS, before the vacuum was turned off.
7. 0,8 μ L secondary antibody was added to 3 mL TTBS/0,5% BSA, and added to the membrane.
8. Step 6 was repeated.
9. The nitrocellulose membrane was removed from the blot holder and kept in TBS before incubation with a detection agent.

3.19.3. Chemiluminescent Detection of Proteins

Materials:

SuperSignal[®] West Pico Chemiluminescent Substrate

Luminol/enhancer

Stable Peroxide Buffer

Procedure:

1. The working solution was prepared by mixing Luminol and Peroxide Buffer 1:1.
2. The nitrocellulose membrane was placed in a plastic tray covered by aluminum foil, and incubated with 20 mL working solution for 5 minutes.
3. The visualization and imaging of the membrane was performed with the Azure c400.

3.20. Detection of Surface Antigens by FITC-labelled Secondary Antibody

The detection of surface localized proteins is made possible by the use of antibodies conjugated with different fluorochromes. The secondary antibody used in this study is conjugated with the fluorochrome fluorescein isothiocyanate (FITC) which emits light when exposed to a laser beam. This allows for the visualization of target proteins located on the surface by flow cytometry and confocal laser scanning microscopy.

In a flow cytometer, particles in solution passes through a laser beam consecutively, while a detector registers the scattered light signals, mainly forward scattered (FSC) and side scattered (SSC) light. These two types of scattered light give information about the particle size and granularity respectively. In addition to the scattered light, the detector may also

detect fluorescent signals from surface-located fluorochromes such as FITC, as it is excited by the laser beam. This may provide information about the localization of surface antigens and be used to compare the relative fluorescence between different cell populations.

The detection of surface antigens by confocal laser scanning microscopy follow the same principle as in a flow cytometer where the fluorochrome is excited by a laser beam, and gives a detectable signal.

3.20.1. Staining of Bacterial Cells for Flow Cytometry and Confocal Laser Scanning Microscopy

Materials:

PBS (see Section 2.9)

PBS/1% BSA

Primary antibody Melan-A(A103): sc-200032 (Santa Cruz Biotechnology Inc)

Secondary antibody Polyclonal Rabbit Anti-Mouse Immunoglobulin HRP FITC

Procedure:

1. Recombinant *L. plantarum* was cultured and induced as described in Section 3.17.1.
2. The bacterial cells were harvested 3 hours after induction by centrifugation at 5,000 x g and 4 °C for 5 minutes.
3. The supernatant was discarded, and the cell pellet was washed 2 times with 1 mL cold PBS. The suspension was centrifuged at 5,000 x g and 4 °C for 5 minutes in each washing step. The supernatant was discarded.
4. The cells were resuspended in a mixture of 50 μ L PBS/1% BSA and 1 μ L primary antibody. The cells were incubated for 30 minutes at room temperature.
5. The cells were centrifuged at 5,000 x g and 4 °C for 1 minute to remove superfluous antibody.
6. The cells were washed with 3 x 600 μ L PBS/1% BSA. The cells were centrifuged at 5,000 x g and 4 °C after each wash, and the supernatant discarded.
7. The cells were resuspended in 50 μ L PBS/1% and 0,8 μ L secondary antibody, and incubated in the dark for 30 minutes at room temperature.
8. Steps 5 and 6 were repeated.

9. Before analysis the pellet was resuspended in 100-200 μL PBS. The flow cytometry analysis was carried out with the MacsQuant[®] Analyser and MacsQuantify[™] software, while the Immunofluorescent microscopy was performed with a Zeiss LSM 700 Laser Scanning Confocal Microscope.

3.20.2. Treatment with Lysozyme

The glycoside hydrolase lysozyme is an enzyme that hydrolyzes the 1,4-beta linkages between N-acetylmuramic acid and N-Acetyl-D-glucosamine in peptidoglycan found in the gram-positive bacterial cell wall. To investigate whether the epitope specific for the antigen was embedded in the cell wall, and thus unavailable for hybridization, the bacterial cells were treated with lysozyme before staining for flow cytometry and confocal laser scanning microscopy.

Materials:

Lysozyme 100 mg/mL

PBS (see Section 2.11)

Procedure:

1. Recombinant *L. plantarum* was harvested and washed as described in 3.17.1. Approximately 10^9 cells were harvested, and the amount of culture needed was calculated using Figure 7.1 in Appendix.
2. The cell pellet was resuspended in 300 μL PBS, before 200 μL lysozyme was added. A control sample was prepared by resuspending the pellet in 500 μL of PBS. The samples were incubated at 37 °C for 30 minutes.
3. After the incubation, the cells were washed with 3 X 1 mL PBS, and centrifuged at 5,000 x g and 4 °C for 2 minutes between each wash.
4. The lysozyme treated cells were immediately stained for flow cytometry.

3.21. Trypsin Digestion of Surface Localized Protein

Trypsin is a serine protease which cleaves peptide chains after lysine and arginine. The only exception is when these amino acids are followed by a proline, which will limit the enzyme's access to the cleavage site. The characteristics of this enzyme enable the prediction of specific peptide fragments to be located in the solution following trypsin digestion of a target protein. The peptide composition of the bacterial cell wall was investigated through trypsin digestion with subsequent MS/MS analysis, in order to determine whether the target protein was located on the surface.

Materials:

2% Acetonitrile/0,1% TFA

70% Acetonitrile/0,1% TFA

0,1% TFA

DTT

LC-MS grade Methanol

MRS without Tween-20 (Section 2.10)

PBS

PBS/ 40% Sucrose

Protein LoBind Tubes

Strata C18-E (55 μ m, 70A) Column, 1 mL

Trypsin/Lys-C Mix, Mass Spec Grade

Procedure:

1. The bacterial cells were cultured and induced as described in Section 3.17.1 with the exception that the bacteria were cultivated in MRS without Tween-20.
2. 3 hours after induction, the cells were harvested by centrifugation at 3,500 x g for 10 minutes. OD₆₀₀ was used to harvest approximately the same number of cells.
3. The supernatant was discarded, and the pellet was washed with 3 x 10 mL PBS.
4. After the final wash step, the pellet was resuspended in 1,4 mL PBS/40% Sucrose, and transferred to 2 mL Protein LoBind tubes.
5. 100 μ L DTT was added to each tube, before the addition of 5 μ g Trypsin.
6. The tubes were incubated in a shaking incubator for 2 hours at 37 °C.

7. After the first incubation, the tubes were centrifuged at 3,500 x g for 10 minutes, and the supernatant was transferred to new Protein LoBind tubes.
8. 1 µg Trypsin was added to each tube, which were incubated at 37 °C. over night.
9. TFA was added to a final concentration of 0,1% to stop the trypsination
10. Sucrose and salts were removed through the use of a Strata C18-E Column. To equilibrate the column 200 µL LC-MS grade Methanol was added, followed by 200 µL Acetonitrile/0,1 % TFA and finally 400 µL 0,1% TFA.
11. The sample was applied twice, before the column was washed with 2 X 200 µL 0,1% TFA.
12. 300 µL 70% Acetonitrile/0,1% TFA was used to eluate the sample into 2 mL Protein LoBind Tubes.
13. The samples were centrifuged in a vacuum centrifuge for 1 hour to remove the acetonitrile and TFA.
14. The pellet was resuspended in 10 µL 2% Acetonitrile/0,1% TFA, and the samples were stored at 4 °C until analysis.

3.22. Mass Spectrometry

The mass spectrometry analysis was carried out by senior engineer Morten Skaugen (KBM, NMBU). Peptides generated from the tryptic digest were analyzed through a Reverse phase (C18) nano online liquid chromatographic MS/MS analysis using a HPLC system. A nanoelectrospray ion source was used to couple the LC system with a LTQ-Orbitrap mass spectrometer. Mass spectra were acquired in the positive ion mode, and peptide samples were analyzed by collision induced dissociation (CID) in the LTQ ion trap.

Scaffold (version Scaffold_4.7.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 94,0% probability to achieve an FDR less than 1,0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 95,0% probability to achieve an FDR less than 1,0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Al et al Anal. Chem. 2003;75(17):4646-58).

3.23. Production of the recombinant protein modMART1_DC in *Escherichia coli* BL21

The pNIC-CH expression vector (vector map shown in Figure 7.2 in Appendix) was used to overexpress the recombinant protein for the purpose of purification. *modMART1_DC* was cloned into the vector by the means of Ligation-Independent Cloning (see Section 3.12) and the plasmid was transformed into One Shot BL21 Star (DE3) Chemically Competent *E. coli*. The pNIC-CH vector contains the gene *sacB* which encodes for a levansucrase that produces a product from sucrose that is lethal to *E. coli*. The gene is flanked by two *BfuAI* restriction sites on each side, which allows for the digestion and cloning of target genes into the vector. The *sacB* gene thus allows for negative selection by the addition of sucrose to the agar plates.

The expression of *sacB* and potentially the target gene is under control of the T7 promoter. The pNIC-CH vector contains a lac operator sequence upstream of the T7 promoter and the *lacI* gene that encodes a repressor that binds to this operator. The expression is thus not initiated before the addition of IPTG that binds to the repressor, and make the expression of the target protein possible. This permits the controlled production of target proteins.

The pNIC-CH vector also contains a C-terminal Histidine-tag that allows for the separation of the produced target protein from other host proteins by Immobilized Metal Ion Affinity Chromatography (IMAC) and Ion Exchange Chromatography (IEC).

3.23.1. Cultivation of *Escherichia coli* BL21

Materials:

IPTG

TB medium (Section 2.10)

Phosphate solution (Section 2.11)

1 L Flask w/ GL45 Sparger

Procedure:

1. 450 mL TB medium was added to a 1 L bottle capped with 1 L GL45 Sparger. The bottles were autoclaved at 115 °C for 15 minutes.
2. 50 mL autoclaved Phosphate Buffer was added to the bottle together with 150 μ L Antifoam 204. Kanamycin was added to a final concentration of 50 μ g/mL. All constituents were added under sterile conditions.
3. 3 mL of an overnight culture of *E. coli* containing the LIC_modMART1_DC plasmid was added to the bottles, which were subsequently connected to the LEX-48 Bioreactor.
4. The oxygen flow was activated, and the bottles were incubated overnight at room temperature.
5. To induce the expression, IPTG was added to a final concentration of 0,5 M the following day, and the bottles were incubated overnight at room temperature.
6. The cells were harvested the next day. See Section 3.23.2 for details.

3.23.2. Harvesting of *E. coli* BL21

Materials:

NiNTA Buffer A

Samples prepared in Section 3.23.1

Procedure:

1. The cultivated cultures from Section 3.23.1 were harvested by centrifugation at 5,000 x g in a JA-14 rotor in the Avanti™ J-25 Centrifuge.
2. The supernatant was carefully decanted. 50 mL of the supernatant was poured back into the centrifugation tubes and used to resuspend the pellet.
3. The suspension was then transferred to 50 mL CELLSTAR® Tubes, and centrifuged at 5,000 x g for 15 minutes.
4. The supernatant was discarded, and the pellet was stored at -80 °C
5. After 3 hours at -80 °C, the tubes were transferred to -20°C, and stored overnight.
6. The frozen pellet was resuspended in 30 mL NiNTA Buffer A, and sonicated for 3 minutes at 30% amplitude and 5 seconds on/off cycles.

7. The sonicated solution was centrifuged at 15,000 x g for 15 minutes, and the supernatant was transferred to a clean tube.
8. A SDS-gel was run with both the pellet fraction and the supernatant fraction to check for the production and solubility of the target protein.
9. The supernatant was stored at 4°C until further analysis.

3.24. Immobilized Metal Ion Affinity Chromatography (IMAC)

Immobilized Metal Ion Affinity Chromatography is a technique typically used to separate a target protein from other proteins in a solution. The effective separation is based on the affinity of the protein to a specific metal ion. Proteins with an affinity for the metal ion will bind reversibly to a column prepacked with the metal ion while other proteins will pass through. The target protein is eluted by adding a solution that contains a compound that replaces the binding of the protein to the column.

In this study, the recombinant modMART1_DC gene was cloned into the pNIC-CH vector. This vector contains a region coding for a Histidine-tag located immediately after the cloning site of the recombinant gene. Due to the imidazole ring in the histidine, this amino acid has a high affinity for Ni²⁺. This property may be used to separate target proteins expressed in bacteria from other expression host proteins by using a Ni²⁺ column.

The ÄKTA Pure Chromatography System was used to perform IMAC, together with the Ni²⁺ affinity HisTrap High-Performance (HP) 5 x 5 mL column. A buffer containing a high concentration of imidazole was used as the elution buffer.

Materials:

NiNTA Buffer A (see Section 2.11)

NiNTA Buffer B (see Section 2.11)

HisTrap HP 5 x 5 mL column

20 % Ethanol

Procedure:

1. The HisTrap HP 5 x 5 mL column was mounted onto the Äkta system. The system was washed with dH₂O to remove ethanol residues.
2. After the initial wash, the system was run with NiNTA Buffer A at a flow rate of 1,5 mL/min for 10 minutes, and the system was auto-zeroed.
3. The sample prepared in Section 3.23.2 was loaded onto the column with a flow rate of 1 mL/min, and the system was subsequently flushed with Buffer A to remove unbound protein.
4. When the baseline had returned to zero, the target protein was eluted with a linear Buffer B gradient.
5. The different fractions corresponding to the elution peak were collected and the proteins were visualized on an SDS-PAGE gel.

3.25. Ion Exchange Chromatography

Ion Exchange Chromatography is a technique used to separate proteins according to their affinity for an ion exchanger resin. The ion exchanger resin usually contains cellulose or agarose beads modified with either positively or negatively charged functional groups, thus giving the resin either a negative or positive charge. Therefore, the nature of the resin determines whether an anion or cation exchange is possible.

The binding affinity of the sample proteins to the resin is determined by the isoelectric point (pI) of the protein and the pH of the buffer used. If the buffer has a greater pH value than the isoelectric point of the protein, the protein will carry a negative net charge, and will therefore bind to an anion exchanger resin. The opposite is true for a cation exchange resin.

By applying an ion gradient, it is possible to separate the proteins according to their affinity to the resin. Proteins with strong ionic interactions, and thus high affinity, will eluate at a later time point than proteins with low affinity. Through monitoring the UV emittance, it is possible to separate target proteins from other impurities.

As the SDS-PAGE analysis of the purified protein from the IMAC procedure showed some impurities, the IEC was carried out in an attempt to remove these.

Materials:

HiTrap DEAE Sepharose FF, 5 mL column

Binding Buffer: MES Buffer A (see Section 2.11)

Elution buffer: MES Buffer B (see Section 2.11)

Procedure:

1. The HiTrap DEAE Sepharose FF, 5 mL column was connected to the Äkta purifier chromatographic and the system was washed with dH₂O.
2. After the initial wash, the system was washed with 50 % Elution Buffer until the UV baseline stayed constant.
3. The protein sample (2 mL) was diluted in 20 mL Binding buffer and added to the HiTrap DEAE Sepharose FF, 5 mL column. The column was subsequently connected to the system, and the sample was loaded onto the column with a flow rate of 1 mL/min.
4. Unbound protein was washed out by the addition of 50 % Buffer A until the UV baseline had stabilized.
5. The target protein was eluted with a linear Buffer B gradient with a flow rate of 3 mL/min.
6. The different fractions corresponding to the elution peak were collected and the proteins were visualized on an SDS-PAGE gel.
7. After SDS-PAGE verification, the fractions containing the target protein were concentrated, and the concentration was determined with Bradford Protein Assay (see Section 3.25).

3.26. Ultrafiltration and Buffer Exchange of Purified proteins

Ultrafiltration was performed after the IMAC procedure (Section 3.24) and the IEC procedure (Section 3.25). The filtration was performed simultaneously with a buffer exchange with a 20 mM Tris-HCl pH 8 Buffer, to remove the imidazole and salt used to eluate the target proteins.

Materials:

Vivaspin® 20, Ultrafiltration unit 3,000 MWCO

Protein Storage Buffer (see Section 2.11)

Procedure:

1. The fractions corresponding to the eluted target protein from Section 3.24 or 3.25 were combined in the ultrafiltration unit.
2. The solution was centrifuged at 4°C at 4,500 x g in a swing-rotor centrifuge until almost all of the solution except 1 mL had passed through the filter.
3. A total of 40 mL 20 mM Tris-HCl pH 8 Buffer was added to the column in consecutive runs.
4. The sample was centrifuged until 1 mL remained. The remainder was transferred to a sterile Eppendorf tube and stored at 4°C.
5. SDS-PAGE was carried out in order to determine the purity of the sample.

3.27. Quick Start™ Bradford Protein Assay

The Bradford Protein Assay is a method used to determine protein concentration through the binding of the dye Coomassie Brilliant Blue G-250 to proteins. The dye has a cationic, anionic and neutral form, with the cationic being the most abundant under acidic conditions. The cationic form is double-protonated and has an absorbance maximum (A_{\max}) of 470 nm. However, binding of the dye to proteins triggers a shift in A_{\max} from 470 nm to 595 nm. By measuring the absorbance at 595 nm in a spectrophotometer, it is possible to determine the protein concentration of an unknown sample with the use of a protein standard curve. In this experiment a Bovine Serum Albumin standard curve was used to determine the protein concentrations of different samples.

Materials

Protein Assay Dye Reagent Concentrate

Polystyrene cuvettes 1,5 mL

Sample Buffer: 25 mM Bis-Tris propane pH 9,5

Concentrated protein solution

Procedure:

1. The protein samples to be measured was prepared by adding 10 μl of the concentrated protein solution to 790 μl sample buffer, to a total volume of 800 μl .
2. 200 μl Protein Assay Dye Reagent was added to the samples. A control sample was prepared by adding 200 μl Protein Assay Dye Reagent to 800 μl sample buffer.
3. The samples were incubated at room temperature for 5 minutes, before the absorbance at 595 nm was measured. The mean sample concentration was calculated from three replicates.

4. Results

The antigen used in this study was the modMART1_DC protein. The antigen is a fusion protein consisting of the modified MART-1 protein and a dendritic cell -binding peptide (DC-peptide) (see Fig. 1.4). In previous studies, the native MART-1 protein has been shown to be able to elicit the generation of anti-melanoma cytotoxic T cells, making the antigen a good candidate for cancer vaccines and immunotherapies (Chodon et al. 2014; Valmori et al. 2000). In addition, the dendritic cell-binding peptide identified by Curiel et al. (2004) has shown increased internalization of fusion antigens into dendritic cells (Mohamadzadeh et al. 2009).

