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Use of anchoring motives for anchoring of Mycobacterium tuberculosis antigens on the surface of Lactobacillus

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Abstract

This thesis describes the work that was done to investigate the use of *L. plantarum* WCFS1 as a live delivery vehicle for the delivery of the *M. tuberculosis* fusion antigen TB10.4-HspX (TH) that is C-terminal fused with a dendritic cell binding peptide (DC-seq). The fusion antigen TH has been shown to elicit immune response from tuberculosis patients with active and latent tuberculosis infection, making the fusion antigen a promising candidate as a multistage vaccine against tuberculosis. The coupling to the DC-seq may enhance the immune response by potentially increasing uptake by dendritic cells. Lactic acid bacteria (LAB) are commonly used in food production, abundant in the environment and are a natural inhabitant in the human gastro-intestinal tract (GIT). These characteristics and their status as "generally regarded as safe" (GRAS), make LAB attractive candidates as oral delivery vehicles for biomolecules to mucosal sites. *L. plantarum* WCFS's ability to survive passage through stomach and in the GIT for more than six days in its active form, make this bacterium well suited as an oral delivery vehicle.

Two non-GM approaches and one GM approach have been conducted in this study. In the non-GM approaches, TH_DC-seq and TH_DC-seq fused to a peptidoglycan binding LysM-anchor was produced intracellularly in *E. coli* or in *L. plantarum* WCFS1 with subsequent extraction and binding to wild type *L. plantarum* WCFS1. Getting adequate amounts of soluble fusion proteins for purification turned out to be challenging and thus, binding assays were done using the soluble fraction since no purified extracts were obtained. Both LysM_TH_DC-seq and TH_DC-seq seemed to be able to bind to *L. plantarum* WCFS1.

In the GM approach, TH_DC-seq was constructed for direct display on the cell surface of recombinant *L. plantarum* WCFS1 using three different anchors (LysM-anchor, LPxTG-anchor and lipobox-anchor). Production was confirmed except in the case of TH_DC-seq that was coupled to the LPxTG-anchor. Correct anchoring could not be confirmed, as fusion proteins on the cell surface of live cells could not be detected.

In conclusion, this study shows that production of TH_DC-seq is possible in both *E. coli* and *L. plantarum* WCFS1. Binding assays indicate that using *L. plantarum* WCFS1 as delivery vehicle for TH_DC-seq is a promising strategy for development of a novel vaccine against tuberculosis.

Sammendrag

Oppgaven omhandler forsøk gjort for å undersøke bruk av *L. Plantarum* WCFS1 som levende vektor for levering av *M. tuberculosis* fusjonsantigenet TB10.4-HspX (TH) som er C-terminalt fusert til et peptid (DC-seq) med affinitet for dendrittiske celler. Det er blitt vist at fusjonsantigenet TH kan initiere immunrespons hos tuberkulosepasienter med aktiv eller latent infeksjon, noe som gjøre TH til en lovende kandidat for utvikling av en multifasevaksine mot tuberkulose. Fusering til DC-seq kan forsterke immunresponsen ved å potensielt øke opptak av fusjonsantigenet av dendrittiske celler. Melkesyrebakterier er ofte brukt i matproduksjon, vanlig å finne i miljøet og er en del av den naturlige floraen i det humane fordøyelsessystemet. Disse karaktertrekkene og deres status som "generally regarded as safe" (GRAS), gjør melkesyrebakteriene til gode kandidater som leveringsvektorer for oral levering av biomolekyler via slimhinner. *L. Plantarum* WCFS1 er velegnet som en oral leveringsvektor fordi bakterien kan overleve passeringen forbi magesekken og i mer enn seks dager i mage-tarm-kanalen.

To ikke-genmodifiserte tilnærminger og én genmodifisert tilnærming har blitt utført i dette prosjektet. I de ikke-genmodifiserte tilnærmingene ble TH_DC-seq og TH_DC-seq fusert til et peptidoglykanbindene LysM-anker, produsert i *E. coli* eller *L. plantarum* WCFS1. Produseringen etterfølges av proteinekstraksjon og bindingsekperiment til villtype *L. plantarum* WCFS1. Å få tilstrekkelig mengde av løselig fusjonsprotein til rensing viste seg å være vanskelig, dermed ble bindingsstudiene utført med den løselige fraksjonen ettersom ingen renset ekstrakt kunne anskaffes. Både LysM_TH_DC-seq og TH_DC-seq synes å kunne binde seg til *L. plantarum* WCFS1.

I den genmodifiserte tilnærmingen ble TH_DC-seq konstruert til å bli direkte forankret på celleoverflaten til rekombinant *L. plantarum* WCFS1 ved hjelp av tre ulike anker (et LysM-anker, et LPxTG-anker og et lipoboks-anker). Produksjon av disse fusjonsproteinene var bekreftet, med unntak av TH_DC-seq som var fusert med LPxTG-ankeret. Riktig forankring på celleoverflaten kunne ikke bli bekreftet da fusjonsproteinene kunne ikke bli detektert på celleoverflaten av levende celler.

I konklusjon kan man med arbeidet gjort i dette prosjektet vise at det er mulig å produsere TH_DC-seq med både *E. coli* og *L. plantarum* WCFS1. Bindingsstudiene viser at bruk av *L. plantarum* WCFS1 som leveringsvektor av TH_DC-seq er en lovende strategi for utvikling av en ny tuberkulosevaksine.

Abbreviations

CM	Cytoplasmic membrane
DC-seq	Peptide sequence that shows affinity for dendritic cells
GIT	Gastro-intestinal tract
GM	Genetically modified
IBs	Inclusion bodies
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LysM	Lysine motif domain
PEP	The Protein Engineering and Proteomics group at NMBU
PG	Peptidoglycan
SppIP	peptide pheromone
TB	Tuberculosis
TH	TB10.4-HspX, two antigens from *M. tuberculosis*

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1 Introduction

With increasing emergence of resistant tuberculosis (TB) infections and the suboptimal effective BCG (Bacillus Calmette–Guérin) vaccine as the only available clinical vaccine, there is a worldwide need for new effective vaccines and therapies against TB infections (Tortora, Funke, & Case, 2010; *WHO | Global tuberculosis report 2014*, 2014). In this respect, oral vaccination is of interest since it can offer a simpler administration and avoidance of risks associated with systemic vaccination, as well as the potential to induce mucosal immune response (Lavelle & O'Hagan, 2006).

This thesis describes work aimed at attaching *Mycobacterium tuberculosis* antigens to the surface of *Lactobacillus plantarum* WFCS1, a food grade lactic acid bacteria, as a delivery vehicle in an oral vaccine against TB.

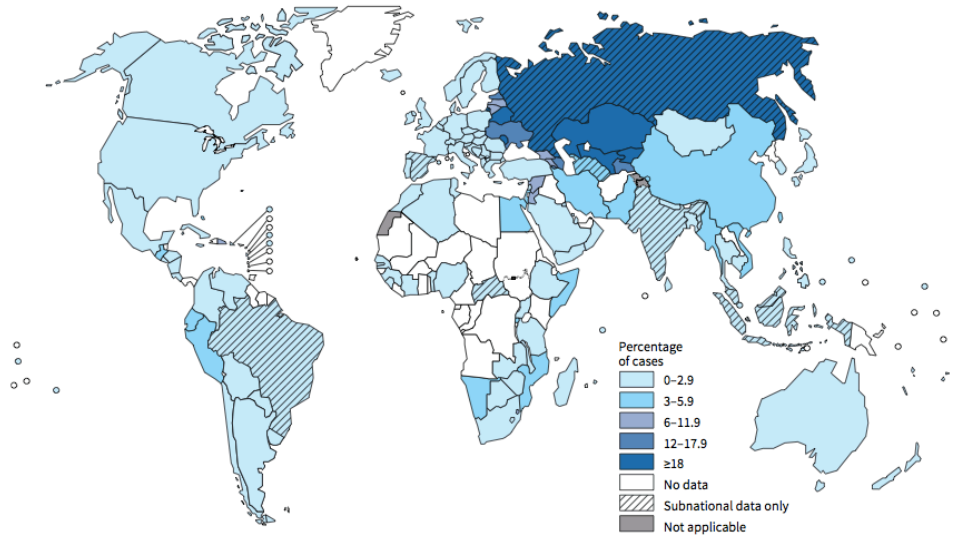
1.1 Tuberculosis

The obligate aerobe bacillus *M. tuberculosis* causes tuberculosis (TB), an airborne infectious disease that mainly affects the respiratory system (pulmonary TB). In the lung, *M. tuberculosis* primary invades alveolar macrophages, but can also be taken up by local dendritic cells (DCs) (Ottenhoff & Kaufmann, 2012; Tortora et al., 2010). *M. tuberculosis* can delay priming of T-cell responses in the local draining lymph nodes of the lungs by at least 1-2 weeks. This gives time to build up a critical mass of the bacilli that can develop into two types of population: latent, non-replicating cells that present a potential reservoir (latent infection); and metabolically active and replicating bacteria that stimulate immune response that give protection or cause TB pathology (active infection).

Worldwide, TB is the second leading cause of death of infectious diseases, and the African Region has the highest rate of new cases and deaths relative to population (*WHO | Global tuberculosis report 2014*, 2014). It has been estimated by the World Health Organization (WHO) that 2013 brought 9 millions new cases of TB and 1.5 millions TB deaths, and that about one in every four of the TB deaths was HIV positive. Although a

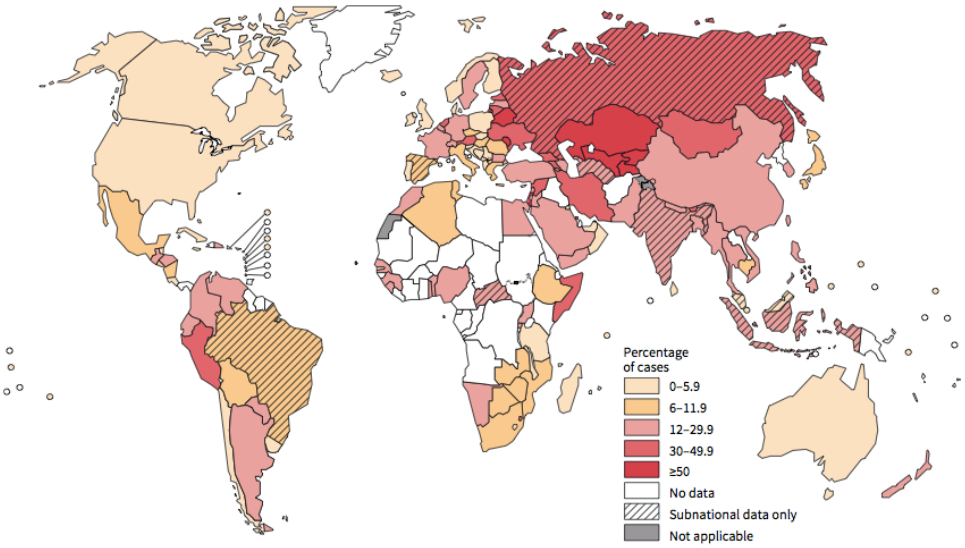
relatively small proportion of people infected with *M. tuberculosis* will develop TB, the incidence rate is much higher for HIV positive people. Given that most TB deaths are preventable with diagnosis and treatment, WHO states that the current TB mortality is unacceptably high.

With a slow generation time of 20 hours and a tendency to develop latent infections, treatment for pulmonary TB lasts for a minimum of six months (Tortora et al., 2010). WHO's recommended standard regimen for new pulmonary TB cases lasts for six months and consists of two fixed-dose combinations of antibiotics, both containing rifampicin (*WHO | Guidelines for treatment of tuberculosis*, 2010). The long run of treatment often causes compliance problems and may therefore contribute to development of antibiotic resistance. For 2013, global estimation of multidrug-resistant TB (MDR-TB) among new TB cases and retreatments were 3.5% and 20.5%, respectively (*WHO | Global tuberculosis report 2014*, 2014). This trend has remained unchanged between 2008 and 2013, but the gap between the number of diagnosis and the number of treatment seems to have widened from 2012 to 2013. Countries such as Russia and other eastern European countries are experiencing serious epidemics of MDR-TB (figure 1.1 and figure 1.2). The "End TB Strategy" approved by WHO in May 2014, aims to reduce TB deaths by 95% and TB incidence by 90% by 2035 compared to 2015.



[a] Figures are based on the most recent year for which data have been reported, which varies among countries.

Figure 1.1 Percentage of new TB cases with MDR-TB(This figure was taken from *WHO | Global tuberculosis report 2014*, 2014)



[a] Figures are based on the most recent year for which data have been reported, which varies among countries. The high percentages of previously treated TB cases with MDR-TB in Bahrain, Bonaire, Israel, Saint Eustatius and Saba, and Sao Tomé and Principe refer to only a small number of notified cases (range: 1–8 notified previously treated TB cases).

Figure 1.2 Percentage of TB retreatment with MDR-TB (This figure was taken from *WHO | Global tuberculosis report 2014*, 2014)

1.2 The antigens from *M. tuberculosis* as vaccine candidates used in this study

In the search for antigens to be used in vaccines, many *M. tuberculosis* proteins have been evaluated and over a dozen are in clinical trials (Andersen & Woodworth, 2014; Ottenhoff & Kaufmann, 2012). In the present study, we have focused on a fusion protein consisting of TB10.4 and HspX antigens. Studies indicates that these antigens might play important roles in mycobacterium-specific functions such as intracellular survival and virulence (Skjøt et al., 2000, 2002; Yuan et al., 1998). A vaccine study that utilised fusion antigens of TB10.4 and HspX showed that the vaccine induces immune response in human T cells from both latent and active TB patients, showing potential as a good candidate for a multistage tuberculosis vaccine (Niu et al., 2011).

The protein TB10.4 (10kDa) is a low-mass *M. tuberculosis* protein identified from culture filtrates and belong to a family of low-mass proteins (14-23 kDa) encoded by the *esat6* gene family (Skjøt et al., 2000, 2002). TB10.4 can be further categorized to the TB10.4 sub-family consisting of TB10.3 (10kDa), TB10.4 (10kDa) and TB12.9 (13kDa). TB10.4 that has been used in this study, is produced in the virulent strain *M. tuberculosis* H37Rv but not in the avirulent strain *M. tuberculosis* H37Ra, suggesting its importance for *M. tuberculosis* virulence (Rindi, Lari, & Garzelli, 1999). TB10.4 contains several strong T-cell epitopes throughout the peptide sequence, with the dominant one in the N-terminus (amino acids 1-18). Epitope mapping of TB10.4 has shown that patients with active infection recognize a broader panel of epitopes than BCG vaccinated donors (Skjøt et al., 2002).