The *modMART1_DC* gene was codon-optimized for *L. plantarum* and ordered from GenScript, USA. The gene was delivered in the pUC_57 vector (see Section 2.7 for plasmid description). In this study, *modMART1_DC* was cloned into plasmids that would either lead to secretion or anchoring of the target protein on the surface of *L. plantarum*. A total of six vectors were constructed, which all were derivatives of the pSIP-vector which allow for inducible gene expression by the addition of an inducer peptide, SppIP (Sørvig et al. 2003; Sørvig et al. 2005). All constructs were characterized in terms of growth rate and surface exposure of the antigen.

It was also considered interesting to quantify the amount of antigen produced by each bacteria harboring the different constructs, as it is vital to know how much antigen is administered during putative vaccine trials. To be able to get a quantitative measurement of antigen production, *modMART1_DC* was cloned into an expression vector for protein production. *modMART1_DC* was subsequently purified to obtain sufficient protein for a semi-quantitative assay.

4.1. Construction of a Plasmid for Cell Wall Anchoring of the Antigen

To achieve cell wall anchoring of the antigen, the gene was fused to a cell wall binding LPXTG motif. The pLp3050_Ag85E6_cwa2 plasmid (Øverland 2013) was used to construct the plasmid for cell wall anchoring of the MART1-antigen. In this vector, the gene is fused N-terminally to a signal peptide (*Lp_3050*), and C-terminally to a cell wall anchor containing the

LPXTG motif (*cwa2*). Through this motif the anchor covalently binds the C-terminus of the protein to the cell wall.

The synthetic modMART1_DC gene was delivered in the pUC57 vector with the *Sall* and *HindIII* restriction sites flanking each terminus of the gene. However, in the pUC57 vector, the DC motif is located C-terminally, and to ensure that the dendritic cell binding motif would protrude from the cell, the motif was moved from the C-terminus to the N-terminus of the protein. This was achieved by a 5-step overlap-extension PCR with pUC57_modMART1_DC as the initial template (Fig 4.1). The PCR product of each run was used as the template for the subsequent reaction as the five forward primers, MART1DC_F1 to MART1DC_F5, (see Section 2.7 Table 2.8 and 2.9) were designed to consist of partially overlapping 3'-ends that gradually elongated the template on the 5'-end. As the Ag85E6 gene in the pLp3050_Ag85E6_cwa2 vector is flanked by a *Sall* and a *MLuI* restriction site, the *Sall* restriction site was therefore introduced by the last forward primer (MART1DC_F5), and the *MLuI* restriction site by the reverse primer (MARTMLuR), in order to be able to clone the insert into the vector. Agarose gel electrophoresis was used for verification of each PCR reaction (data not shown).

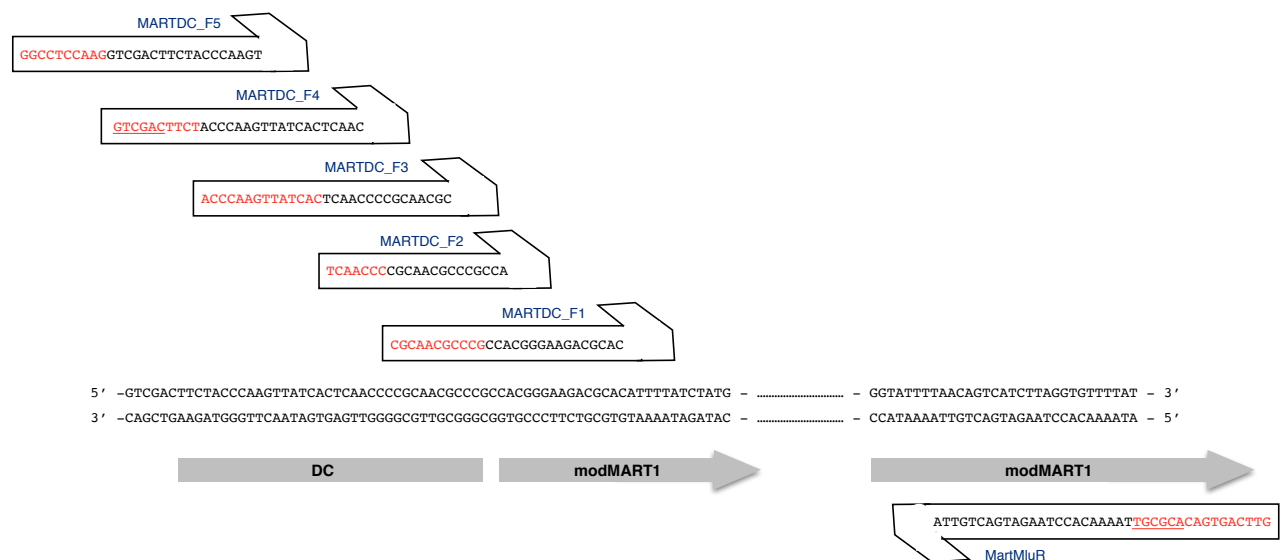


Figure 4.1 Schematic overview of the step-wise elongation of the 5'-end to move the DC-motif N-terminally. The overlap-extension PCR was achieved by the use of 5 forward primers that gradually incorporated the DC-sequence on the 5'-end. Each elongating sequence is denoted in red. Incorporated restriction sites, *Sall* and *MLuI* is underlined in the sequence. Primer names are given in blue.

Subsequently, the resulting PCR-product and the vector pLp_3050_Ag85E6_cwa2 were digested with the restriction enzymes *SalI* and *MluI*, before ligation and transformation into One Shot® TOP10 Chemically Competent *E. coli* (Fig. 4.2). After sequence verification by GATC Biotech, the plasmid was transformed into electrocompetent *L. plantarum* (Section 3.14).

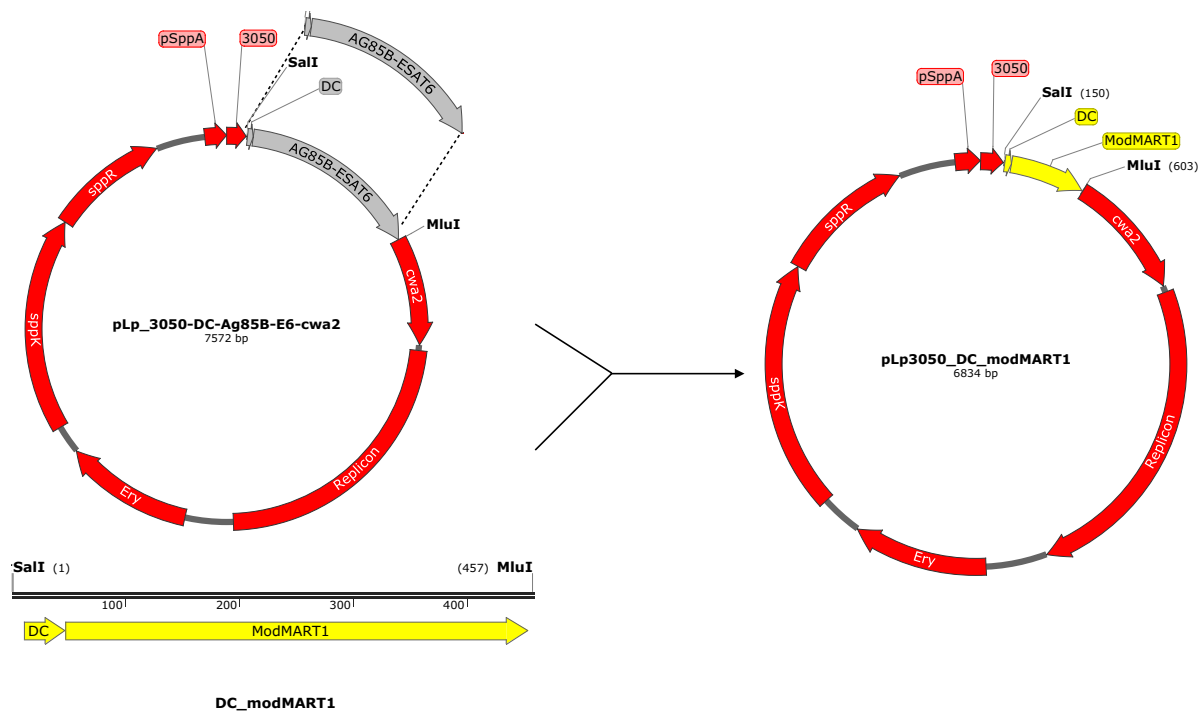


Figure 4.2 Cloning strategy for the construction of pLp_3050_DC_modMART1_cwa2 for covalent cell wall anchoring of the antigen. The PCR product of the overlap extension PCR (See Fig. 1) and the vector, pLp_3050_DC_modMART1_cwa2, were digested with *SalI* and *MluI* and subsequently cloned yielding the pLp_3050_DC_modMART1_cwa2 plasmid

4.2. Construction of Plasmids for Membrane Anchoring of the Antigen

In order to anchor the modMART1_DC antigen to the cell membrane, two lipoprotein anchors were selected. These anchors were derived from the *Lp1261* and *Lp1452* genes in *L. plantarum* WCFS1, and have successfully been used several times to anchor heterologous proteins to the cell membrane (Kuczkowska et al 2015; Fredriksen et al 2012). Thus, two plasmids for membrane anchoring of the modMART1_DC antigen were constructed, pLp_1261_modMART1_DC and pLp1452_modMART1_DC. These two lipoprotein anchors

attach the protein N-terminally to the cell membrane (see Section 1.7.2), hence the DC motif was kept on the C-terminus of the gene.

In-Fusion Cloning (Section 3.11) was used to generate the modMART1_DC gene insert for the vector containing the *Lp1452*-derived lipoanchor. In-Fusion1452_R primer (Section 2.7) were designed to introduce an *EcoRI* restriction site. The pUC57_modMART1_DC was used as the template for the In-Fusion amplification PCR (Fig 4.3) with the forward primer InFusion1452_F and reverse primer, InFusion1452_R (see Section 2.7).

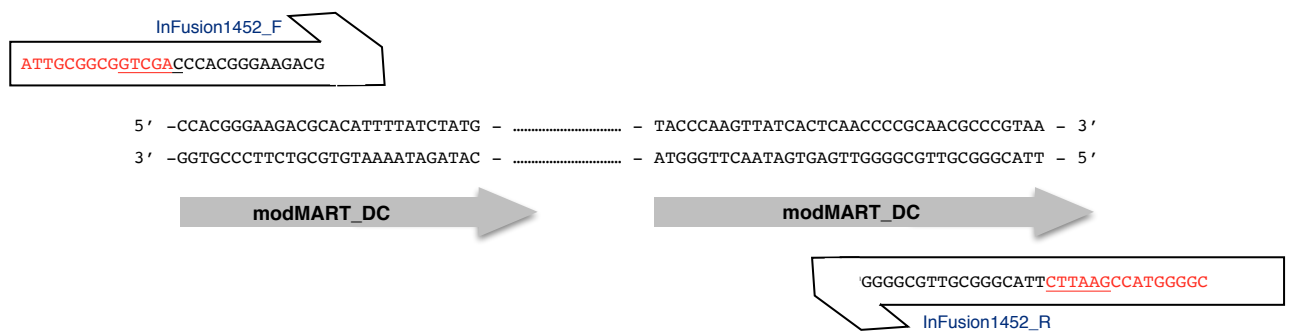


Figure 4.3 Generation of gene insert for InFusion cloning. The gene insert for InFusion cloning was generated by PCR amplification with the primers InFusion1452_F and InFusion1452_R. The primers contain vector complementary overhangs and introduce restriction sites that will ensure the successful cloning into the vector. The vector complementary overhangs are denoted in red, restriction sites are underlined. Primer names are given in blue

The 476 bp PCR product was cloned into the *Sall* and *EcoRI* predigested pLp1452_Invasin vector, yielding the pLp_1452_modMART1_DC (Fig 4.4). The plasmid was transformed into One Shot® TOP10 Chemically Competent *E. coli*, and the sequence was verified through sequencing by GATC Biotech, before transformation into *L. plantarum*.

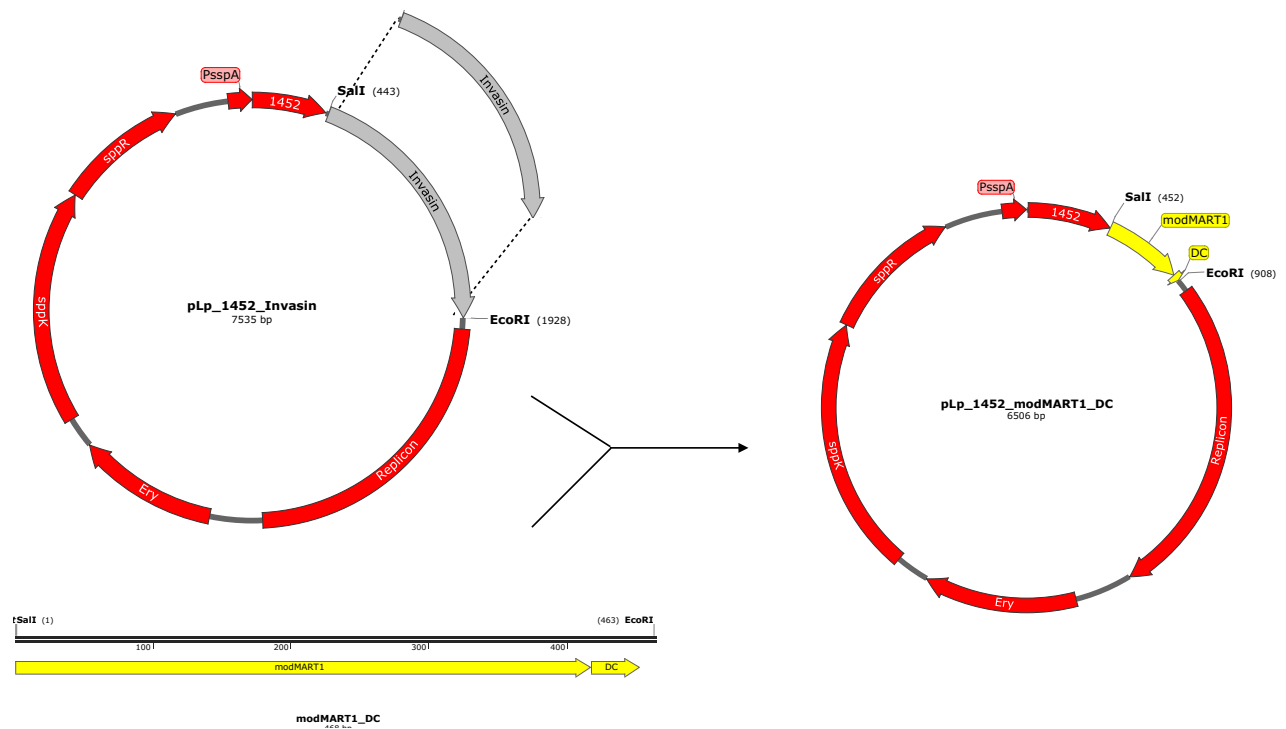


Figure 4.4 InFusion cloning strategy for the construction of pLp_1452_modMART1_DC for membrane-anchoring of the antigen. The vector is linearized by restriction digestion, and mixed with the gene insert generated from a PCR with vector complementary overhangs generated by InFusion primers (Table 2.8) and the InFusion 5X HD Enzyme Premix that contains enzymes for cloning

To construct pLp1261_modMART1_DC, modMART1_DC was cloned into the pLp_1261-Ag85B-ESAT6 plasmid (Tjåland 2011). The Ag85B-ESAT6 gene is flanked by a *SalI* restriction site at the C-terminus and a *HindIII* restriction site at the N-terminus, similarly to the modMART1_DC gene in the pUC57 vector. Therefore, pLp_1261-Ag85B-ESAT6 and pUC57_modMART1_DC were initially digested with *SalI* and *HindIII*, ligated and transformed into *E. coli* TOP10. Several attempts at this cloning procedure failed as no transformants harbored the correct plasmid, as verified by colony-PCR (Section 3.4.2). Furthermore, an attempt at In-Fusion cloning with the In-Fusion primers InFusion1261_F and InFusion1261_R (Section 2.7) yielded the same negative result. However, the successful construction was achieved through restriction digestion of the pre-constructed plasmid pLp1452_modMART1_DC and pLp_1261-Ag85B-ESAT6 with *BamHI* and *SalI*, before subsequent ligation and transformation into *E. coli* TOP10 (Fig. 4.5). After sequence verification by GATC Biotech, the plasmid was transformed into *L. plantarum*.

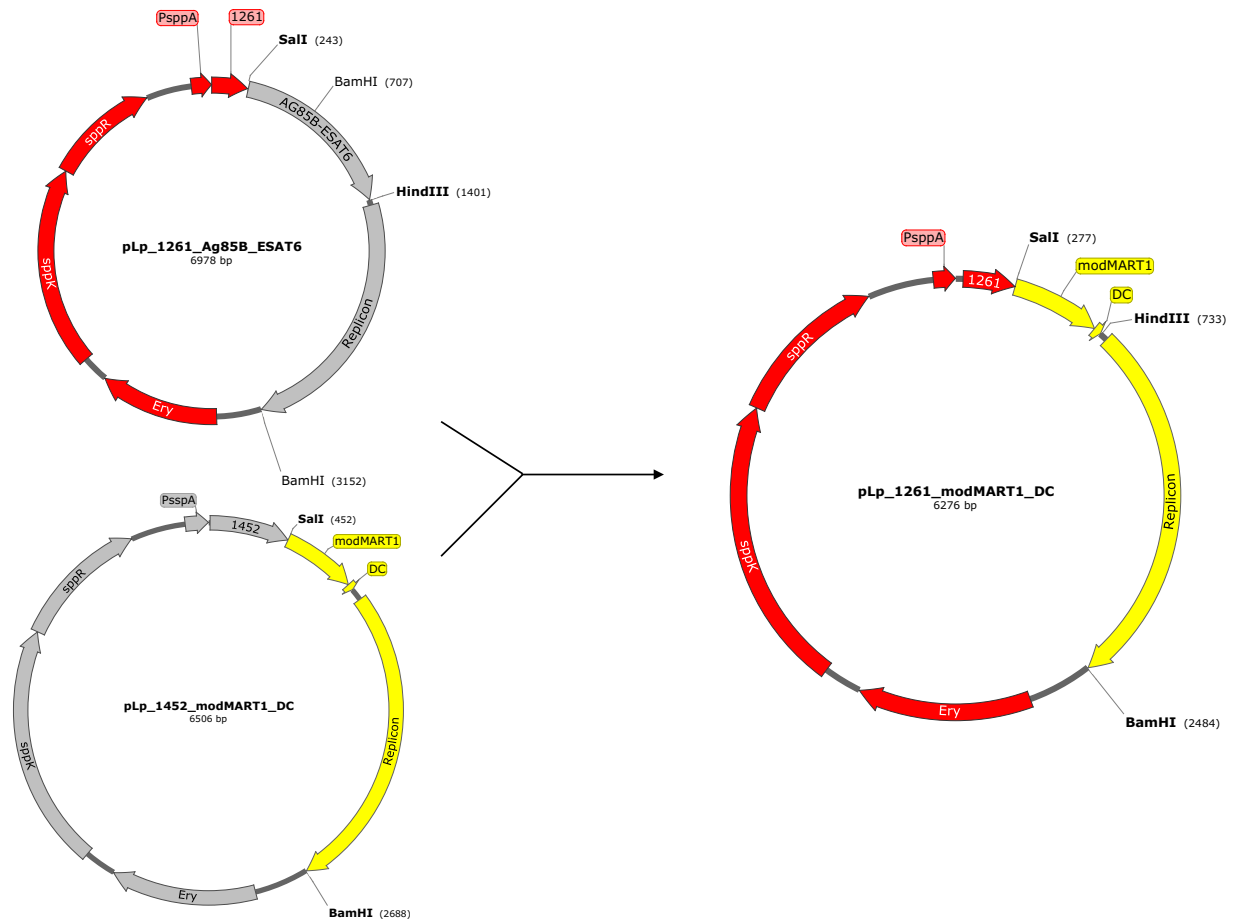


Figure 4.5 Cloning strategy for the construction of pLp_1261_modMART1_DC. The plasmids pLp_1261_Ag85B_ESAT6 and pLp_1452_modMART1_DC was digested with the restriction enzymes SalI and BamHI. Fragments were separated with agarose gel-electrophoresis, and fragments of correct size were ligated, yielding the pLp_1261_modMART1_DC construct.

4.3. Construction of Non-covalently bound Antigen to the Cell Wall

The non-covalent binding of proteins to peptidoglycan of the bacterial cell wall is often achieved through a LysM Domain in Gram-positive bacteria. One plasmid for non-covalent binding of the antigen to the cell wall was constructed in this study, the pLp_3014_modMART1_DC construct. The antigen is fused to the full length Lp_3014-protein, which is a putative transglycosylase with an N-terminal LysM domain. The modMART1_DC gene was excised with the restriction enzymes SalI and HindIII from the pUC57_modMART1_DC plasmid. The plasmid pLp_3014-Ag85E6-DC (Målbakken 2014) was digested with the same restriction enzymes, and subsequently the fragments were ligated and transformed into *E. coli* TOP10 (Fig. 4.6). After sequencing, the plasmid was successfully transformed into *L. plantarum*.

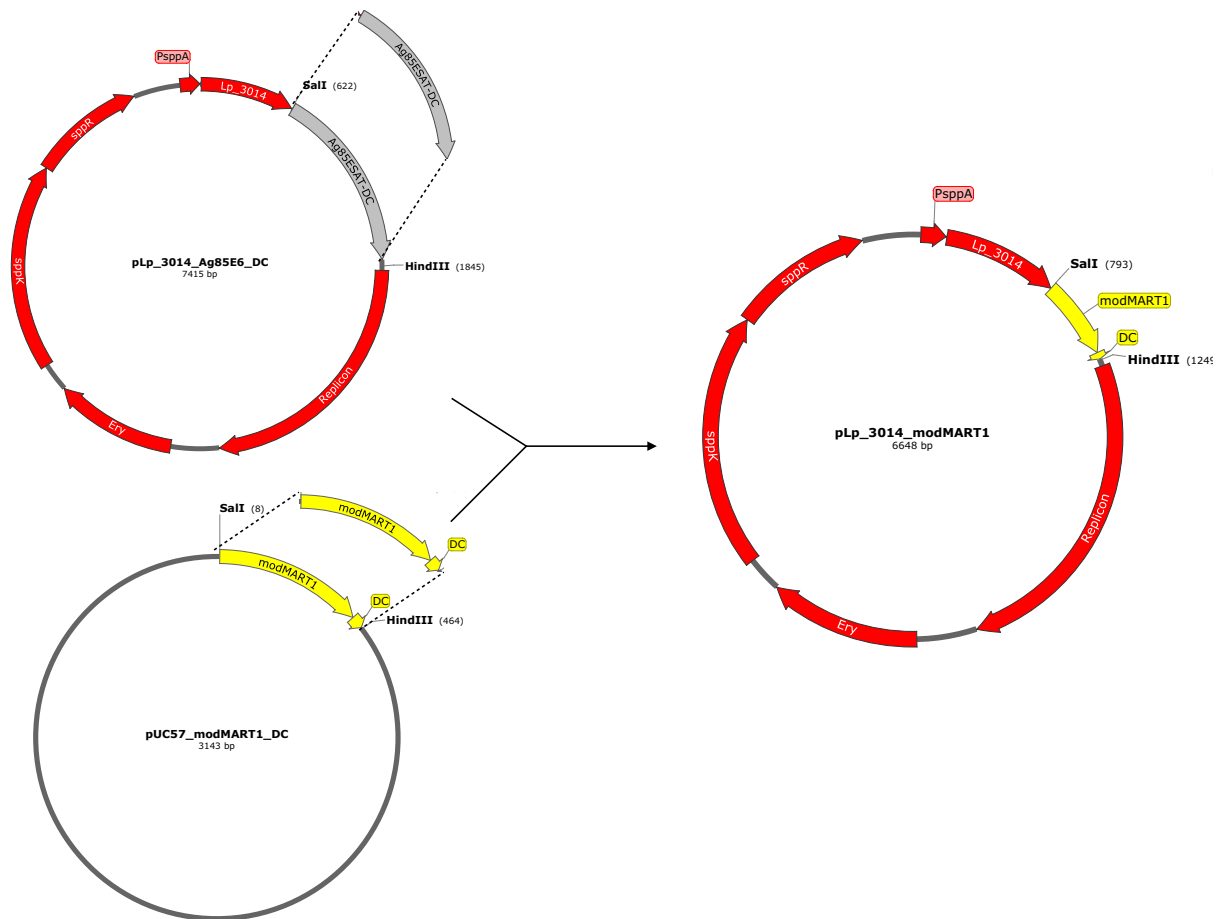


Figure 4.6 Cloning strategy for the construction of pLp_3014_modMART1_DC for cell wall anchoring through a LysM Domain. The *modMART1_DC* gene was excised from the pUC57-vector with the restriction enzymes SalI and HindIII. The vector pLp_3014_Ag85BE6_DC was digested with the same enzymes, and the fragments were ligated, yielding the pLp_3014_modMART1_DC construct.

4.4. Construction of Plasmids for Secretion of *modMART1_DC*

The *modMART1_DC* gene was fused to two different signal peptides without an anchoring motif to generate two plasmids for secretion of the *modMART1_DC* antigen. The signal peptides used were the *Lp_3050* and *Lp_0373* proteins which both have been utilized as signal peptides for secretion in previous studies (Øverland 2013, Fredriksen 2007).

The plasmid pLp_3050-DC-Ag-E6-cwa2 was digested with SalI and HindIII to generate the vector backbone lacking the gene and the cell wall anchor. *modMART1_DC* was excised from the pUC57_ *modMART1_DC* vector using the same restriction enzymes, and was subsequently cloned into the *Lp_3050* containing vector (Fig.4.7). Thus, generating a plasmid containing the *modMART1_DC* gene fused to a signal sequence (*Lp3050*) that would

translocate the protein from the cytosol to the exterior. The same strategy and the same restriction enzymes were used for the construction of the pLp0373_modMART1_DC plasmid, where the pLp_0373-Ag85E6-cwa2 functioned as the vector backbone to the modMART1_DC insert.

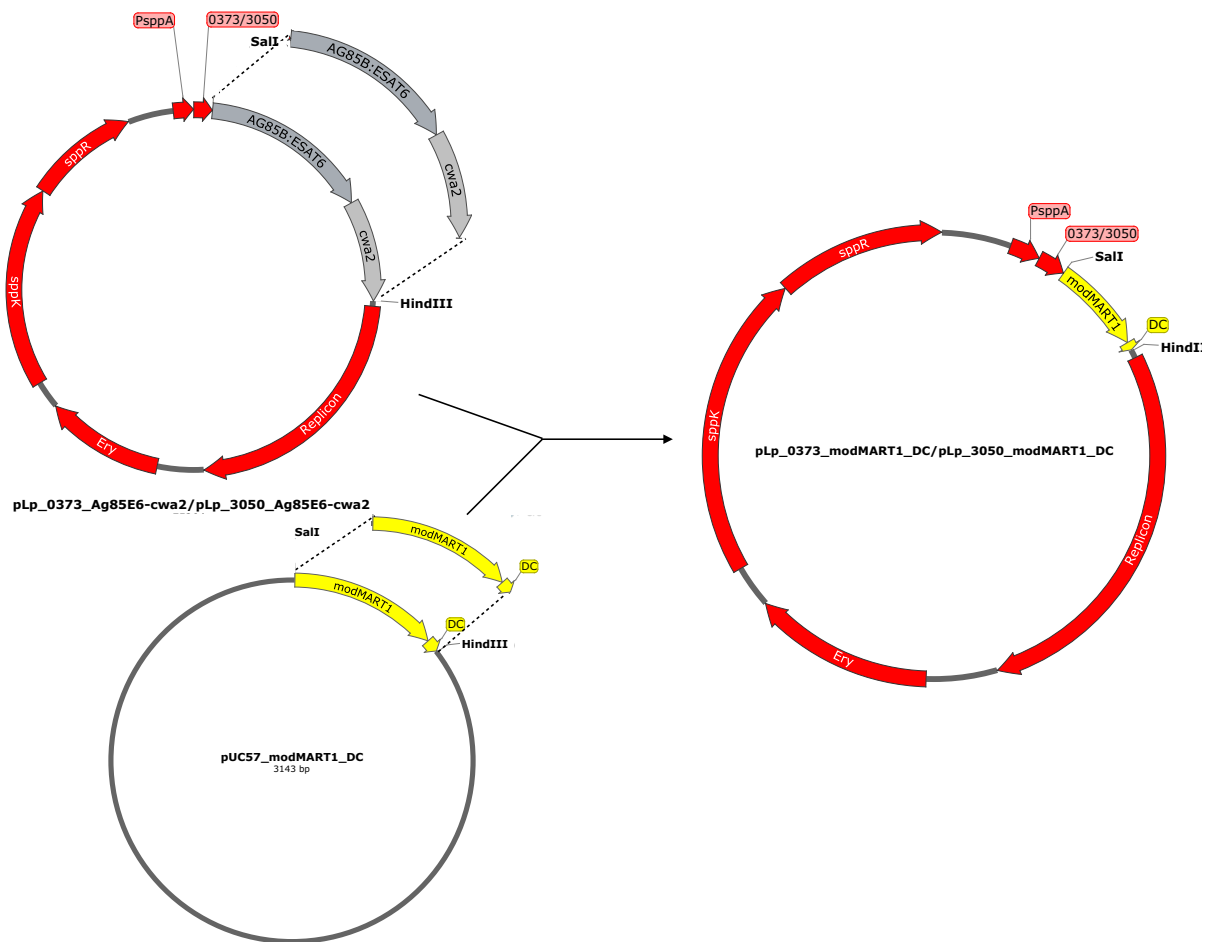


Figure 4.7 Cloning strategy for the construction of pLp_0373_modMART1_DC and pLp_3050_modMART1_DC for secretion of the antigen. The *modMART1_DC* gene was excised from the pUC57-vector with the restriction enzymes SalI and HindIII. The vectors pLp_0373_Ag85BE6_cwa2 and pLp_3050-Ag85BE6_cwa2 were digested with the same enzymes, and the fragments were ligated, yielding the pLp_0373_modMART1_DC and pLp_3050_modMART1_DC construct.

4.5. Construction of plasmid for production and purification of modMART1_DC

To be able to produce and purify the recombinant protein, the *modMART1_DC* gene was cloned into the pNIC-CH expression vector by the Ligation-Independent Cloning procedure described in Section 3.12. The pNIC-CH vector had previously been digested and treated with

the T4 Polymerase by Dr. Lasse Fredriksen. To generate the gene insert for the LIC procedure, PCR amplification with Q5[®] High-Fidelity 2 x Master Mix (Section 3.4.1) with the forward primer pNIC_LIC_F and reverse primer pNIC_LIC_R was performed (see Section 2.7 Tables 2.7 & 2.8 for primer sequence information and description). These primers have a chimeric sequence, consisting of bases complementary to *modMART1_DC* and to the pNIC-CH vector. The pUC57_*modMART1* vector was used as the template for amplification. During PCR, the insert complementary ends of the chimeric primers hybridize to *modMART1_DC*, and chimeric PCR-products with both a vector and insert complementary sequence are generated. After amplification, the PCR fragments were treated with T4 polymerase to generate the vector complementary overhangs and subsequently cloned into the predigested pNIC-CH vector as described in section 3.12, before transformation into *E. coli* BL21.

Agarose gel electrophoresis analysis was performed of the PCR amplification fragments and transformation colonies with the pNIC_LIC_F and pNIC_LIC_F primers to verify successful PCR and to find candidates for sequencing by GATC Biotech (Fig 4.8).

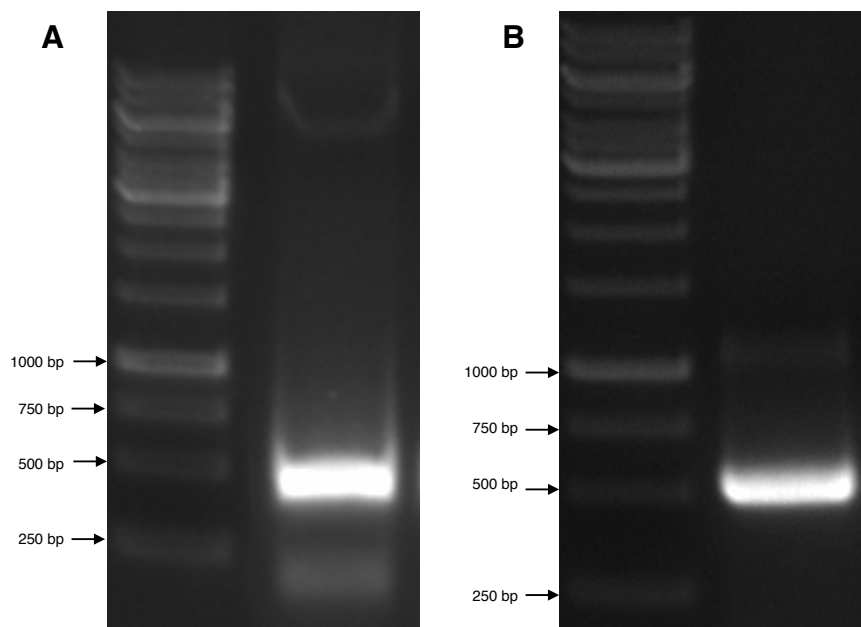


Figure 4.8 Agarose gel electrophoresis of PCR fragments. (A) Agarose gel electrophoresis of PCR fragment from insert generation. Predicted fragment size was 476 bp. (B) Agarose gel electrophoresis of PCR fragment generated from colony PCR. Predicted fragment size was 476 bp

4.6. Growth Curve Analysis of *L. plantarum* harboring different Plasmids

Previous research has shown that the production of heterologous proteins may impede the bacterial growth (Kuczkowska et al 2015; Myrbråten 2016). To investigate the potential effect of recombinant protein production on the growth of *L. plantarum* harboring different plasmids for modMART1_DC production, the growth rates after induction with SppIP was determined by measuring the absorbance (OD₆₀₀) of the bacterial cultures every hour for 8 hours. *L. plantarum* carrying the pEV plasmid was used as a control since it lacks a gene for expression.

The samples for the growth curve analysis were prepared by diluting overnight cultures of *L. plantarum* in pre-warmed MRS to an OD₆₀₀-value of 0,10 and before incubation at 37 °C without shaking. When the cultures reached an OD₆₀₀ of ~ 0,3, the production of the recombinant proteins was induced by the addition of 25 ng/mL SppIP. All the *L. plantarum* carrying the different plasmids showed similar growth up to the time of induction (data not shown).

The *L. plantarum* carrying the empty vector (pEV) displayed a higher growth in comparison to the bacteria producing the modMART1_DC protein (Fig 4.9). Amongst the latter, *L. plantarum* carrying the plasmid pLp_1261_modMART1_DC showed the highest growth which anchors modMART1_DC to the cell membrane. Interestingly, the bacteria with the other lipoprotein anchor, Lp1452, showed nearly no growth at all. The remaining constructs exhibited a steady increase in absorbance and reached nearly the same OD₆₀₀ values after 8 hours (Fig. 4.9). However, bacteria harboring plasmids that only contained a signal peptide (pLp3050_modMART1_DC and pLp0373_modMART1_DC) showed a slightly higher growth than all the bacteria with anchored antigens, with the exception of pLp1261_modMART1_DC.