HspX or (also named Acr1) is a 16 kDa heat shock protein that is highly expressed in *M. tuberculosis* during hypoxic condition and latency (Siddiqui, Amir, & Agrewala, 2011). It is an ATP-independent chaperone that prevents aggregation of denatured protein and consequently also assists in the refolding process by other chaperones. Direct correlation has been observed between the rate of depletion of HspX and loss of tolerance to anaerobiosis (Wayne & Lin, 1982). *Acr1* is the gene coding for HspX and activation of *acr*-promoter is rapidly induced *in vivo* on entry to macrophage and *in vitro* under oxygen limitation (Yuan et al., 1998). Overexpression of HspX in exponential phase *in vitro* slows the growth rate of *M. tuberculosis* as well as the post-stationary phase autolysis in aging culture, indicating that synthesis of HspX comes at the expense of growth (Hu,

Movahedzadeh, Stoker, & Coates, 2006; Yuan et al., 1998). The needed hypoxic condition to induce HspX expression seems unlikely to cause protein instability and suggest that hypoxia may serve as a signal for hostile environment and expression of HspX may play a protective role for stress factors other than hypoxia (Yuan et al., 1998). The immunostimulatory effect of HspX lies in the N-terminus of the protein and mainly induces Th1 response (detection of IFN-γ release) (Siddiqui et al., 2011). It has also been shown that recombinant BCG overexpressing HspX increases efficacy of BCG and survival of immunocompromised mice, showing potential as candidate for a vaccine that boost protection against TB (Shi et al., 2010).

1.3 Lactic acid bacteria

Lactic acid bacteria (LAB) is a group of Gram-positive, anaerobic or microaerophilic cocci or bacilli (Berlec, Ravnikar, & Štrukelj, 2012; Chapot-Chartier & Kulakauskas, 2014). Phylogenetically related genera are *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weisela*. One of their shared features is the production of lactic acid as a product of carbohydrate metabolism. LAB is widely used in fermentation of food and they are nutritionally demanding and auxotrophic for several amino acids and nutrients. Thus, they easily colonize nutrient rich environments like meat, dairy products, plant fermentations, oral cavity, genital tracts and mammalian gastrointestinal tract (GIT).

LAB is recognized as suitable vector candidates for oral delivery of biomolecules to mucosal surfaces for several reasons. Food and Drug Administration (FDA) has defined LAB as GRAS (generally regarded as safe) and some of the *Lactobacilli* species are considered as probiotic (Berlec et al., 2012; Chapot-Chartier & Kulakauskas, 2014). Probiotics are defined as "live microorganisms which, when consumed in adequate amounts as part of food, confer a health benefit on the host". LAB is able to provoke or modulate an adaptive immune response as well as a tolerogenic one. *L. plantarum* has for example been studied to deliver antigens to the GIT. To avoid use of genetically modified (GM) bacteria, proteins can be fused with cell wall binding domains (CWBDs) and then

anchored to the LAB surface. This approach has been exploited with LysM- and SLH-domains (LysM = Lys motif; SLH = S-layer homologous) to develop oral vaccines.

1.3.1 Lactobacillus plantarum WCFS1

The ability of *L. plantarum* to survive in human GIT has stimulated research in using the species as delivery vehicle for therapeutic compounds and vaccines (Berlec et al., 2012; Kleerebezem et al., 2003). *L. plantarum* WCFS1 has become one of the model strains for LAB research since its first publication of its genome by Kleerebezem et al. (2003), followed by reseqencing and reannotations by Siezen et al. (2012) (Kleerebezem et al., 2003; Siezen et al., 2012). *L. plantarum* WCFS1 was isolated from a single colony of *L. plantarum* NCIMB8826 originating from human saliva (Kleerebezem et al., 2003). It has been shown that this strain can survive passage through stomach in an active form and is able to persist for more than six days in the human GIT. Over 240 putative extracellular proteins have been predicted from the genome sequence and several proteins are predicted as cell-surface proteins. The predicted surface anchors include N- or C-terminal transmembrane anchors, lipoprotein anchors, LPxTG-anchors and other cell wall binding domains like LysM (see below).

A study exploring the human intestinal response to *L. plantarum* WCFS1 has shown that this strain can induce time-dependent transcriptional changes in intestinal mucosa of healthy humans, and gene expression profile analysis indicates that this strain can trigger pro-inflammatory responses (Troost et al., 2008). Healthy participants that have been exposed to *L. plantarum* WCFS1 for six hours showed an overall upregulation of genes involved in cell growth, proliferation and development (Troost et al., 2008).

1.4 Anchoring motives from cell surface proteins used in this study

Analysis of 13 genomes of lactobacilli, including *L. plantarum* WCFS1 has revealed that these species contain genes encoding for 4 of 7 known secretion pathways in Gram-positive bacteria, namely secretion (Sec), fimbrilin-protein exporter (FPE), peptide efflux ABC and holin systems (Kleerebezem et al., 2010). Sec pathway is the major secretion pathway in Gram-positive bacteria and surface anchors used in this study are all derived from putative extracellular proteins that are subject to Sec secretion in *L. plantarum* WCS1. The Sec translocase consists of a membrane-embedded protein-conducting channel

(SecYEG) and an ATPase motor protein (SecA). All proteins secreted through Sec pathway contain a N-terminal signal peptide (SP). The SP typically consists of three regions – N, H and C. The N-region is a positively charged N-terminus, the H-region is a stretch of 15-25 hydrophobic residues and the C-region is the C-terminus that may contain a cleavage site for Type-I or Type-II signal peptidase (SPase).

Surface-associated proteins of *Lactobacillus* can be divide into four subcategories according to their anchoring mechanisms (Figure 1.3): (1) single hydrophobic N- or C-terminal domain in transmembrane anchors that integrates into the cytoplasmic membrane (CM); (2) lipoproteins that are N-terminally anchored to long-chain fatty acids of the CM; (3) Covalent binding to peptidoglycan (PG) layer through a C-terminal LPxTG-motif; and (4) non-covalent binding to cell wall using various cell wall binding domains (Boekhorst, Wels, Kleerebezem, & Siezen, 2006; Kleerebezem et al., 2010).

Boekhorst et al. (2006) repredicted 223 extracellular proteins (Figure 1.3) in *L. plantarum* WCFS1 (Boekhorst et al., 2006). A large majority of these are predicted to contain motives associated with cell surface anchoring: 48 contain a N-terminal lipobox that covalently attach Cys residue to a lipid in CM; 27 contain C-terminal LPxTG motif covalently attached to PG by sortase; 10 proteins contain one or more LysM domain; 71 proteins contain N-terminal transmembrane anchors; 10 proteins contain C-terminal transmembrane anchors. Only 57 were predicted to be secreted proteins.