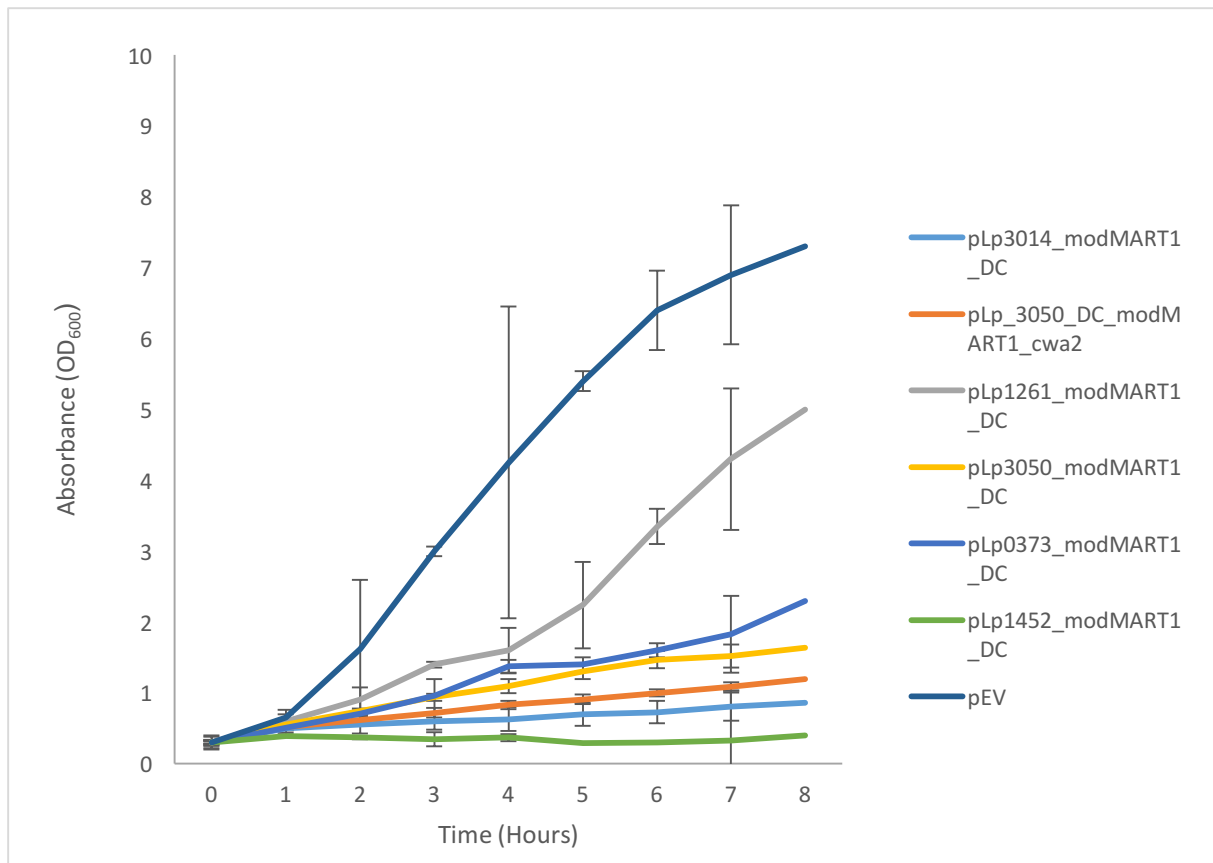


Figure 4.9 Growth Curves of recombinant *L. Plantarum* harboring plasmids for antigen production. *The bacterial cultures were induced with SppIP when the absorbance (OD_{600}) reached 0,3 (0 Hours). After induction the absorbance was measured every hour for a total of 8 hours. The absorbance values at each time point is the average of 3 independent experiments.*

4.7. Western blot analysis of antigen production

Western blot analysis was used to verify the production of modMART1_DC after induction with SppIP. The bacteria were cultivated according to Section 3.17.1, before harvesting 3 hours after induction. Cell-free protein extracts were obtained by cell disruption using glass beads as described in section 3.17.2. To ensure that approximately the same number of cells from each culture was used in the experiment, the samples were adjusted according to the OD_{600} measured at the time of harvest. Western blot analysis was also carried out on the culture supernatants obtained during harvesting of the cells to investigate the presence of the antigen in the supernatants. The culture supernatants were precipitated with trichloroacetic acid (TCA) as described in section 3.17.3 prior to the procedure, as almost no amount of protein was observed during SDS-PAGE analysis of undiluted samples.

A Western blot of cell free protein extracts and precipitated culture supernatants from *L. plantarum* harboring the empty vector pEV (2 and 3) and from bacteria carrying plasmids for anchoring of the antigen (4 through 11) is presented in Figure 4.10. There is a slight discrepancy between the theoretical molecular weight of the protein and the molecular weight observed in the Western blot for some of the constructs. The cell wall anchor and the LysM Domain anchor have a lower estimated molecular weight than what is observed in the blot. The lipoprotein anchor 1261 was in accordance to the estimated molecular weight. The protein bands from these constructs, shows a similar intensity, indicating equal production of the protein. However, the antigen anchored with the lipoprotein anchor 1452 is not detected in this particular blot, though previous blots have showed that the antigen is successfully produced (data not shown). No antigen was detected in the culture supernatants through western blotting as illustrated in Figure 4.10.

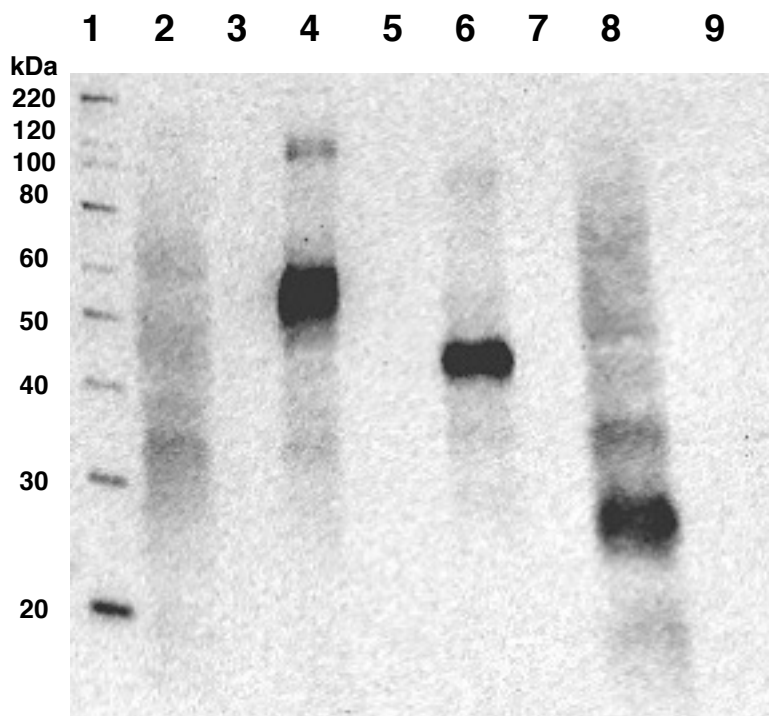


Figure 4.10 Western blot of cell-free protein extracts and TCA precipitated culture supernatants from *L. plantarum* harboring plasmids for anchoring of the antigen to the cell surface. (1) MagicMark™ XP Western Protein Standard; (2) pEV Cell Free Extract; (3) pEV culture supernatant (SN); (4) pLp3050_DC_modMART1_cwa2 (44 kDa) Cell Free Extract; (5) SN; (6) pLp3014_modMART1_DC (40 kDa) Cell Free Extract; (7) SN; (8) pLp1261_modMART1_DC (26 kDa) Cell Free Extract; (9) SN. Theoretical molecular weight is shown in parentheses.

The Western blot of cell free protein extracts and culture supernatants treated with TCA/DOC from the two constructs with only a N-terminal signal sequence is shown in Figure 4.11. Since the antigen is only translationally fused to signal peptide that transports the protein out of the cell, the antigen should be located in the culture supernatant. Surprisingly, no antigen was detected in either the untreated supernatants, or the supernatants precipitated with TCA and DOC even though the production had been verified. Methanol/Chloroform precipitation and Acetone precipitation yielded the same result (data not shown).

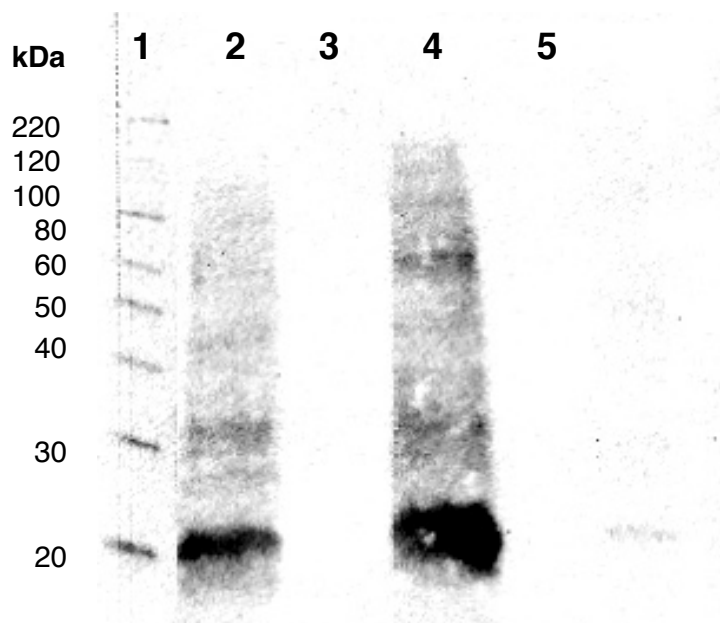


Figure 4.11 Western blot of cell free extracts and TCA precipitated culture supernatants from *L. plantarum* harboring plasmids for secretion of the antigen. (1) MagicMark™ XP Western Protein Standard; (2) pLp3050_modMART1_DC (23 kDa) Cell Free Extract; (3) pLp3050_modMART1_DC culture supernatant (SN); (4) pLp0373_modMART1_DC (23 kDa) Cell Free Extract; (5) SN. Theoretical molecular weight is shown in parentheses.

4.8. Detection of Antigen on the Surface of *L. plantarum* by Flow Cytometry

Flow cytometry of bacterial cells was performed to investigate whether the antigen was successfully translocated to and exposed on the bacterial surface. Bacterial cultures of *L. plantarum* carrying the different plasmids were induced and harvested according to section 3.17.1. The harvested cells were incubated with the primary antibody targeting the MART1 antigen, and subsequently with the secondary antibody conjugated to the fluorochrome FITC for fluorescent detection by flow cytometry (See section 3.20.1 for details).

The data generated from the flow cytometry analysis may be presented in a histogram where the y-axis represents the number of bacterial cells sampled, and the x-axis represents the relative fluorescence of FITC as measured by the flow cytometer. Hence, a greater shift to right signifies a higher fluorescent signal. The histograms generated from the flow cytometry analysis of the FITC-stained bacteria is presented in Figure 4.12. The y-axis was normalized in the MacsQuantify™ Software, in addition to smoothing of the curves. The histograms are representative of 4 independent experiments.

As expected, no fluorescent signal was detected from the bacteria harboring the empty vector, pEV (Fig. 4.12). A shift in the fluorescence signal was observed for the lipoprotein anchor construct pLp1452_modMART1_DC, while the highest fluorescent signal was observed for the cell wall anchor construct pLp3050_DC_modMART1_cwa2. However, no shift was observed for the peaks of the lipoprotein anchor pLp1261_modMART1_DC and the LysM domain anchor pLp3014_modMART1_DC, indicative of no antigen hybridization on the bacterial surface. The flow cytometry histogram of the two constructs fused to a signal peptide showed the same trend with no shift compared to the empty vector (Fig. 4.12 B).

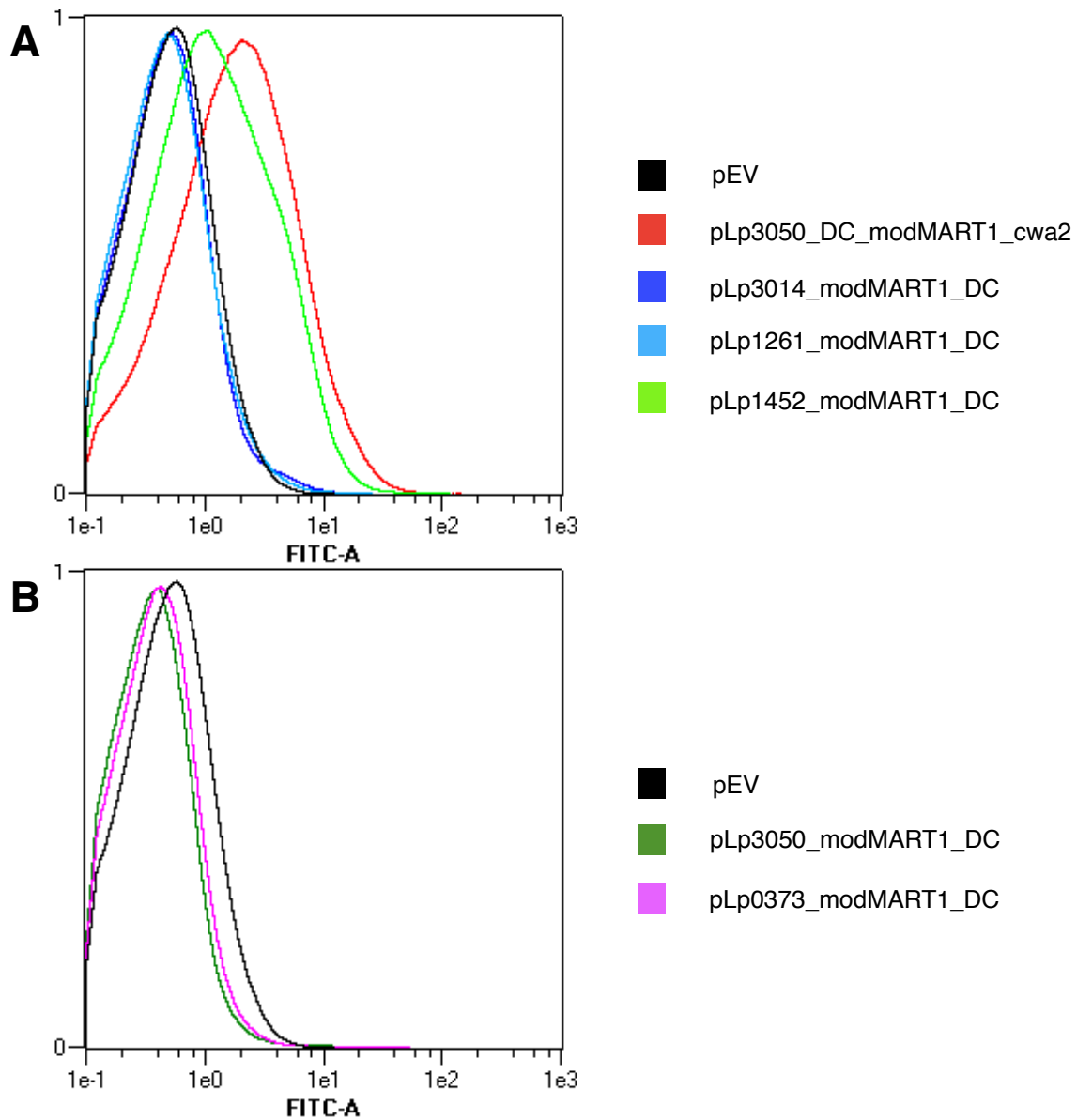


Figure 4.12 Flow Cytometry analysis of FITC stained recombinant *L. plantarum*. The results are shown as histograms with the relative fluorescence (x-axis) plotted against number of events (y-axis). Construct color coding is denoted in the figure. (A) Histogram showing the results from the flow cytometry analysis of the constructed plasmids for anchoring of the modMART1 antigen to the cell surface (B) Histogram showing the results from the flow cytometry analysis of the constructed plasmids for secretion of the modMART1 antigen.

4.9. Detection of Antigen on the Surface of *L. plantarum* after Treatment with Lysozyme

Due to varying lengths and properties of the anchors used, the antigen may become embedded in the cell wall, thus making it inaccessible for antigen hybridization. The result is a weaker fluorescent signal or no signal at all after staining with the primary antibody and secondary antibody conjugated to the fluorochrome. To investigate whether the flow cytometry results were affected by the inaccessibility of the antigen epitope, the bacterial cells were treated with lysozyme prior to staining. Lysozyme degrades the peptidoglycan of the bacterial cell wall, potentially revealing embedded proteins such as the target antigen.

Figure 4.13 shows the results from the lysozyme treatment experiment with an overlay of the untreated and lysozyme treated cells. The curves of the untreated cells (Black) were in accordance to the curves previously observed (Fig. 4.12). A small fluorescent shift was observed for the lysozyme treated empty vector, pEV, however, as it is so small it is not likely to be of significance to the other results.

Of the two constructs that previously gave a fluorescent signal without lysozyme treatment, only the bacterial cell harboring the pLp_3050_DC_modMART1_cwa2 gave a greater fluorescent shift after treatment with lysozyme. The lysozyme treatment of the lipoprotein anchor, Lp1452, and the LysM Domain anchor Lp_3014, yielded no significant shift in the observed fluorescence in comparison to the untreated cells. However, a significant shift is seen in the fluorescent signal of the bacterial cells harboring the pLp1261_modMART1_DC, indicating that the antigen may be shielded from antigen hybridization by the bacterial cell wall in untreated cells.

Interestingly, a significant shift in the fluorescence intensity was also observed in the constructs lacking an anchoring motif, pLp3050_modMART1_DC (Fig. 4.13 G) and pLp_0373_modmART1_DC (Fig. 4.13 F), indicative of hidden antigen in the cell wall.

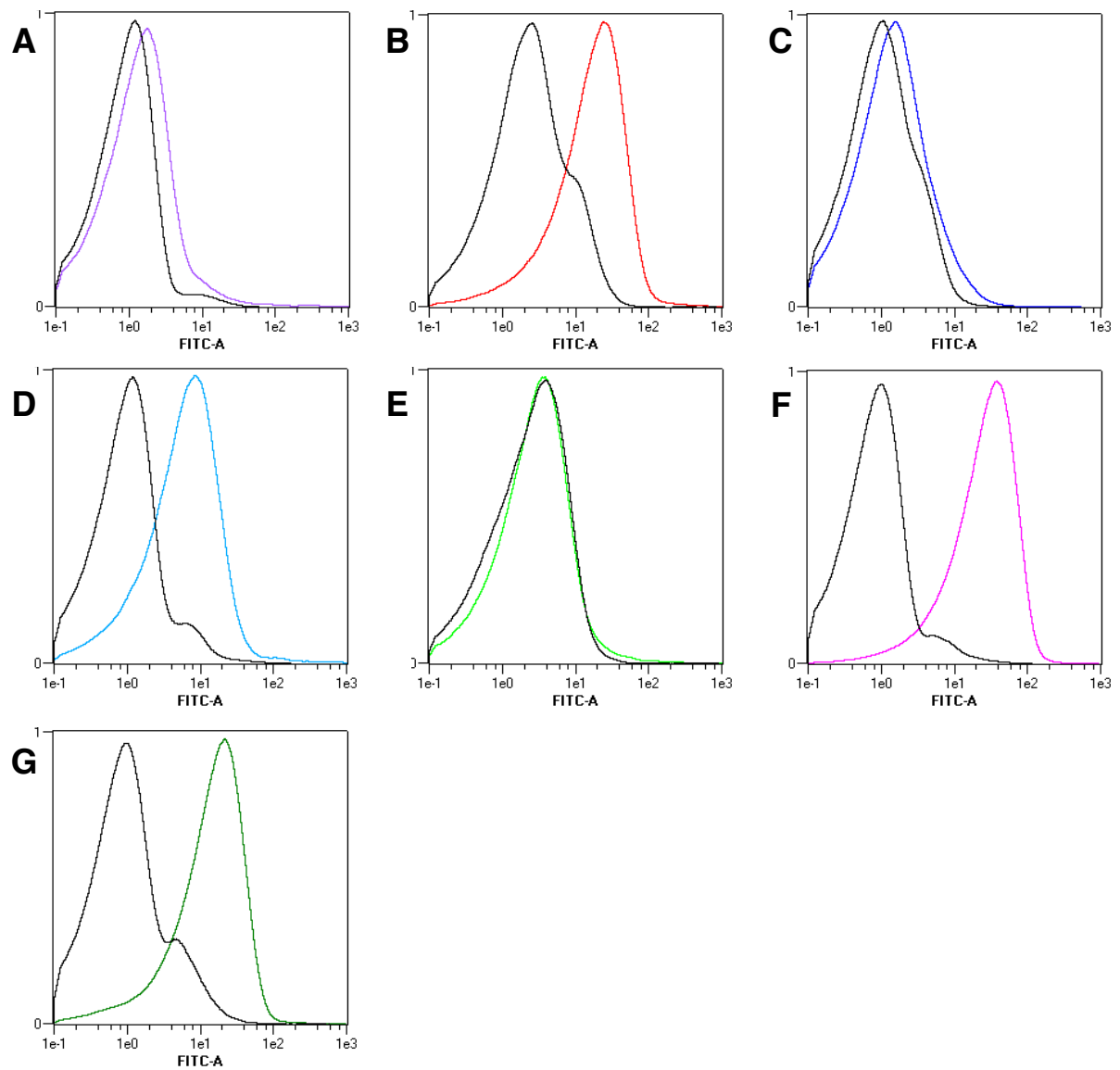


Figure 4.13 Flow Cytometry analysis of FITC stained recombinant *L. plantarum*. The results are shown as histograms with the relative fluorescence (x-axis) plotted against number of events (y-axis). The results are presented as an overlay of fluorescent signals from lysozyme treated (Curves shown in color) and untreated cells (Curves shown in black). (A) pEV; (B) pLp3050_DC_modMART1_cwa2; (C) pLp3014_modMART1_DC; (D) pLp1261_modMART1_DC; (E) pLp1452_modMART1_DC; (F) pLp0373_modMART1_DC (G) pLp3050_modMART1_DC.

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

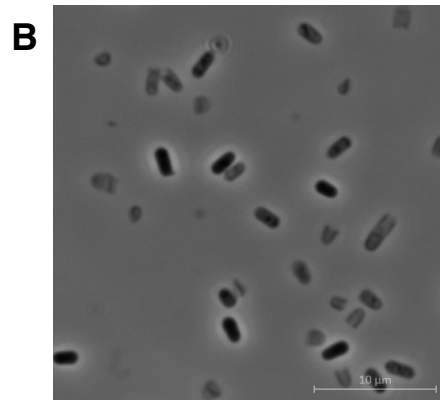
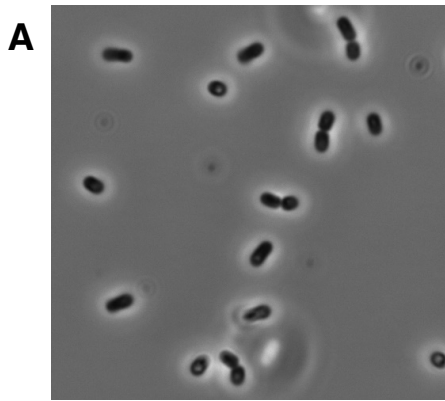
Immunofluorescent microscopy was used to further investigate the presence of the antigen on the surface of both untreated and lysozyme treated *L. plantarum*. The lysozyme treatment was carried out as described in section 3.20.2 prior to staining, while the untreated bacterial cells were stained according to section 3.20.1.

The immunofluorescence analysis was performed on untreated cells in 4 biological replicates, while analysis of lysozyme treated cells were performed 2 times on cells harvested from the same bacterial culture. The result of the microscopy analysis is shown in Figure 4.14. These are the same samples used to produce the flow cytometry data presented in Figure 4.14.

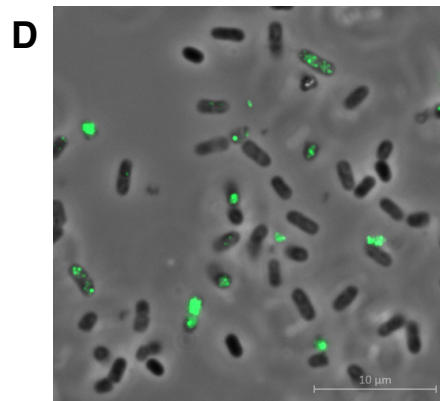
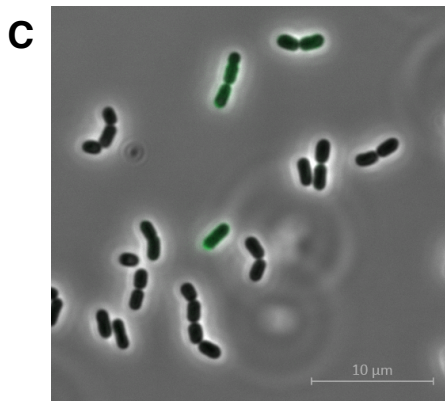
The fluorescent signals detected by the microscope are largely consistent with the findings of the flow cytometry analysis (Fig. 4.13). No fluorescent signal was detected from the control bacteria harboring the pEV vector. In accordance with Figure 4.13, the highest number of fluorescent cells were seen amongst the bacteria harboring the pLp_3050_DC_modMART1_cwa2 and pLp_1452_modMART1_DC, prior to lysozyme treatment (Fig. 4.14 C & D, I & J). The increased fluorescent intensity seen after lysozyme treatment of pLp_3050_DC_modMART1_cwa2 was also observed in the immunofluorescent microscopy analysis. An increased number of fluorescent cells were also seen after lysozyme treatment of the bacteria harboring the pLp_1261_modMART1_DC (Fig. 4.14 G and H).

In the case of the secretion constructs, pLp_0373_modmART1_DC and pLp3050_modMART1_DC, there were a high number of observed fluorescent cells after lysozyme treatment as opposed to no observed fluorescent bacteria prior to degradation by lysozyme (Fig. 4.14 K & L, and M & N respectively).

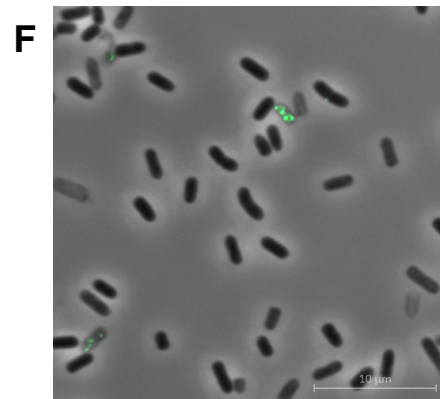
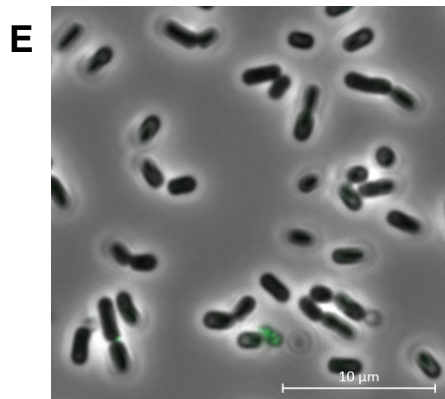
pEV



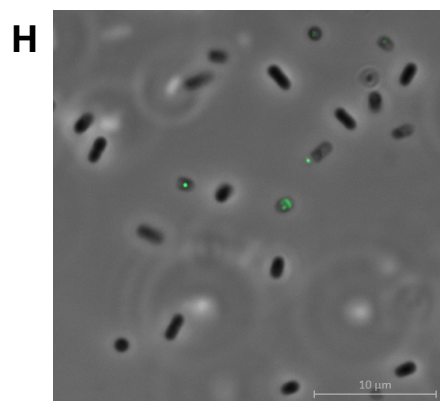
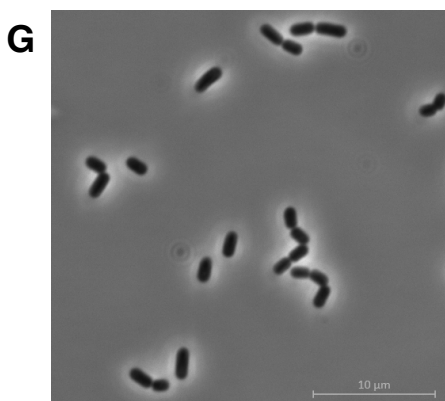
pLp_3050_DC_modMART1_cwa2



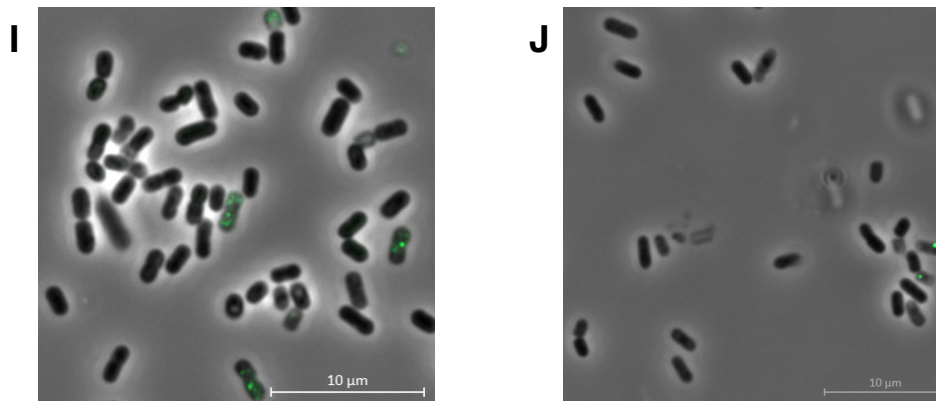
pLp_3014_modMART1_DC



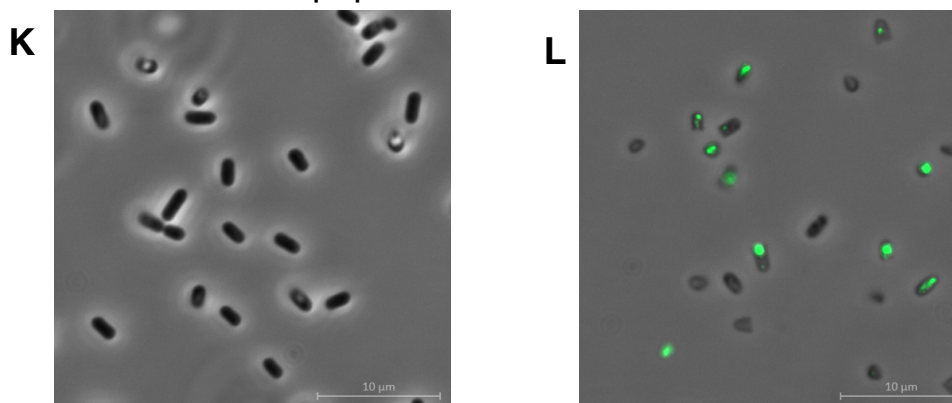
pLp_1261_modMART1_DC



pLp_1452_modMART1_DC



pLp_0373_modMART1_DC



pLp_3050_modMART1_DC

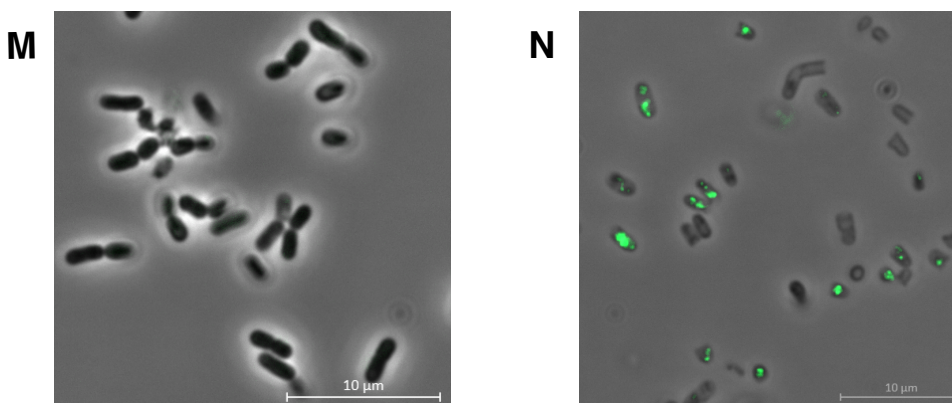


Figure 4.14 Immunofluorescent microscopy of FITC stained recombinant *L. plantarum* with and without lysozyme treatment. The untreated cells are located in the left panels, while the lysozyme treated cells are in the right panels. (A) Untreated pEV; (B) Lysozyme treated pEV (C) Untreated pLp3050_DC_modMART1_cwa2; (D) Lysozyme treated pLp3050_DC_modMART1_cwa2 (E) Untreated pLp3014_modMART1_DC; (F) Lysozyme treated pLp3014_modMART1_DC; (G) Untreated pLp1261_modMART1_DC; (H) Lysozyme treated pLp1261_modMART1_DC (I) Untreated pLp1452_modMART1_DC; (J) Lysozyme treated pLp1452_modMART1_DC (K) Untreated pLp0373_modMART1_DC (L) Lysozyme treated pLp0373_modMART1_DC (M) Untreated pLp3050_modMART1_DC; (N) Lysozyme treated pLp3050_modMART1_DC

4.11. Trypsin Digestion of the Bacterial Cell Wall

To further investigate the presence of the antigen on the cell surface, the bacterial cell wall was shaved using trypsin on viable cells, as described in section 3.20.1. Trypsin acts by cleaving peptide chains following the carboxylic C-terminus of the basic amino acids lysine and arginine. Only peptides derived from the cell wall are obtained as long as no lysis occurs. The amino acid sequence of the recombinant modMART1_DC protein was therefore analyzed with the PeptideCutter tool from ExPASy to predict sites for trypsin digestion. The predicted resulting peptide fragments consisting of more than one amino acid are listed in Table 7.1 in Appendix.

Bacteria harboring the different plasmids were grown in MRS without Tween-20. Since trypsin digestion of the bacteria carrying the pEV vector would not give any information about the surface localization of the antigen, it was omitted from the experiment. Instead, bacteria harboring a vector containing a gene for cytosolic expression of the Ag85ESAT6 antigen was included, as the identification of the Ag85ESAT antigen in the samples would indicate lysis. An initial trypsin digestion using 5 μ g trypsin did not result in the detection of any peptide fragments from the modMART1_DC protein (data not shown). As this could be due to an insufficient concentration of the enzyme, the experiment was repeated with an increased trypsin amount of 10 μ g. Table 4.1 shows the number of peptide fragments identified in the samples from each construct in this experiment while Figure 4.15 gives an overview of which fragments were identified.

Table 4.1 Number of peptide hits. Number of tryptic peptide fragments identified by LC-MS/MS after trypsin treatment of *L. plantarum* harboring plasmids for anchoring and secretion of modMART1_DC

Plasmid	Protein			
	modMART1_DC		Ag85ESAT6	
	No. of peptide hits	Total coverage (%)	No. of peptide hits	Total coverage (%)
pLp_Ag85ESAT6_cyt1	-	-	0	-
pLp_3050_DC_modMART1_cwa2	1	10	-	-
pLp_3014_modMART1_DC	2	17	-	-
pLp_1452_modMART1_DC	0	0	-	-
pLp_1261_modMART1_DC	3	25	-	-
pLp_0373_modMART1_DC	3	27	-	-
pLp_3050_modMART1_DC	4	54	-	-

Peptide fragments corresponding to the predicted tryptic digest fragments were observed in all constructs apart from the tryptic digest of pLp_1452_modMART1_DC.

MPREDAHFYGYPKGHGHSYTTAEAAAGIGILTVILGVLLLLIGCWYCRRRMGYRALMDKSL
HVGTQCALTRCPQEGFDHRDSKVSLQEKNCEPVPNAPPAYEKLSAEQSPPPYSPYTTAE
LAGIGILTVILGVLFYPSYHSTPQRP

Figure 4.15 Identified predicted tryptic fragments. Identified predicted tryptic fragments of a length greater than 3 amino acids is denoted in blue. Unidentified peptide fragments are denoted in red. The beginning of a new fragment is marked in bold.

None of the remaining identified peptides corresponded to the putative transmembrane region of the modMART1_DC protein. In the two bacterial samples harboring plasmids for secretion of the antigen, differing degrees of predicted peptide fragments were identified. Trypsin digestion of the pLp_0373_modMART1_DC sample yielded no fragments from the N-terminus of the protein, while the pLp_3050_modMART1_DC digestion resulted in the detection of one such fragment. No peptide fragments from the Ag85ESAT6 antigen were observed in the control sample. This may indicate that no lysis had occurred during the sample processing, however, the spectra of all peptides detected indicated a high number of identified proteins.