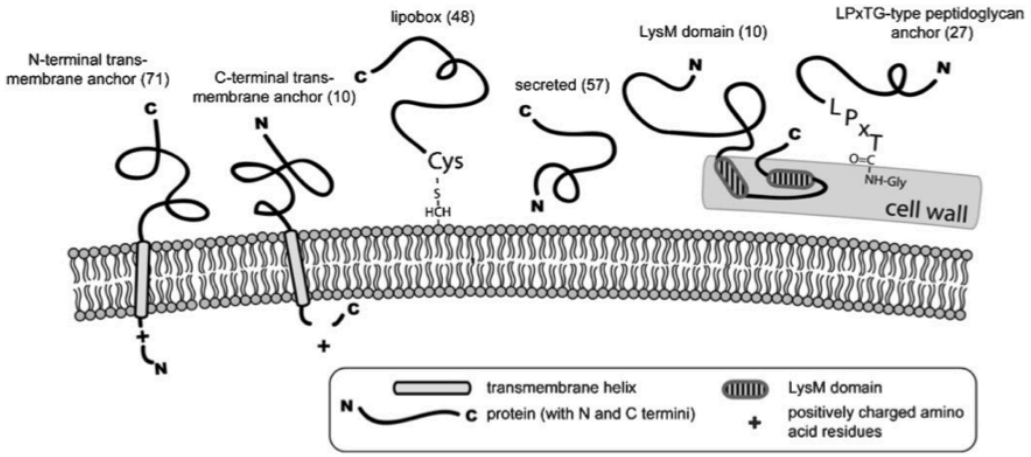


Figure 1.3 Extracellular proteins of *L. plantarum* This picture shows various types of extracellular proteins from *L. plantarum* WCFS1 predicted by Boekhorst et al. 2006. Numbers in parentheses indicates the number of each type of the proteins predicted. (This figure was taken from Boekhorst et al. 2006)

1.4.1 Lysine motif – anchoring of cell surface proteins

The lysine motif (LysM) is thought to have a general PG-binding function and can be found in many extracellular proteins that are involved in cell-wall metabolism (Kleerebezem et al., 2010; Visweswaran, Leenhouts, van Roosmalen, Kok, & Buist, 2014). Prediction analysis of *L. plantarum* WCFS1 secretome by Boekhorst et al. (2006) has shown that predicted proteins with LysM domains also contain enzymatic domains with functions that are related to biosynthesis and degradation of polysaccharides (Boekhorst et al., 2006). This indicates that different enzymes employ a common method for anchoring to the PG.

One copy of LysM typically contains 44-65 amino acid residues. Sequences from over 4,500 species from prokaryotes, eukaryotes and viruses have been registered in the Pfam database (www.pfam.org; entryPF01476). LysM is usually found in the N- or C-terminus, and multiple LysM sequences are often separated by small linker sequences that are Ser-, Thr and Asn-rich (Visweswaran et al., 2014). Binding to PG occurs relatively fast and with high affinity (Andre, Leenhouts, Hols, & Dufrêne, 2008). LysM has specificity towards oligomers of *N*-acetylglucosamine (GlcNAc) (Ohnuma, Onaga, Murata, Taira, & Katoh, 2008; Visweswaran et al., 2014). Mesnage et al. (2014) showed that LysM-domain primary recognizes GlcNAc-X-GlcNAc (X=GlcNAc or MurNAc, MurNAc = *N*-acetylmuramic acid) (Mesnage et al., 2014). Fusion proteins with LysM domains can thus be isolated through binding to PG or other N-acetylglucosamine containing glycans. This property of LysM has been utilized to display proteins on cell surfaces, by either direct display in expression host cell or through binding extracted LysM-containing proteins to non-GM cells.

The LysM domains that are used in this study are derived from the genes *lp_3014* and *lp_2162* of from *L. plantarum* WCFS1 (figure 1.4) (Zhou, Theunissen, Wels, & Siezen, 2010). *Lp_3014* is a putative extracellular transglycosylase containing one copy of N-terminal LysM. *Lp_2162* is a putative gamma-D-glutamate-meso-diaminoimelate muropeptidase with two copies of N-terminal LysM.

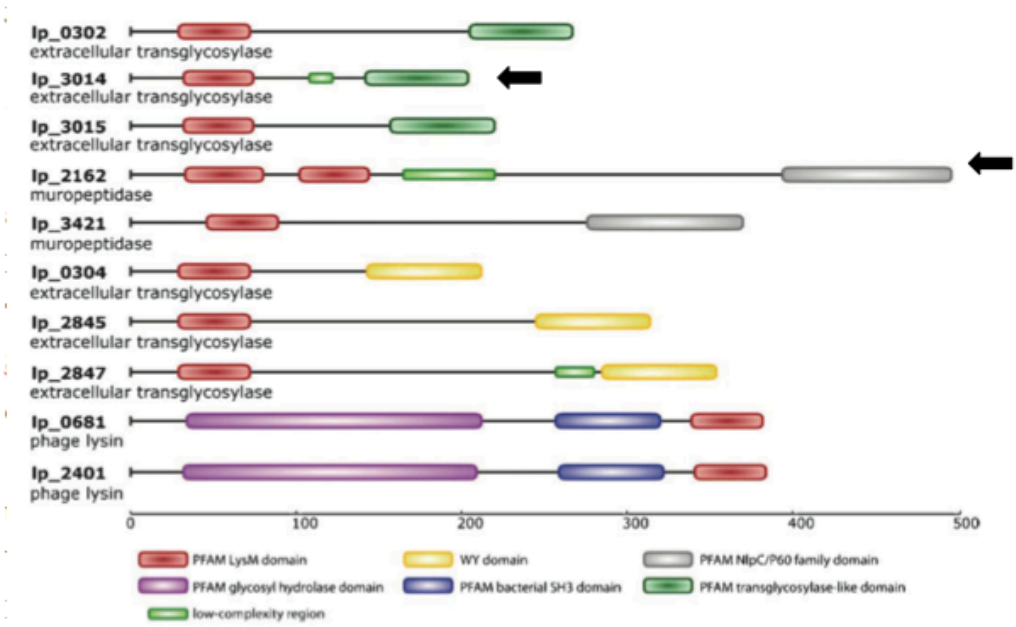


Figure 1.4 Predicted *L. plantarum* proteins with LysM domains (This figure was taken from Boekhorst et al. 2006)

1.4.2 LPxTG – anchoring of cell surface proteins

LPxTG-anchored proteins contain a C-terminal LPxTG motif and are anchored covalently to PG in a reaction catalysed by sortase (SrtA) (Boekhorst, de Been, Kleerebezem, & Siezen, 2005). These proteins typically contain a N-terminal SP with Type-I SPase cleavage site and the LPxTG motif at the C-terminus. After secretion, SrtA cleaves the LPxTG motif between T and G and covalently attaches the threonine carboxyl group to the PG.

The LPxTG motif that is used in this study are derived from the gene *lp_2578* that encodes a collagen-binding adherence protein from *L. plantarum* WCFS1(Zhou et al., 2010).

1.4.3 Lipobox – anchoring of cell surface proteins

Lipoproteins are the second largest group of cell-surface associated proteins of *Lactobacillus*. These proteins contain the N-terminal lipobox motif L-(A/S)-(A/G)-C (Hutchings, Palmer, Harrington, & Sutcliffe, 2009; Kleerebezem et al., 2010). The Cys residue undergoes lipidation by lipoprotein diacylglyceryl transferase, generating a

thioether linkage. After that, Type-II SPase cleaves between the (Ala-/Gly-) and Cys-residue and covalently anchors the mature protein to a lipid in the cytoplasmic membrane.

The lipobox motif that is used in this study is derived from the gene *lp_1261* that encodes for a substrate binding oligopeptide ABC transporter from *L. plantarum* WCFS1 (Zhou et al., 2010).

1.5 Peptide sequences with affinity for dendritic cells

Dendritic cells (DCs) have the ability to induce and direct adaptive immune responses and tolerance (Merad, Sathe, Helft, Miller, & Mortha, 2013), thus the extent of a vaccine's interaction with DCs may affect the efficacy of the vaccine. Classical DCs (cDCs) form a small group of tissue hematopoietic cells and reside in most lymphoid and non-lymphoid tissues. They have the ability to sense tissue injuries and present phagocytized antigens to T-cells. cDCs are further divided into different subsets that differently regulate T-cell responses. It is now known that DCs arise from a distinct hematopoietic lineage and transcriptome analysis suggests that cDC form a transcriptional entity that is distinct from other leukocytes.

Antigens delivered by LAB are too large to enter systemic circulation through epithelial absorption in the GIT (Berlec et al., 2012). Instead, they are sampled by DCs that extrudes in-between intestinal epithelial cells and M-cells on Peyer's patches in the small intestine of the GIT. Through sampling by DCs, antigens are presented to basal immune cells and can elicit local and distal immune responses, as well as systemic immune responses (Berlec et al., 2012; Purchiaroni et al., 2013).

Curiel et al. (2004) have shown that specific peptide sequence with affinity for human DC could be coupled to hepatitis C virus antigen and that this enhances the immune response. The team screened a phage display peptide library of 12-mer peptide sequences with high affinity for human DCs. A 12-mer peptide sequence (FYPSYHSTPQRP; abbreviated as DC-seq) from the Curiel et al. (2004) study, which has previously shown successful for DC targeting when used in lactobacilli (Fredriksen, Mathiesen, Sioud, & Eijsink, 2010; Mohamadzadeh, Duong, Sandwick, Hoover, & Klaenhammer, 2009), has been used in this study to promote the immune response to antigens presented by lactobacilli to DCs.

1.6 Outline of this study

This study is a sub-project of an on-going main project in the Protein Engineering and Proteomics (PEP) group at NMBU, where the goal is to develop oral vaccines using LAB as delivery vehicle. The main objective of this study is to contribute to the development of a better tuberculosis vaccine that is more effective and convenient than the BCG vaccine.

The main strategy explored in this study was to produce fusion proteins consisting of a LysM-anchor, the fused *M. tuberculosis* antigens TB10.4 and HspX (TH), and a DC-binding sequence (DC-seq) and bind the fusion proteins to PG-layer of *L. plantarum* WCFS1 for the purpose of developing a non-GM oral vaccine. The experimental work done in this study can be divided into three main steps: (1) construction and intracellular expression of fusion proteins consisting of LysM, TH and DC-seq in *E. coli* and *L. plantarum* WCFS1; (2) purification of fusion proteins; (3) investigate binding efficacy of LysM-containing fusion proteins to wild type *L. plantarum* WFCS1.

Due to challenging problems with intracellular production and purification using the commercial pET system (Novagen, 2011) and pSIP system (Sørvig et al., 2003), an alternative strategy based on generation of GM strains for delivery of antigens has also been commenced. In this side-study, TH and DC-seq has been fused with anchors and expressed in *L. plantarum* WCFS1 using the pSIP system. In this way, the antigens are hopefully displayed on cell surface of *L. plantarum* WFCS1. The anchors used in this side-study were a lipobox-anchor, a LPxTG-anchor and a LysM-anchor.

2 Materials

For a list of materials not described in the tables below, see appendix A.

2.1 Bacterial strains and plasmid constructs

Table 2.1 Bacterial strains

	Source
***Escherichia coli* strains:**	
One Shot® BL21 Star™ (DE3)	Invitrogen™
E.coli TOP10	Invitrogen™
Stellar™ Competent Cells	Clontech Laboratories
***Lactobacillus plantarum* strains:**	
L. plantarum WCFS1	Kleerebezem et.al. 2003
L. sakei Lb790	Sørvig et.al. 2003
L. rhamnosus GG	ATCC 53103

Table 2.2 Vector systems

Expression vector system	Description	Source
pET-16b	N-terminal His-tag Restriction sites for cloning: SalI and XhoI	Genscript
pLp	Restriction sites for cloning: NdeI, SalI and EcoRI	Derivative of the pSIP system from pSIP400-series (Sørvig et al., 2003)
pUC	Restriction sites for cloning: SalI and EcoRI	Merck

Table 2.3 Plasmid constructed in this study

Plasmid Constructed in this Study	Description
pET16b_2162S_TH_ DC-seq	pET-16b plasmid that expresses a fusion protein consisting of 2162S-LysM (has two N-terminal LysM domains from *lp_2162*), TH (*M. tuberculosis* fusion antigens used in this study) and DC-seq (the sequence that has affinity for DC). Designed for intracellular protein production and do not contain signal peptide.
pET16b_2162S_TH	pET-16b plasmid that expresses a fusion protein consisting of 2162S and TH. Designed for intracellular protein production and do not contain signal peptide.
pET16b_3014_TH_ DC-seq	pET-16b plasmid that expresses a fusion protein consisting of 3014 (full-length sequence of *lp_3014* that has one N-terminal LysM domains and the transglycosylase sequence), TH and DC-seq. Designed for intracellular protein production and do not contain signal peptide.
pET16b_3014S_TH_ DC-seq	pET-16b plasmid that expresses a fusion protein consisting of 3014S (truncated sequence of *lp_3014* that only includes one copy of N-terminal LysM domains), TH and DC-seq. Designed for intracellular protein production and do not contain signal peptide.
pET16b_3014S_TH	pET-16b plasmid that expresses a fusion protein consisting of 3014S and TH. Designed for intracellular protein production and do not contain signal peptide.
pET16b_TH_DC-seq	pET-16b plasmid that expresses a fusion protein consisting of TH and DC-seq. Designed for intracellular protein production and do not contain signal peptide.
pET16b_TH	pET-16b plasmid that expresses a fusion protein consisting of TH. Designed for intracellular protein production and do not contain signal peptide.
pLp_His3014_TH_ DC-seq	pSIP plasmid that expresses a fusion protein consisting of a in-house constructed N-terminal His6-tag, 3014, TH, DC-seq. Designed for intracellular protein production and do not contain signal peptide.
pLp_0373_TH_ DC-seq _2578	pSIP plasmid that expresses a fusion protein consisting of 0373 (N-terminal SP from *lp_0373*), TH, DC-seq and 2578 (C-terminal LPxTG anchor from *lp_2578*).
pLp_1261_TH_ DC-seq	pSIP plasmid that expresses a fusion protein consisting of 1261 (N-terminal lipobox from *lp_1261*), TH and DC-seq.
pLp_3014_TH_ DC-seq	pSIP plasmid that express a fusion protein consisting of 3014S, TH and DC-seq.
For more details of the parts of the fusion proteins see section 1.3, section 1.5 and section 1.6	