4.12. Production and purification of modMART1_DC in *E. coli* BL21

In a potential vaccine trial, it is important to know the amount of antigen that is administered. To investigate how much of the modMART1_DC antigen was produced by bacteria harboring the different plasmids, *modMART1_DC* was cloned into the pNIC-CH vector and transformed into an overexpression host to produce the protein in excess as described in Section 3.12 and 3.23. The aim was to produce and purify enough protein to be able to run a semi-quantitative analysis of antigen produced. *E. coli* has previously been used as an expression host for the production of the MART1-protein with the aim of using the purified protein for research (Bardliving et al. 2013; Kawakami et al. 1997).

An initial small scale pilot study of the protein expression step (see Section 3.23.1 and 3.23.2) was carried out to investigate whether it was possible to produce and purify the modified protein, or if the protein would aggregate and form inclusion bodies. A 15 mL overnight culture of *E. coli* BL21 pNIC-CH_modMART1_DC was induced with 0,5 M IPTG. The culture was then allowed to grow overnight, before harvesting by centrifugation and subsequent sonication. After centrifugation, the pellet fraction and the supernatant fraction were analyzed with SDS-PAGE. The analysis indicated that the target protein was located in the supernatant fraction, however, in small amounts (Fig. 4.16).

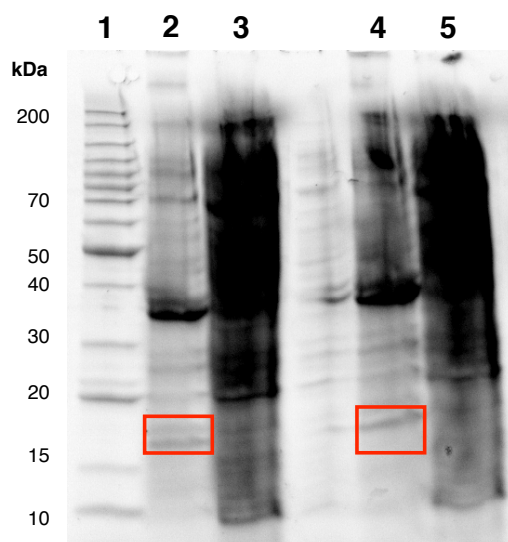


Figure 4.16 Expression of modMART1_DC in *E. coli* BL21. SDS-PAGE analysis of pellet and supernatant fractions after harvesting. Protein of interest is indicated by a red rectangle. (1) The BenchMark™ Protein Ladder (2) Supernatant fraction of sonicated cells from cell culture induced with 0,5 M IPTG. (3) Pellet fraction of sonicated cells from cell culture induced with 0,5 M IPTG; (4) Supernatant fraction of sonicated cells from cell culture induced with 0,5 M IPTG; (5) Pellet fraction of sonicated cells from cell culture induced with 0,5 IPTG.

Based on these results, a large-scale production and subsequent purification was carried out. The cultivation and harvesting of the bacteria was done according to Section 3.23.1 and 3.23.2. An SDS-PAGE analysis was performed of the supernatant and pellet fractions, which yielded the same results as in the pilot study (data not shown).

4.12.1. Ion Metal Affinity Chromatography (IMAC) and Ion Exchange Chromatography (IEC)

To separate the modMART1_DC protein from other expression host proteins, Ion Metal Affinity Chromatography (IMAC) was performed with a Ni²⁺ affinity HisTrap High-Performance (HP) 5 x 5 mL column. After the cultivation and harvesting of *E. coli* BL21, the supernatant resulting from the centrifugation of the sonicate was loaded onto the column as described in section 3.24.

When applying the elution buffer, a broad elution peak of ~ 400 mAU was observed, indicating a low yield of the target protein (Fig 4.17).

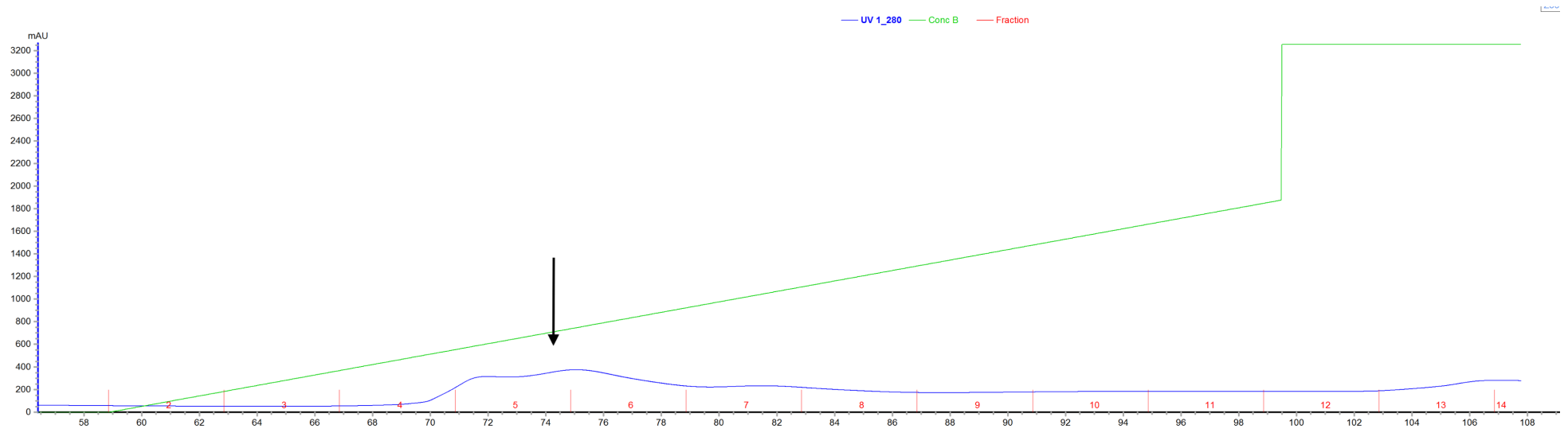


Figure 4.17 Ion Metal Affinity Chromatography used for purification of modMART1_DC. The x-axis is representative of the volume of buffer eluted. Fraction numbers are shown in red, and each fraction corresponds to 4 mL eluate. UV absorbance was measured at 280 nm, and the light blue line represents the measured absorbance intensity of eluted proteins. The protein of interest eluted in fractions 4-8, and the elution peak is indicated with an arrow

The fractions corresponding to the elution peak and the flow-through were analyzed by SDS-PAGE which indicated that the target protein was eluted in the collected fractions (Fig. 4.18 4-8). However, impurities of both higher and lower molecular weights can be observed in the same fractions, indicating that the purification had not been entirely successful. Therefore, Western blot analysis was performed to see whether it was the actual recombinant protein, or an impurity of the same size. The resulting Western blot (Fig. 7.3 in Appendix) indicated that it was the target protein, but protein bands of higher molecular weights were also observed. However, the estimated molecular weight of these protein bands may correspond to dimerization of the protein.

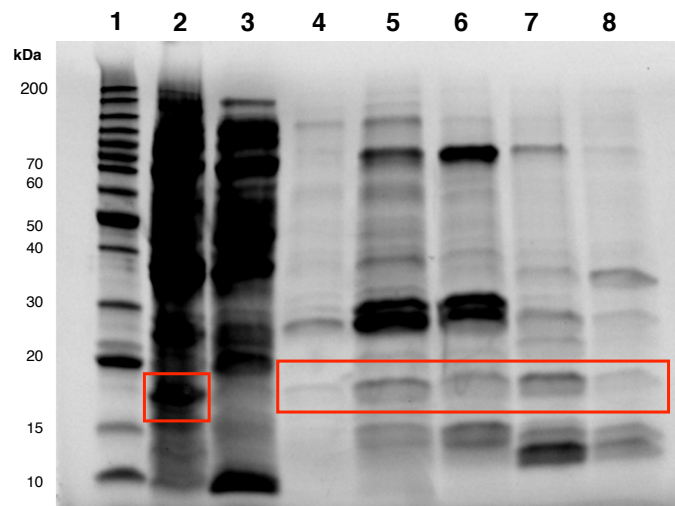


Figure 4.18 SDS-PAGE analysis after Ion Metal Affinity Chromatography (IMAC). (1) The BenchMark™ Protein Ladder (2) Pellet fraction from sonication; (3) Flow through fraction IMAC; (4) Elution Fraction 4; (5) Elution Fraction 5; (6) Elution Fraction 6; (7) Elution Fraction 7; (8) Elution Fraction 8.

In an attempt to further purify the protein, Ion Exchange Chromatography (IEC- see Section 3.25) was carried out. Figure 4.19 shows the chromatogram from the procedure.

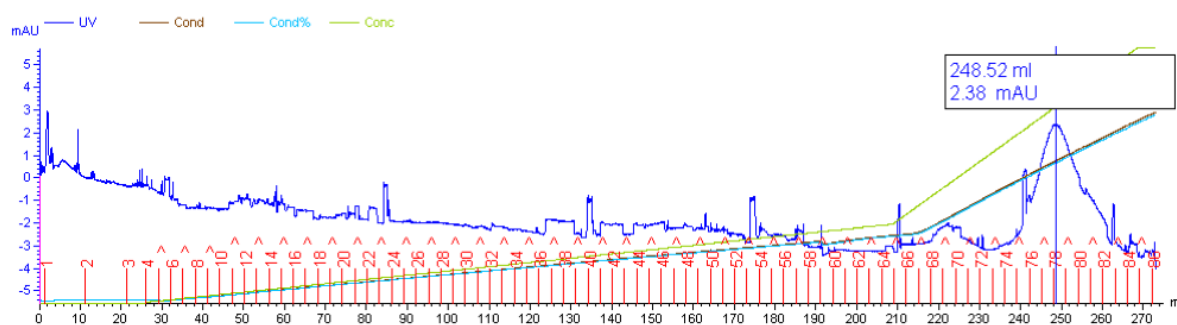


Figure 4.19 Ion exchange chromatography used for further purification of modMART1_DC. The x-axis is representative of the volume of buffer eluted. Fraction numbers are shown in red, and each fraction corresponds to 3 mL eluate. UV absorbance was measured at 280 nm, and the blue line represents the measured absorbance intensity of eluted proteins. The protein of interest eluted in fractions 76-82.

The protein did not elute until the linear salt gradient reached 80%, indicative of a high affinity of the protein to the ion exchange resin. However, a lesser amount of protein was eluted than during the IMAC purification. The fractions corresponding to the elution peak (Fractions 76-82) was analyzed by SDS-PAGE, and protein bands of the correct size (17 kDa) was observed (Figure 4.20) The amount of eluted protein in the different fractions was consistent with the low amount of protein seen in the chromatogram (Figure 4.19). Two protein bands with higher molecular weight were also observed in fraction 78 and 79, and these fractions were processed separately. However, none of the other impurities seen after IMAC purification was observed after IEC.

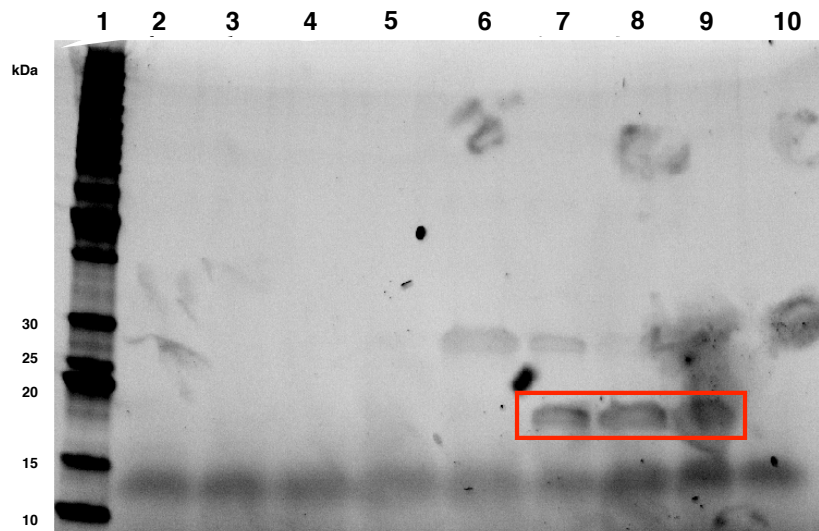


Figure 4.20 SDS-PAGE analysis of proteins after purification with Ion Exchange Chromatography. (1) The BenchMark™ Protein Ladder (2) Flow through fraction 1; (3) Flow through fraction 2; (4) Elution Fraction 76; (5) Elution Fraction 77; (6) Elution Fraction 78; (7) Elution Fraction 79; (8) Elution Fraction 80; (9) Elution Fraction 81; (10) Elution Fraction 82.

The fractions with only one protein band of the correct molecular weight were concentrated according to Section 3.26. Protein concentrations were measured with the Quick Start™ Bradford Protein Assay using three parallels. The protein concentration of the concentrated sample from the fractions with only one protein band was 0,258 mg/mL, and for the sample from the fractions with two visible bands was measured to be 0,142 mg/mL.

4.12.2. Semi-quantitative Western blot analysis

The purified and concentrated protein was to be used in a semi-quantitative estimation of the amount of modMART1_DC produced by the recombinant bacteria. The assay was performed as described by Myrbråten (2016) where a Western blot of a serial dilution of the target protein was used to produce a standard curve by the AzureSpot program. The standard curve is then used to estimate the amount of protein in lysed samples. However, in the initial Western blot, no target protein was observed after 2X serial dilutions of the concentrated sample with concentrations ranging from 4 to 0,25 $\mu\text{g/mL}$ (data not shown). Undiluted concentrated protein was therefore used as the starting sample in another series of 2X dilutions (concentrations ranging from 258 to 32,25 $\mu\text{g/mL}$). In addition, the undiluted concentrated protein sample from the purification fractions containing two bands (Fig. 4.20 Lane 6 & 7) was included to see whether it was possible to detect the modMART1_DC protein in this sample. Protein bands of the correct size (17 kDa) were observed on the SDS-

PAGE gel for all dilutions of the concentrated protein sample (Fig. 4.21 Lane 5-8) Two protein bands were observed for the undiluted protein sample stemming from the purification fractions containing two bands (Fig 4.21 Lane 9) in accordance with the purification results (Fig. 4.20 Lane 6 & 7). In the resulting western blot, weak bands were observed for both the undiluted samples (Fig. 4.22 Lane 5 & 9), but no bands were observed for the serial dilution of the concentrated protein sample. Due to time constraints the experiment was discontinued.

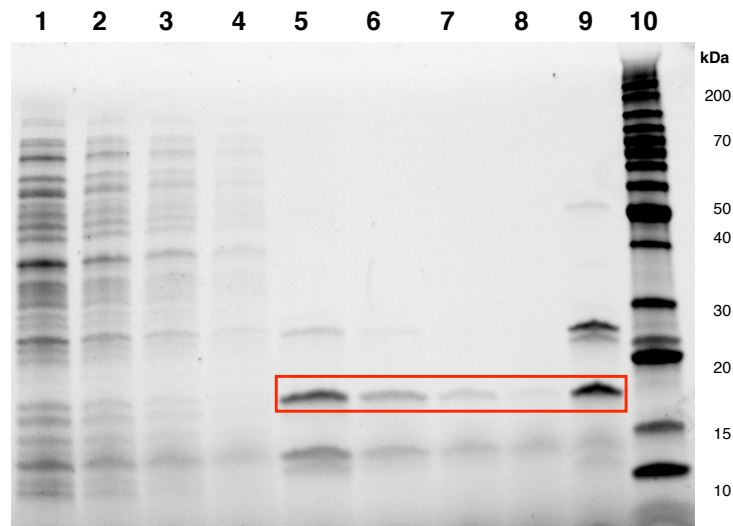


Figure 4.21 SDS-PAGE gel prior to Western blotting. (1) Undiluted Cell-free protein extract from pLp_3050_DC_modMART1_cwa2 ; (2) 2-fold dilution of Cell-free protein extract from pLp_3050_DC_modMART1_cwa2 ; (3) 4-fold dilution of Cell-free protein extract from pLp_3050_DC_modMART1_cwa2 ; (4) 8-fold dilution of Cell-free protein extract from pLp_3050_DC_modMART1_cwa2 ; (5) Undiluted concentrated protein from purification ; (6) 2-fold dilution of concentrated protein from purification; (7) 4-fold dilution of concentrated protein from purification; (8) 8-fold-dilution of concentrated protein from purification; (9) Undiluted concentrated protein that showed multiple bands after purification; (10) The BenchMark™ Protein Ladder

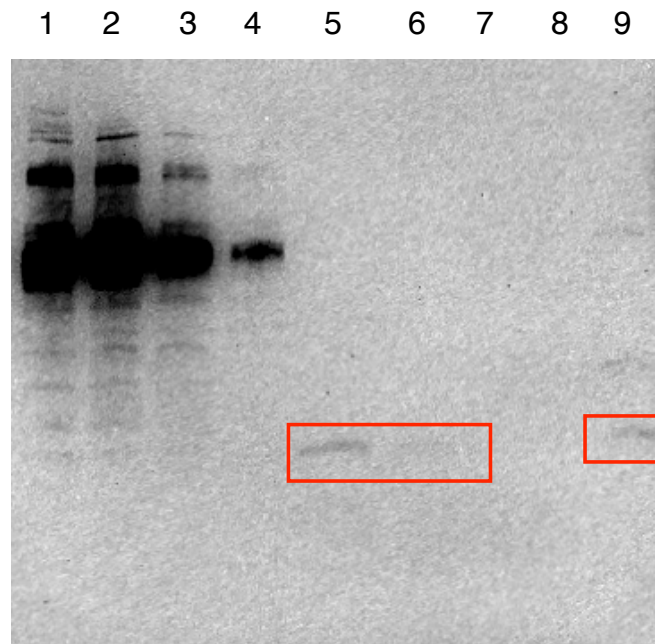


Figure 4.22 Western blot of serial dilutions of cell-free protein extract from pLp_3050_DC_modMART1_cwa2 and concentrated purified modMART1_DC protein. (1) Undiluted Cell-free protein extract from pLp_3050_DC_modMART1_cwa2 (2) 2-fold dilution of Cell-free protein extract from pLp_3050_DC_modMART1_cwa2 ; (3) 4-fold dilution of Cell-free protein extract from pLp_3050_DC_modMART1_cwa2 ; (4) 8-fold dilution of Cell-free protein extract from pLp_3050_DC_modMART1_cwa2 ; (5) Undiluted concentrated protein from purification (6) 2-fold dilution of concentrated protein from purification; (7) 4-fold dilution of concentrated protein from purification; (8) 8-fold-dilution of concentrated protein from purification; (9) Undiluted concentrated protein that displayed multiple bands after purification.

5. Discussion

5.1. Antigen and Construction of plasmids

The MART-1 antigen have previously been shown to be able to elicit the generation of cytotoxic T cells *in vivo* (Chodon et al. 2014; Lienard et al. 2009; Ribas et al. 2011; Wang et al. 1999). Abdel-Wahab et al. (2003) showed that a modified antigen containing an alanine to leucine (A27L) substitution, elicited a higher immune response. The modified epitope was therefore fused C-terminally to the native MART-1 protein (Fig. 1.4). The gene was also fused to a dendritic cell (DC) binding peptide identified by Curiel et al. (2004). The specific DC targeting of antigens has shown increased internalization and immune response (Mohamadzadeh et al. 2009). The fusion protein was denoted modMART1_DC.

Several strategies were employed to elucidate the use of *L. plantarum* as vaccine delivery vectors, and *modMART1_DC* was successfully cloned into six different plasmid vectors with or without anchoring mechanisms; two constructs for lipoprotein anchoring, pLp_1452_modMART1_DC and pLp_1261_modMART1, two constructs for cell wall anchoring pLp_3050_DC_modMART1_cwa2 (covalent anchoring) and pLp_3014_modMART1_DC (non-covalent binding), and two constructs only with a N-terminal signal sequence for secretion of the antigen.

5.2. Growth Rate of *L. plantarum* harboring different plasmids

The production of heterologous proteins may pose as a significant stress factor by subjecting the bacteria to metabolic strain that cause stalling of other cellular processes (Lulko et al. 2007). Anchoring of proteins to the cell surface is dependent on the successful secretion of the protein through the cell membrane and subsequent retention at the surface (section 1.6 and 1.7.) The inducible pSIP-system used in this study has previously been shown to initiate high expression of heterologous proteins (Sørvig et al. 2003). However, in addition to placing strain on the metabolic machinery of the bacteria, ineffective secretion of the produced protein may cause congestion of the secretion apparatus and eventually leads to adverse effects for the bacterial cell (Michon et al. 2016).

The growth impeding effect on *L. plantarum* from production of heterologous proteins for secretion or anchoring has been demonstrated in previous studies (Fredriksen et al. 2012; Myrbråten 2016; Solberg 2015; Tjåland 2011; Øverland 2013). The growth analysis of bacteria harboring the different *modMART1_DC*-containing plasmids show the same tendency as in these studies, with a hampered growth of all the bacteria with plasmids for secretion or anchoring of the *modMART1_DC* protein in comparison to the bacteria carrying the empty vector, pEV (Fig. 4.9). Already 2 hours after induction, a difference in growth rate was observed between all the recombinant *Lactobacillus* compared to pEv. Out of the six plasmids constructed, the bacteria carrying the lipoprotein anchor Lp_1261 showed the highest growth after induction with SppIP. Interestingly, heterologous production and anchoring showed the most detrimental effect on the bacteria harboring the other lipoprotein anchor, Lp_1452, where no growth was observed after induction. The fact that it is the same type of anchor as Lp1261, and as the bacteria harboring the pLp_1452_ *modMART1_DC* demonstrated no deviation in the growth rate prior to induction, the production and anchoring of the *modMART1_DC* protein through the lipoprotein anchor Lp1452 appeared to cause significant stress to *L. plantarum*. The observed difference in growth seen between bacteria with the two lipoprotein anchors is consistent with another study using these two anchors for the anchoring of the fusion protein CCL3Gag (Kuczkowska et al. 2015). However, higher growth rates have been observed for the Lp_1452 in a study by Fredriksen et al. (2012), implying that the nature of the heterologous protein in combination with the anchor is of importance for efficient secretion and thus reducing the pressure subjected to the bacteria.

The bacteria harboring plasmid for production of secreted *modMART1_DC* showed a slightly better growth ability than the bacteria with cell wall anchoring and LysM Domain, but still not as good as the bacteria with the Lp_1261 anchor. This could be indicative of high protein production and congestion as mentioned earlier.

5.3. Western Blot Analysis of Antigen Production

The production of *modMART1_DC* was verified through Western blot analysis of cell-free protein extracts for all plasmid constructs (Figure 4.10 and Figure 4.11). Produced antigen was detected for all the bacteria harboring plasmids for anchoring of the antigen to the cell surface (either to the membrane or the cell wall). While the protein band corresponding to the

pLp_1261_modMART1 displayed the correct molecular weight, protein bands of higher molecular weight was observed in the samples derived from pLp_3014_modMART1_DC and pLp_3050_DC_modMART1_cwa2. The observed molecular weight was approximately 10-20 kDa higher than the theoretical weight, thus a dimerization of the proteins is not likely. However, the observed increase in molecular weight could be due to linked cell wall fragments resulting from insufficient separation during cell lysis as observed in other studies (Liew et al. 2012; Myrbråten 2016; Nguyen et al. 2016).

The production of the protein was also verified for the constructs with only a N-terminal signal sequence (Fig. 4.11). Clear protein bands are seen around 20 kDa in the cell-free protein extract samples corresponding to bacteria harboring the pLp_3050_modMART1_DC and pLp_0373_modMART1_DC which corresponds to the theoretical molecular weight (23 and 23 kDa, respectively).

It was expected that the secreted antigen would be present in the culture supernatants of bacteria harboring the pLp_3050_modMART1_DC and pLp_0373_modMART1 constructs, as modMART1_DC was only fused to a N-terminal signal peptide. However, when investigating the undiluted culture supernatant of these constructs, no antigen was detected (Data not shown). The production of the antigen had previously been verified through Western blotting, and as the absence of the antigen in the supernatants could be due to a low secretion efficiency, three different protein precipitations were carried out. Nevertheless, no antigen was detected after precipitation with either trichloroacetic acid (TCA), Methanol/Chloroform or Acetone precipitations. To rule out the possibility that errors during the precipitation step was the cause of this discrepancy, Bovine serum albumin (BSA) was added to each sample prior to precipitation. The resulting SDS-PAGE gel (see Fig. 7.4 in Appendix for an example) showed the presence of proteins of different sizes, hence the precipitations seemed to have been carried out correctly.

After the precipitation, the precipitated proteins needed to be solubilized. This is normally achieved through the SDS-PAGE analysis preparation described in Section 3.18. However, some proteins with low solubility may be resistant to the standard sample preparation, making them ineligible for SDS-PAGE analysis. In retrospect, to investigate the possibility that the protein remained insoluble, urea could have been added to the samples prior to the SDS-PAGE analysis to enhance the solubility.

The protein precipitation experiments indicated that the absence of secreted antigen in the culture supernatant could be due to unsuccessful secretion by the signal peptides. The successful secretion is dependent on the nature of the protein such as the amino acid composition. In a study on the secretion efficiency of *Lactococcus lactis* by Langella and Le Loir (1999), it was found that the introduction of negative net charge of the 10 first amino acids of the mature NucA protein significantly enhanced the secretion efficiency. The modMART1_DC protein has an estimated neutral charge on these amino acids which may interfere with the secretion efficiency. However, the Lp_3050 signal peptide was used successfully to target the antigen to the cell wall in the LPxTG cell wall anchor as shown in the flow cytometry analysis. These results indicate that it should be at least partially functional in *L. plantarum*, and that an interference by the modMART1_DC protein itself could be a more plausible explanation.

While the full protein structure of the native MART-1 protein has yet to be determined, the amino acid sequence harbor a hydrophobic region between residue 27 and 48, corresponding to a transmembrane domain (Kawakami et al. 1997). Furthermore, Rimoldi et al. (2001) showed that the native MART-1 protein is embedded into membranes with the C-terminus protruding into the cytosol, and the N-terminus into the lumen of the melanosomal exocytic compartment in eukaryotic cells. The antigen epitope recognized by T cells is part of the hydrophobic region, and a sequence coding for a modified epitope which had shown a higher immunogenicity was included together with the unmodified epitope in the *modMART1_DC* gene (Figure 1.4), thus giving the modMART1_DC two hydrophobic regions. Little is known about whether a eukaryotic transmembrane protein display the same properties in a bacterial cell, however, based on the results from the protein precipitations, it could be speculated that something is retaining the modMART1_DC protein either intracellularly or in the cell wall. In the latter case, the protein may be trapped in the cell wall by non-specific binding. The cell wall of Gram-positive bacteria carries a net negative charge that could impair the complete secretion of the protein into the environment (Bolhuis et al. 1999; Freudl 2005). The modMART1_DC protein carries a net positive charge, but as the protein structure is not known it is not possible to ascertain if the positively charged residues is exposed on the surface, and thus may be prone to interact with the teichoic acids of the cell wall.

5.4. Detection of Surface Localized Antigen by Flow Cytometry and Immunofluorescent Microscopy

Flow cytometry analysis was carried out on induced bacterial cells in order to investigate the successful secretion and surface exposure of the antigen on the bacterial surface. The bacteria were incubated with a primary antibody that would bind to surface exposed modMART1, and subsequently with a secondary antibody conjugated with fluorescein isothiocyanate (FITC) prior to analysis. The relative fluorescence was measured by a flow cytometer. In the initial flow cytometry analysis only two constructs displayed a shift in the relative fluorescence as compared to the negative control, pEV, the pLp_3050_DC_modMART1_cwa2, and pLp_1452_modMART1 (Fig. 4.12), with the cell wall anchor demonstrating the most prominent shift. While the bacteria harboring pLp_1452_modMART1_DC had a severely impaired growth in the growth analysis (Figure 4.9), the flow cytometry analysis showed a shift in the observed fluorescence. Therefore, this impaired growth is most likely due to overproduction and following secretion stress.

Since *L. plantarum* is a Gram-positive bacterium, the cell is covered by a thick cell wall. Due to the nature and length of the different anchors, the antigen may be shielded from antigen hybridization during staining with a primary and secondary antibody, thus yielding a negative flow cytometry result. This shielding may be the result of the anchor lengths, but also molecular interactions that retain the protein closer to the plasma membrane.

In the case of the lipoprotein anchor with the Lp_1261 lipobox motif, it has previously been postulated that the relative short anchor (75 residues) may cause the antigen to be embedded in the cell wall (Nguyen et al. 2016; Øverland 2013). It would, however, be expected that the pLp_3014_modMART1_DC that anchor the antigen to the cell wall through a LysM domain would have given a shift in the observed relative fluorescence as the antigen is attached to the cell wall and not the membrane, hence the antigen should be more exposed for antibody hybridization.

Since the production of the antigen had been verified in all constructs (Figure 4.10 and 4.11), the cells were treated with lysozyme in order to disrupt the cell wall. This disruption had the potential of revealing hidden antigen, and the increase in relative fluorescence after disruption would confirm the potential shielding effect. Flow cytometry analysis of bacteria after

lysozyme treatment increased the observed fluorescent shift of the Lp_1261(lipoprotein anchor) construct, thus indicating that the antigen was shielded from antibody hybridization by the cell wall (Fig. 4.13 D). This shift was in accordance to previous studies of lysozyme treated bacterial cells with antigens anchored through Lp_1261 (Øverland 2013).

The bacteria harboring pLp_3050_DC_modMART1_cwa2 (covalent cell wall anchor) also showed a significant shift in the measured relative fluorescence after treatment with lysozyme (Fig. 4.13 B) Since a shift already had been observed for this construct, it is likely that interactions in the cell wall may obstruct the complete protruding into the environment. However, no shift in the observed relative fluorescence was detected of the bacteria harboring the pLp_3014_modMART1_DC plasmid (non-covalent attachment; LysM domain) after lysozyme treatment (Fig. 4.13 C). This indicated that the protein may be retained inside the cell by insufficient secretion. Similar results regarding the 3014-anchor were observed by Urdal (2013), where no fluorescence was observed in the bacteria harboring this mechanism. However, degradation products of the antigen were visible in the Western blot of the intracellular extract and in the culture supernatants, which lead to the conclusion that the antigen was most likely secreted poorly and subjected to proteolytic degradation inside the cell (Urdal 2013). The degradation of overly expressed heterologous proteins have been observed in several other expression hosts (Bolhuis et al. 1999; Durmaz et al. 2015; Le Loir et al. 2005). In contrast to Urdal's observations, no intracellular degradation products were observed for the pLp_3014_modMART1_DC construct in this current study, thus not compatible with a potential protease degradation intracellularly. As the protein is visible in the other constructs, the degradation of modMART1_DC by extracellular proteases may not be a feasible explanation. In addition, no antigen or degradation products is observed in the precipitated culture supernatants (Fig. 4.10 Lane 7), further indicating that the antigen is retained inside the cell in the case of the LysM domain construct.

Interestingly, a significant shift was observed after lysozyme treatment for the bacteria carrying the pLp_3050_modMART1_DC and pLp_0373_modMART1_DC plasmids for secretion. Further indicative of that the modMART1_DC protein was in fact retained in the cell either as part of the membrane or in the cell wall (Fig. 4.13 F and G). Previous studies have shown that a shift in fluorescent intensity is seen in constructs with proteins targeted for secretion, indicating poor secretion efficiency or that the protein may be retained in the membrane. However, in these studies, a shift in fluorescent intensity of the untreated bacterial

cells were generally also observed compatible with secretion of the proteins (Tjåland 2011; Øverland 2013). These previous observations may also indicate that modMART1_DC is not secreted successfully, and retained closer to the plasma membrane. However, it is not possible to ascertain the exact location of the protein in these constructs, as to whether it is embedded in the membrane or non-covalently trapped in the cell wall of *L. plantarum* based on the lysozyme treatment. Nevertheless, this entrapment may be potentially beneficial in a potential vaccine vector as discussed later.

The results from the immunofluorescent microscopy analysis of the bacterial cells were largely consistent with the flow cytometry data of both untreated and lysozyme treated cells (Fig. 4.14). The microscopy of the untreated pLp_1452_modMART1_DC revealed an uneven distribution of the antigen on the bacterial surface, while the results after the lysozyme treatment were indistinguishable. In the case of the pLp_3050_DC_modMART1_cwa2, the patchy distribution of the antigen was visible in the lysozyme treated cells. An intense fluorescence was also observed in the lysozyme treated bacteria harboring secretion plasmids (Fig. 4.14 L & N). These results, in combination with the results from the flow cytometry analysis, further supported the conclusion that modMART1_DC remains trapped in the membrane or in the cell wall of *L. plantarum* when not associated with an anchoring motif.

5.5. Trypsin Digestion of the Bacterial Cell Wall

In a study by Bøhle et al. (2011), tryptic digest of the cell wall (shaving) was performed to investigate surface proteins of *Enterococcus faecalis* V583. They identified 69 proteins, of which 3 were predicted as transmembrane. Of these proteins, only fragments of the predicted extracellular parts were identified, thus a trypsin digestion of bacteria harboring plasmids for secretion of modMART1_DC could provide insights into whether the protein was embedded in the cell membrane. In addition, the shaving procedure had the potential of providing supplementary information of successful translocation of the antigen to the cell surface in the bacteria with anchoring mechanisms, previously confirmed by the flow cytometry data.

The spectrum resulting from all proteins detected after trypsin digestion, showed a significant number of peptides present, which may indicate leakage from certain cells (Personal communication, Senior engineer Morten Skaugen). However, a lysis control was included in

this experimental set-up, the pCytAg85B-E6 (Tjåland 2011), that produce the tuberculosis antigen Ag85BESAT6 intracellularly, and no fragments corresponding to this protein were identified in the MS/MS analysis. Fragments from a total of 830 proteins were detected in all the samples combined. Some of the proteins identified were predicted cytoplasmic proteins, however, the presence cytosolic proteins to a certain extent is not uncommon in tryptic digest of bacteria, and it has been postulated that some of the proteins assigned as primarily cytosolic may in fact have extracellular purposes as well (Bøhle et al. 2011; Dumas et al. 2009; Espino et al. 2015; Schaumburg et al. 2004; Severin et al. 2007). Peptide fragments stemming from the modMART1_DC peptide were, nevertheless, identified. The spectra corresponding to these fragments were distinct, with limited interference of the fragment peaks, thus confirming the presence of the antigen in the samples. The absence of the Ag85ESAT6 antigen in the control sample may indicate that the modMART1_DC antigen was located on the surface of the cell in the other samples, as confirmed by the flow cytometry analysis, however, precautions must be made in interpreting the data as a high number of proteins were detected in the experiment.

Regarding the topology of the protein, the data were therefore inconclusive. If only predicted peptide fragments of either the N-terminal or C-terminal side of the transmembrane had been identified, this could have shed light on whether the transmembrane region had been inserted into the membrane of *L. plantarum* harboring the plasmids for secretion. However, predicted fragments on both sides of the transmembrane area were observed in the tryptic digest of bacteria harboring the pLp_3050_modMART1_DC construct, but not in the bacteria carrying the pLp_0373_modMART1_DC with a peptide threshold of 95 %. It is possible to regulate the stringency of peptide identification in the Scaffold software. By applying a peptide threshold of 95 %, the peptide identification certainty must be 95 % before the program will include these as a hit. Under less stringent conditions, the predicted EDAHFIYGYPK trypsin peptide on the N-terminus of the transmembrane region also appeared for the pLp_0373_modMART1_DC with a low peak interference. As lysis remains a possibility, it is not possible to say if these fragments are derived from intracellular protein or protein localized on the bacterial surface.

Interestingly, the predicted trypsin fragment containing the putative transmembrane region, GHGHSYTTAEAAAGIGILTVILGVLLIGCWYCR, did not appear in any of the samples when the peptide threshold was decreased. The absence of the predicted trypsin digested

transmembrane peptide fragment in all the bacteria and the possibility of cell lysis is somewhat of a conundrum, as the trypsin fragment containing the transmembrane region would be expected to have been identified not only in lysed samples, but also in the bacteria harboring plasmids for anchoring. It would therefore be of great interest to fine-tune the shaving procedure to exclude the possibility of lysis, to further investigate the localization of the modMART1_DC protein.