Table 2.4 Insert gene and constructs from other sources

Insert genes and constructs from other sources	Description	Source
2162S_AgESAT	2162S (has two N-terminal LysM-domains from *lp_2162*) fused with two *M. tuberculosis* antigens (Ag85 and ESAT-6)	PEP group, NMBU
3014S_AgESAT	3014S (truncated 3014 LysM with one N-terminal LysM-domain) fused with two *M. tuberculosis* antigens (Ag85 and ESAT-6)	PEP group, NMBU
pET16bChiA	pET-16b plasmid that expresses chitinase A	Anne Grethe Hamre, NMBU
pEV	pSIP plasmid with no insert gene	PEP group, NMBU
pLp_0373_OFA_cwa2	pSIP plasmid that expresses a fusion protein with 0373-N-terminal SP, OFA (37-kDa oncofetal antigen from mammalian tumors) and cwa (2578-LPxTG-anchor)	(Fredriksen et al., 2010)
pLp_1261_Inv	pSIP plasmid that expresses a fusion protein consisting of 1261-lipobox-anchor fused with invasin	(Fredriksen et al., 2012)
pLp_3014_Inv	pSIP plasmid that expresses a fusion protein consisting of 3014-LysM-anchor fused with invasin	(Fredriksen et al., 2012)
pUC_TH_DC-seq	Commercial pUC plasmid that expresses a fusion protein consisting of with TH and DC-seq	Merck

2.2 Primers and restriction enzymes

Table 2.5 Primer sequence (for description, see Table 2.6)

Primers sequence	Sequence
Seq F	GGCTTTTATAATATGAGATAATGCCGAC
Seq RR	AGTAATTGCTTTATCAACTGCTGC
pET SeqF	CCCCTCTAGAAATAATTTTGTTTAACTTT
pET SeqR	GCAGCCAACTCAGCTTC
Mtb.HspX SeqF	GGTGATATGGCTGGTTATGC
pET 3014 F	TCGAAGGTCGT CATATG GACTCAACTTACACCGTTAAGAGC
pET Mtb.HspX F	TCGAAGGTCGT CATATG TCTCAAATTATGTACAACTATCCTG
pET Mtb.HspX R	CAGCCGGATC CTCGAG TTAGTTAGTTGAACGAATTTGAATGTGC
pET Mtb.HspX_DC R	CAGCCGGATC CTCGAG TTATGGTCTTTGTGGAGTAGAGT
pET 2162 F	TCGAAGGTCGT CATATG GCCTCAATCACTGTAAAAGCAAA
pLp 0373_TbH_DC-seq F	TGCTTCATCAGTCGACTCTCAAATTATGTACAACTATCCTG
pLp TbH_DC-seq_2578 R	GTTCAGTGACACGCGTTGGTCTTTGTGGAGTAGAGTGATAT
pLp His3014uSP F	GGAGTATGATCATATGCATCATCACCACCACCATGCTGCTGCTG CTGACTCAACTTACACCGTTAAGAG
pLp TbH_DC R	CCGGGGTACCGAATTCTTATGGTCTTTGTGGAGTAGAGTG
pLp His_TH_DC F	GAGTATGATTCATATGCATCATCACCACCACCATGCTGCTGCTG CTGTCGACATGTCTCAAATTATGTACA

Table 2.6 Primer descriptions. Primers were used for sequencing of plasmids and for recombining plasmids using the In-Fusion® Cloning Kit

Description of primers	Description	Restriction site
Seq F	Forward primer for sequencing pSIP plasmids	
Seq RR	Reverse primer for sequencing pSIP plasmids	
pET SeqF	Forward primer for sequencing pET-16b plasmids	
pET SeqR	Reverse primer for sequencing pET-16b plasmids	
Mtb.HspX SeqF	Forward primer for sequencing pET-16b plasmids containing TH	
	Forward primer for pET-16b plasmids with 3014-LysM.	NdeI
pET 3014 F	Used for In-Fusion® Cloning	
pET MTtbHspX F	Forward primer for pET-16b plasmids with TH.	NdeI
	Used for In-Fusion® Cloning	
pET MTtbHspX R	Reverse primer for pET-16b plasmids with TH.	XhoI
	Used for In-Fusion® Cloning	
pET MtbHspX_DC R	Reverse primer for pET-16b plasmids with TH_DC-seq.	XhoI
	Used for In-Fusion® Cloning	
pET 2162 F	Forward primer for pET-16b plasmids with 2162-LysM.	NdeI
	Used for In-Fusion® Cloning	
pLp 0373_TbH_DC F	Forward primer for pSIP plasmids with LPxTG anchor.	SalI
	Used for In-Fusion® Cloning	
pLp TbH_DC_cwa2 R	Reverse primer for pSIP plasmids with LPxTG anchor.	MluI
	Used for In-Fusion® Cloning	
pLp His3014uSP F	Forward primer for pSIP plasmids with His-tagged 3014-LysM.	NdeI
	Used for In-Fusion® Cloning	
pLp TbH_DC R	Reverse primer for pSIP plasmids with TH_DC-seq.	EcoRI
	Used for In-Fusion® Cloning	
pLp His_TH_DC F	Forward primer for pSIP plasmids with His-tagged TH_DC-seq.	NdeI, SalI
	Used for In-Fusion® Cloning	

Table 2.7 Restriction enzymes and buffer

Restriction enzymes and buffer	Supplier
FastDigest: FastDigest EcoRI FastDigest NdeI FastDigest SalI FastDigest XhoI FastDigest Green Buffer	Thermo Scientific

2.3 Antibodies

Table 2.8 Antibodies

Primary antibodies:	Supplier
Monoclonal Mouse-anti-HspX, alpha crystalline	Acris Antibodies GmbH
Penta-His™ Antibody, BSA-free (100µg), mouse	Qiagen
Polyclonal rabbit antibody to TB10.4	Antibodies-online GmbH
Secondary antibodies:	
Goat Anti-Rabbit IgG-HRP	SouthernBiotech
Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP	Dako
Anti-Mouse IgG FITC-antibody	Sigma-Aldrich

3 Methods

3.1 Buffers and solutions

Buffers

Buffer A (for protein purification)	100 mM Tris- pH8 150 mM NaCl 0.5 mM Imidazol
Buffer B (for protein purification)	100 mM Tris- pH8 150 mM NaCl 300 mM Imidazol
Lysis buffer	16 µl DNase I 16 µl Lysozyme, 1 mg/ml 16 ml Buffer A
10X PBS	80 g NaCl 2.0 g KCl 18.05 g $Na2HPO_4 \bullet 2H_2O$ 2.4 g $KH2PO_4$ Dissolve in dH_2O and adjust pH to 7.4. Adjust volume to 1 l with dH_2O. Sterilize by autoclaving at 121°C in 15 minutes.
10X TBS	1.5 M NaCl 0.1 M Tris-HCl, pH 7.5 Sterilize by autoclaving at 121°C in 15 minutes.
50X TAE	242 g Tris-Base 57.1 ml Acetic acid 100 ml 0.5M EDTA pH8 Adjust volume to 1 l with dH_2O

TEN buffer	5 ml Tris-HCL pH8 1 ml 0.5M EDTA pH8 50 ml 1M NaCl
1M Tris	60.55 g Tris-Base Adjust pH to 8 Adjust volume to 500 ml with dH_2O. Sterilize by autoclaving at 121°C in 15 minutes.

Chemical solutions

0.5 M EDTA	93.05 g EDTA•$2H_2O$ 400 ml dH_2O Adjust pH to 8 Adjust volume to 500 ml with dH_2O. Sterilize by autoclaving at 121°C in 15 minutes.
2M Glucose	36.032 g Glucose (anhydrate) Dissolve in dH_2O and adjust volume to 10 ml
20 % Glycine (w/v)	20 g Glycine Dissolve in dH_2O and adjust volume to 100 ml Sterilize by autoclaving at 121°C in 15 minutes.
0.1 M IPTG (isopropyl β-D-1-thiogalactopyranoside)	0.2383 g IPTG Dissolve in dH_2O and adjust volume to 10 ml.
Lysozyme, 1 mg/ml	100 mg Lysozyme Dissolve in dH_2O and adjust volume to 1 ml.