5.6. Production and purification of modMART1_DC

To quantify the amount of modMART1_DC expressed by *L. plantarum*, the target protein must first be overexpressed and purified, enabling its use in semi-quantitative Western blot. The *modMART1_DC* gene was cloned into the pNIC-CH vector, and the recombinant vector was transformed into the production strain *E.coli* BL21. The MART1-protein has previously been expressed in and purified from *E. coli* (Bardliving et al. 2013; Chen et al. 1996). An initial pilot expression study indicated that the modMART1_DC protein was soluble, however, in small amounts (Fig. 4.16). A larger-scale experiment was therefore performed, followed by purification of the target protein via Immobilized Metal Ion Affinity Chromatography (IMAC; Section 3.24). The result of the purification (Fig. 4.17) and the subsequent SDS-PAGE analysis (Fig. 4.18) indicated a low yield of the target protein, as observed in the pilot study. A significant amount also appeared to be localized in the pellet fraction of the sonicated cells (Fig 4.18 Lane 2), indicating poor sonication or the formation of inclusion bodies by the target protein. The generation of inclusion bodies is a common problem in heterologous protein production, as the extensive production and accumulation of produced protein leads to aggregation. (Fahnert et al. 2004; García-Fruitós 2010; Marston 1986) To counteract this problem, the recombinant protein could have been coupled to a solubilizing agent such as the maltose-binding protein (MBP). This protein was utilized in a previous purification study performed by Kawakami et al. (1997) to yield higher amounts of purified protein. Different solubilizing buffers may also have been used. Bardliving et al. (2013) used a buffer containing 8 M urea, 1% Triton-114 and 2% w/v deoxycholate to solubilize inclusion bodies prior to IMAC purification. However, due to time constraints, these measures were not employed in this study.

Impurities of both lower and higher molecular weights were also observed in the elution fractions (Figure 4.18). The observed protein bands may correspond to other expression host proteins, however, some of the bands may also correspond to dimerization of the protein (~ 30 kDa). The elution of *E. coli* host proteins together with the 6x His-tagged target protein is a well-known problem, as many of the host proteins contain histidine residues on the surface of the tertiary structure, in addition to metal binding motifs that associate with the nickel-column (Robichon et al. 2011). A western blot analysis was carried out to see whether some of the protein could be a result of dimerization and how much were in fact *E. coli* contaminants. An overlay of the resulting Western blot of the SDS-PAGE gel (Fig. 7.3 in Appendix) indicated that the bands corresponding to the correct molecular weight (~18 kDa) was the target protein, and also indicated that some of the protein bands of higher molecular weight could correspond to dimerization. Taken together with the indistinct elution peak, the impurities observed indicated that the sample needed to be further purified before it could be used in a semi-quantitative assay.

Ion Exchange Chromatography (IEC; Section 3.25) was subsequently utilized on the ultrafiltrated fractions to obtain sufficiently purified protein. However, only a small amount eluted during this procedure (Fig. 4.19). The observed elution peak decreased from ~ 400 mAU to ~2,85 mAU indicating a much lower protein yield. This could be due to the insufficient binding of the protein to the IEC column, however, SDS-PAGE analysis of the IEC flow-through (Fig. 4.20 Lane 2 & 3) showed that no protein was present in this fraction.

Nevertheless, weak bands of the correct molecular size were observed in the elution fractions of the IEC. The fractions with a higher molecular band, maybe corresponding to a dimerization of the protein were processed separately. The final concentration of the protein with only one visible band was 0,258 mg/mL, an amount sufficient for semi-quantitative analysis. The semi-quantitative western blot analysis proved difficult carry out, even though protein bands of the correct size were observed in the SDS-PAGE gel before western blotting, these bands were only very weakly present on the nitrocellulose membrane following Western blotting. While it is difficult to speculate as to the reason why such minor amounts of target protein were observed after Western blotting, one possibility may be that the initial concentration of the protein was too low. Another potential reason may be that the target protein, which has a relatively low molecular weight, may have passed through the membrane

during transfer, preventing its subsequent detection (iBlot™ 2 Dry Blotting System User Guide, Publication number: MAN0009112 Rev: D.0). Due to time constraints, the experiment was not repeated with a shorter blotting time that would prevent the passing through of smaller proteins. However, as the initial protein yield after IMAC was relatively low, the repetition of the experiment in its entirety would probably yield a better result.

5.7. Potential use of modMART1_DC-producing *L. plantarum* as a vaccine

The characterization experiments of *L. plantarum* harboring the different plasmid construct showed that the nature of the anchor or lack thereof, is a determining factor for the viability and thus also for the potential use as a vaccine. While the pLp_1452_modMART1_DC showed a significant shift in observed relative fluorescence in the flow cytometry analysis (Figure 4.12), the severely impaired growth eliminates this construct as a potential delivery vector of the modMART1_DC protein as it would be difficult to obtain significant number of viable cells for immunization. It would also most likely not be able to survive in the harsh environment of the intestine, thus making an oral distribution challenging.

The potential of each bacterial construct as a vaccine is a trade-off between exposure and shielding of the antigen. As mentioned, exposed antigens may more readily interact with host cells, but also be subjected to significant mechanical stress that may lead to shearing. Antigens which are more shielded from the environment, may in turn be more unavailable for recognition, but also more likely to reach the mucosal target site of the intestine. In a study by Michon et al. (2016), bacterial cells with more exposed antigen were more readily internalized by dendritic cells *in vitro*, however, bacteria with more shielded antigens, showed the same effect *in vivo*. In relation to this work, a shift in fluorescence was observed for the pLp_3050_DC_modMART1_cwa2 construct in the initial flow cytometry analysis (Figure 4.12) thus indicating a more surface exposed antigen than in the other constructs. On the other hand, the pLp_1261_modMART1_DC displayed a higher shift in the relative fluorescence after lysozyme treatment, indicating a more embedded antigen in correlation with previous observation. This combined with a relative unaffected growth after induction make the bacteria harboring the pLp_1261_modMART1_DC plasmid a good candidate for immunologic analyses. This could also be true for the plasmids deigned for secretion of modMART1_DC, the pLp_3050_modMART1_DC and pLp_0373_modMART1_DC

constructs, if the protein is in fact retained either in the membrane or cell wall of *L. plantarum*.

5.8. Concluding remarks

L. plantarum has been shown to have immunomodulatory effects, and is a good candidate for the delivery of antigens to mucosal sites. This study shows that *L. plantarum* WCFS1 is able to produce the heterologous modMART1_DC fusion protein and that several constructs are promising candidates for further immunological analyses with the aim of inducing immune responses against melanoma.

6. References

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7. Appendices

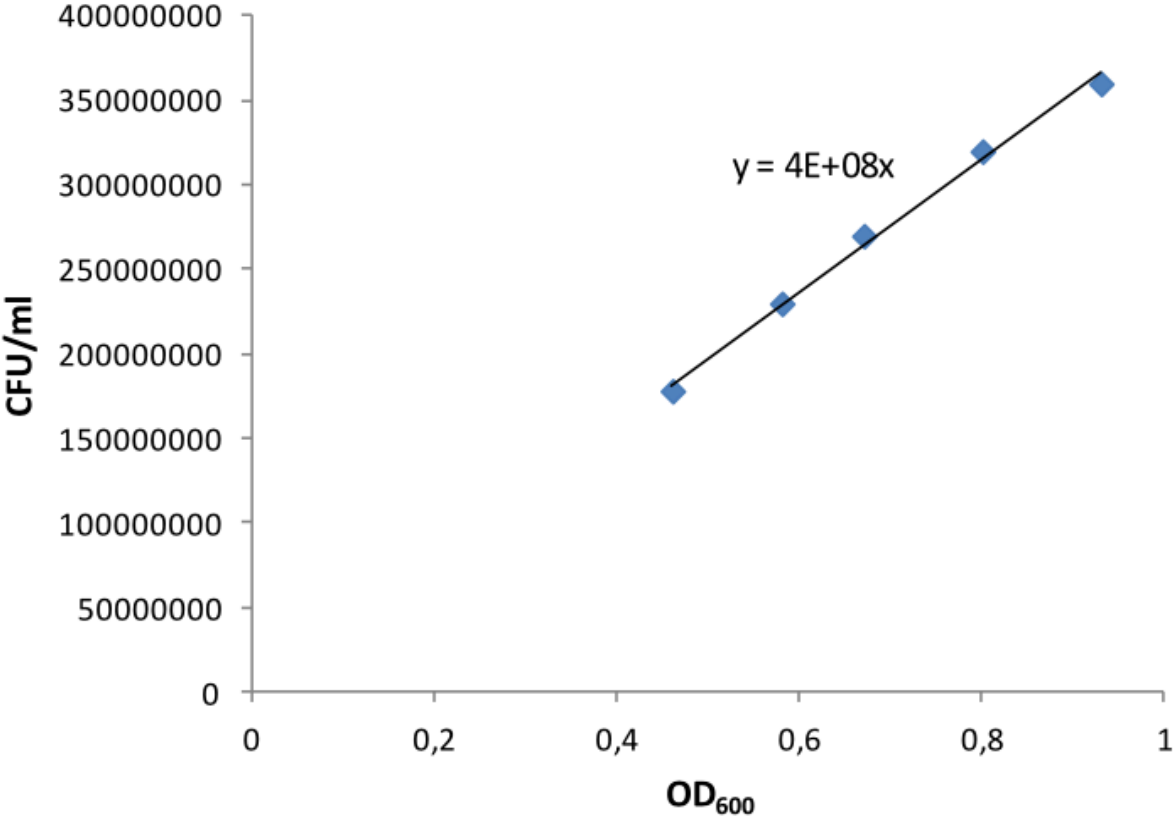


Figure 7.1 Relationship between OD₆₀₀ and CFU/mL of *L. plantarum* harboring the empty vector pEV. Graph used to calculate amount of harvested culture to yield approximately 10⁹ cells for all bacteria harboring plasmids for expression of modMART1_DC. Figure provided by Geir Mathiesen.

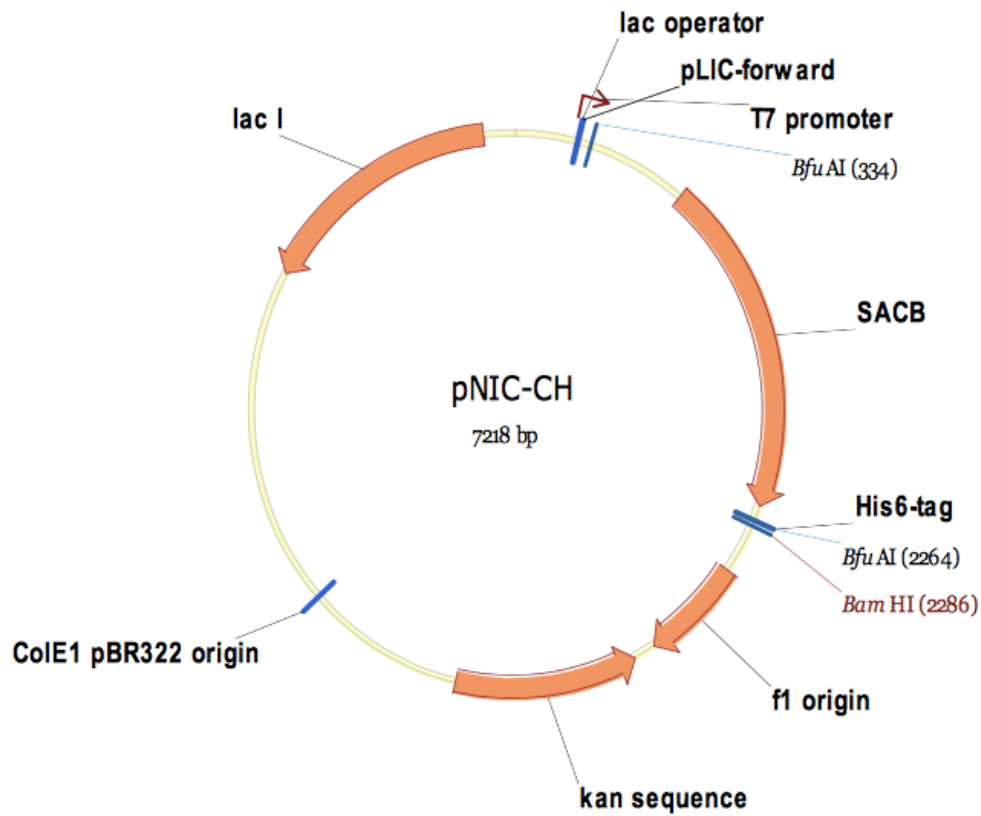


Figure 7.2 pNIC-CH vector map. The figure shows the different constituents of the pNIC-CH vector. Plasmid genes are transcribed from the T7 promoter which the T7 RNA polymerase of the bacterial host. *sacB* allows for negative selection on sucrose, as the gene product produces is lethal to the bacterial host. The restriction sites *Bfu AI* allows for cloning of inserts into the vector upstream of a C-terminal 6xHis-tag that facilitate the purification of the target protein. *apH* confers kanamycin resistance. Figure taken from Opher Gileadi Lab via Addgene (Addgene plasmid 26117).

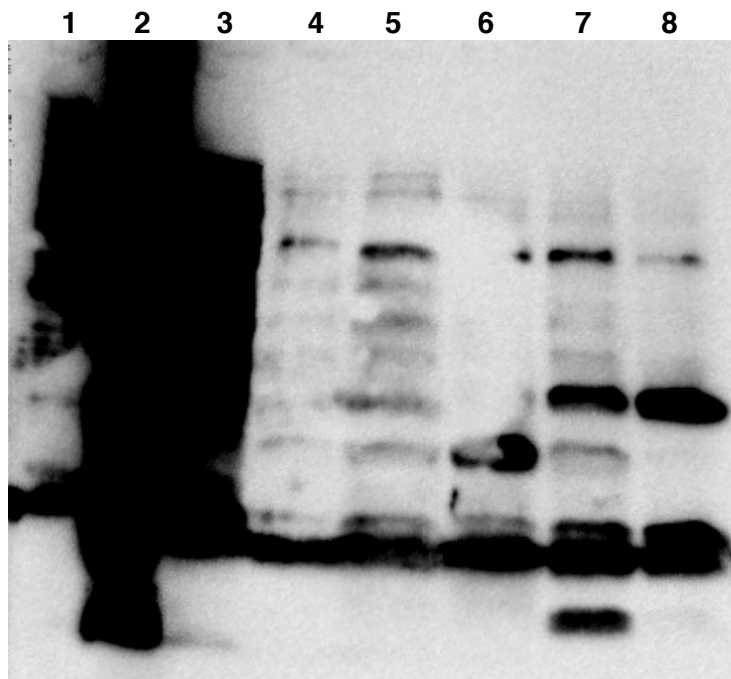


Figure 7.3 Western blot analysis after Ion Metal Affinity Chromatography (IMAC). (1) The BenchMark™ Protein Ladder (2) Pellet fraction from sonication; (3) Flow through fraction IMAC; (4) Elution Fraction 4; (5) Elution Fraction 5; (6) Elution Fraction 6; (7) Elution Fraction 7; (8) Elution Fraction 8.

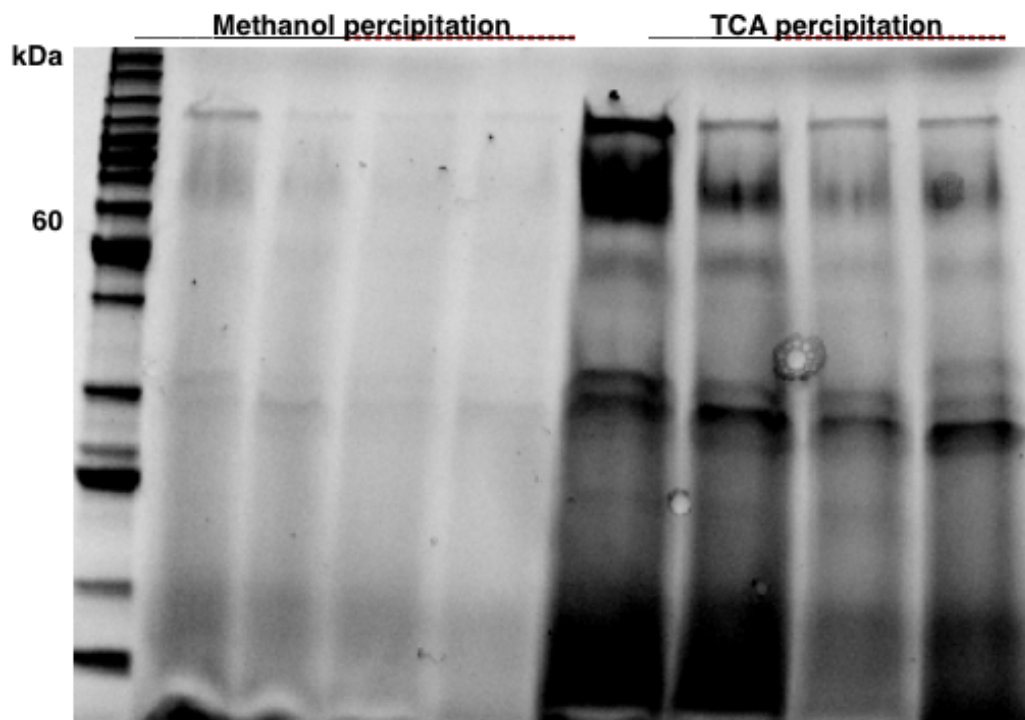


Figure 7.4 Example of an SDS-gel after Methanol and TCA precipitations of culture supernatants. The presence of a protein band around 60 kDa is indicative of the presence of BSA, thus indicative of a successful protein precipitation.

Table 7.1 Predicted tryptic fragments. The table lists the predicted fragments generated from the modMART1_DC protein by the enzyme trypsin.

Position of Cleavage Site	Resulting Peptide Sequence	Peptide length	Cleavage Probability
5	MVDPR	5	100 %
16	EDAHFYGYPK	11	86.6 %
51	GHGHSYTTAEAAAGIGILTVILGVLLIGCWYCR	34	91.2 %
57	NGYR	4	100 %
62	ALMDK	5	100 %
74	SLHVGTQCALTR	12	90.9 %
84	CPQEGFDHR	9	100 %
87	DSK	3	100 %
93	VSLQEK	6	91.1 %
108	NCEPVVPNAPPAYEK	15	94.7 %
151	LSAEQSPPYSPYTTAEELAGIGILTV ILGVLFYPSYHSTPQR	43	36.2 %



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