1 mM $MgCl_2$	0.0508 g $MgCl_2 \bullet 6H_2O$ Dissolve in dH_2O and adjust volume to 250 ml. Sterilize by autoclaving at 121°C in 15 minutes. Store at room temperature.
30 % PEG 1500	40 g Polyethylen Glycol, PEG-1450 Dissolve in dH_2O and adjust volume to 100 ml Sterilize by filtration with 0.45 µm syring filter and distribute in 1.5 ml microtubes.

Antibiotic stock solutions

Ampicillin 50 mg/ml, 10mL	500 mg ampicillin sodium salt 10 ml distilled water Sterilize by filtration with 0,22 µm syringe filter and distribute in 1,5 ml microtubes. Stored at -20°C.
Erythromycin 100mg/ml, 10mL	1000 mg erythromycin 10 ml 96% ethanol Sterilize by filtration with 0.22 µm syringe filter and distribute in 1.5 ml microtubes. Stored at -20°C.
Erythromycin 10mg/ml, 10mL	100 mg erythromycin 10mL 96% ethanol Sterilize by filtration with 0.22 µm syring filter and distribute in 1.5 ml microtubes. Stored at -20°C.

Medium

Brain-Heart-Infusion (BHI), liquid medium	37 g dehydrated BHI broth per 1 litre of distilled water. Sterilize by autoclaving at 121°C in 15 minutes. Store at room temperature
Lysogeny broth / Luria Bertani broth (LB)	10 g Bacto-Tryptone 5 g Bacto-Yeast Extract 10 g NaCl 1 L dH_2O Sterilize by autoclaving at 121°C in 15 minutes. Store at room temperature
Medium plates	1. 15 g agar-agar per 1 litre of liquid broth. Sterilize by autoclaving at 121°C in 15 minutes. For selectivity: Add antibiotic to the solution when it is cooled down to approximately 50-60°C before the medium is poured in petri dishes. 2. Store solidified medium plates at 4°C. Shelf-life depends on type of antibiotic added and recommendation provided by supplier.
MRS (De Man, Rogosa, Sharpe) broth	52 g dehydrated MRS broth per 1 litre of distilled water. Sterilize by autoclaving at 121°C in 15 minutes. Store at room temperature 3.
MRSSM	Dissolve 17.1 g sucrose (0.5M) and 2 g $MgCl_2$ (0.1M) in 100 ml MRS. Sterile filtrate with 0.45 µm syringe filter.

SOC medium

1. Dissolve in 60 ml dH_2O:
   2 g Bacto™ Tryptone
   0.5 g Bacto™ Yeast Extract
   0.057 NaCl
   0.019 g KCl
   0.247 g $MgSO_4$
   Stir with a magnetic stirrer
2. Autoclave at 121°C in 15 minutes and let cool down to room temperature.
3. Add 1 ml 2M glucose (Sterile filtrated) and adjust volume to 100 ml with dH_2O.
4. Store at -20°C in 1.5 ml and 10 ml aliquots.

3.3 Cloning

In-silico construction of plasmids was done using the software SerialCloner 2.6.1. Two different methods were used to recombine plasmid vector. One of the method was to digest plasmids using restriction enzymes and recombine by ligation using DNA ligase. The other method was to PCR-amplify an insert that consisted the insert gene and a 3' and 5' extension that overlap with the plasmid vector, and recombining the linearized vector and insert with In-Fusion® Cloning Kit (more detail in section 3.2.5 and 3.2.8).

3.3.1 Cultivation of bacteria

Cultivation of *Escherichia coli* carrying pET-16b or pUC plasmid

E.coli carrying pET-16b or pUC plasmid was grown either in 10 ml liquid broth (BHI or LB) medium or on medium plates (BHI or LB) containing 1.5% agarose. 200 µg/ml or 100 µg/ml ampicillin was added to liquid media or media plates, respectively. *E.coli* grown in liquid medium were incubated overnight at 37°C in a shaking incubator, whereas *E.coli* grown on medium plates were incubated at 37°C in a incubator without shaking.

Cultivation of *Lactobacillus* spp.

Lactobacillus. spp without plasmid was grown in 10 ml liquid MRS medium. *L. plantarum* WCFS1 carrying pSIP plasmid was grown in 10 ml liquid MRS medium or on MRS plate with 200 µg/ml erythromycin. *Lactobacillus*. spp were all incubated overnight at 30°C in a incubator without shaking.

Long-term storage of bacteria

Liquid medium with bacteria were store at -80°C for long-term storage. Glycerol was added to prevent cell disruption due to ice crystallization.

Material:

Bacterial culture

Glycerol solution, 85% (v/v)

Procedure:

1. Autoclave glycerol solution at 121°C for 15 minutes, chill and store at 4°C until use.
2. Mix 1 ml bacterial culture and 300 µl glycerol solution in a cryogenic tube by pipetting or gentle vortexing. Store at -80°C.

3.3.2 Isolation of plasmid DNA from bacterial cultures

During plasmid DNA (pDNA) isolation, pDNA from *E.coli* was liberated by Sodium-Dodecyl-Sulfate/alkaline lysis. After the cell lysis step, a neutralization step was performed. pDNA will remain soluble after the neutralization step, and when adding the soluble fraction to the binding resin, negatively charged pDNA will bind to the positively charged resin. Contaminations of salts and EDTA and cell debris were washed away by ethanol precipitation and pDNA was eluted with a low ionic buffer solution.

Different kits were used for pDNA isolation from *E.coli* due to the fact that these kits had different efficiency when used for different combination of host cell strains and plasmid constructs. The combinations were as listed:

Plasmid DNA purification kit	**Construct and strains**
NucleoSpin® Plasmid	Construct: pLp plasmids Strains: One Shot® BL21 Star™ (DE3)
JetStar® 2.0 Plasmid Purification Kits	Construct: pET and pUC plasmids Strain: Stellar™ Competent Cells

NucleoSpin® Plasmid

Material:

NucleoSpin® Plasmid Kit

- Resuspension Buffer A1
- Lysis Buffer A2
- Neutralization Buffer A3
- Wash Buffer AW
- Wash Buffer A4
- Elution Buffer AE
- NucleoSpin® Plasmid Columns (white rings)
- Collection tubes

1-5 ml *E.coli* over-night culture

dH_2O

Procedure:

1. Harvest cell pellet from 1-5 ml of *E.coli* culture by centrifugation at 11,000 x g for 30 seconds.
2. Resuspend the cell pellet with 250 µl of Buffer A1.
3. Lyse resuspended cells by adding 250 µl Buffer A2 and invert the tube 6-8 times before incubating for 5 minutes at room temperature.

   NB! Do not vortex to reduce the risk of shearing genomic DNA.
4. After incubation, add 300 µl of Buffer A3 to the suspension to neutralize and precipitate debris. Invert the suspension 6-8 times before centrifugation at 11,000 x g for 5-10 minutes. Repeat if the supernatant is not clear enough.
5. Meanwhile, prepare a NucleoSpin® Plasmid Column and collection tube for each sample. After centrifugation in step 4, pipette a maximum of 750 µl supernatant onto the column and centrifuge at 11,000 x g for 1 minute. Repeat if there is more supernatant left. Discard flow-through.
6. Wash plasmid DNA that is now bound to the silica in the column, for salts with 600 µl Buffer A4 and centrifuge at 11,000 x g for 1 minute. Discard flow-through.

NB! For constructs with low plasmid DNA yields perform a washing step with 500 µl Buffer AW before washing with Buffer A4.

7. After washing step(s), dry the silica membrane by centrifugation at 11,000 x g for 2 minutes and optionally air-dry for 2 minutes.
8. After drying step, transfer the NucleoSpin® Plasmid Column to a 1.5 ml microtube for collection of plasmid DNA. Elute plasmid DNA by adding 25 µl of preheated Buffer AE or dH_2O to the column and incubate for 3 minutes at 50°C before centrifugation at 11,000 x g for 1 minute. Repeat the elution step, making a total elution volume of 50 µl.
9. Measure DNA concentration (section 3.2.3) and proceed to further experiments or store at -20°C.

(Adapted from the NucleoSpin® Plasmid manual from 2012)

JetStar® 2.0 Plasmid Purification Kit, Midiprep

Material:

JetStar® 2.0 Plasmid Purification Kit, Midiprep

Cell Resuspending Buffer E1

Lysis Buffer E2

Precipitation Buffer E3

Equilibration Buffer E4

Wash Buffer E5

Elution Buffer E6

Isopropanol

70% ethanol

E.coli over-night culture

Procedure adapted from supplier's manual:

1. Equilibrate JetStar™ Midi Column by adding 10 ml Equilibration Buffer (E4) onto the column and let it drain by gravity flow. This step takes about 10-15 minutes, so one could proceed with cell harvesting meanwhile.

2. Harvest cells by centrifugation at 12,000 x g for 2-3 minutes. Discard supernatant and add 4 ml of E1 to resuspend the cell pellet. Then transfer the cell suspension to a 15 ml nunc tube.
3. Add 4 or 8 ml (for 25 or 50 ml culture respectively) of Lysis Buffer (E2) and invert gently until the suspension appeared homogenous. Incubated the homogenised suspension for maximum 5 minutes at room temperature.

   NB! Do not vortex to reduce the risk of shearing genomic DNA.
4. Add 4 or 8 ml (for 25 or 50 ml culture respectively) of Precipitation Buffer (E3) to the suspension and immediately inverted until the suspension appeared homogenous.

   NB! Do not vortex to reduce the risk of shearing genomic DNA.
5. Centrifuge the homogenous suspension from step 4 at 12,000 x g for 10 minutes at room temperature. Load supernatant onto the equilibrated column (from step 1) and let it drain by gravity flow.
6. Wash column twice with 10 ml Wash Buffer (E5) and let it drain by gravity flow. Discard flow-through.
7. After washing steps, place a new sterile nunc tube under column for elution. Load 5 ml of Elution Buffer (E6) onto the column and let it drain by gravity flow.
8. Add 3.5 ml of isopropanol to the elution tube and mix well. Distribute the mixture to 6 x 1.5 ml microtubes and centrifuge at 12,000 x g for 30 minutes at 4°C.
9. Discard supernatant from step 8 by vacuum suction and wash with 3 ml 70% ethanol (approximately 500 µl per microtube) and centrifuge at 12,000 x g for 5 minutes at 4°C.
10. Discard supernatant from step 9 by vacuum suction and let the DNA pellet air-dry at 60°C.

    NB! Drying was checked approximately every 2 minutes to avoid overheating of the plastic that can cause DNA to be absorbed into it.
11. Resuspend the DNA pellet in 30 µl dH_2O.
12. Measure DNA concentration (section 3.2.3) and proceed to further experiments or store at -20°C.

(Adapted from the JetStar® 2.0 Plasmid Purification Kit manual from 2010)

3.3.3 Plasmid DNA concentration measurement

The Qubit® dsDNA BR Assay Kit was used to measure plasmid DNA concentrations. This assays is a fluorescence-based quantification method using dye that is selective for double stranded DNA ("Qubit® dsDNA BR Assay Kits," 2011). The assay was performed at room temperature (temperature fluctuations may influence accuracy).

Material:

Qubit® Fluorometer

Qubit® dsDNA BR Assay Kit

- Qubit® dsDNA BR reagent and buffer should be stored at room temperature
- Qubit® dsDNA BR standard #1 and #2 should be stored 4°C

Qubit® Assay tube

Plasmid DNA

Procedure (setup including standards):

1. Make working solution by diluting Qubit® dsDNA BR reagent 1:200 in Qubit® dsDNA BR buffer in a 1.5 ml microtube.

   NB! Make a final volume of working solution so that there will approximately one sample volume in excess.

2. For the assay, make a final volume of 200 µl for each sample or standard for calibration as followed:
   a. Standard: 10 µl of standard and 190 µl of working solution.
   b. Sample with plasmid: 1 or 3 µl of sample and 199 or 197 µl of working solution, respectively.

   Vortex the mixture for about 1-3 seconds and incubates for 2 minutes at room temperature.

3. To measure concentration, choose the option "Use last calibration" and follow instructions on screen.
   a. To calibrate, choose the option "Run a new calibration" and follow instructions on screen.

(Adapted from the Qubit® dsDNA BR Assay Kit manual from 2011)

3.3.4 Double digestion with FastDigest® restriction enzymes – (Thermo Scientific)

Restriction enzymes type II was used to digest plasmid DNA. These types of restriction enzymes recognise their specific nucleotide sequence (usually 4-6 nucleotides long) and digest both DNA strands within the specific sequence. (Pray, 2008a). For an overview of the FastDigest® enzymes used in this study, see section 2.2.

Materials:

FastDigest® Enzymes

FastDigest® Green Buffer

Plasmid DNA

Water bath 37°C

Procedure:

1. Make the reaction mix by combining the components in the following order:

	Volume (µl)
dH_2O	15
10X FastDigest Green Buffer (can be used as electrophoresis loading buffer)	2 (1X final concentration)
Plasmid DNA	1 (up to 1 µg)
FastDigest Enzyme	1
FastDigest Enzyme	1
Total volume (µl)	20

NB! The combined volume of enzymes should not exceed 1/10 of the total volume.

2. Mix gently and spin down.
3. Incubate at 37°C in a water thermostat for the time as recommended by supplier (5-15 minutes).
4. Load the reaction mix directly on an agarose gel for gel electrophoresis (see section 3.2.6).
5. Excise the target gel band for clean-up (see section 3.2.7).

(Adapted from Product information - Thermo Scientific FastDigest EcoRI. Rev 9 2012)

3.3.5 Polymerase chain reaction

The polymerase chain reaction (PCR) is a technique applied for amplifying a specific DNA fragment (Pray, 2008b). The basic components for PCR are: DNA polymerase, template DNA, the four deoxyribonucleotides (substrate for DNA polymerase and raw material for new DNA strands), forward and reverse primers with exposed 3´OH groups and buffer. Primers are DNA sequences that are complementary to the outer end of the target DNA sequence. They have a free 3´OH group at their ends because DNA polymerase can only add new deoxyribonucleotides to a pre-existing 3´OH group. The forward and reverse primers are complementary to the sense and anti-sense DNA strands respectively. PCR are performed in test tube (PCR-tube) placed in a thermocycler (PCR machine). In general, each cycle of PCR contains of three main, temperature-dependent steps (figure 3.1):

1. Denaturing of double DNA strands in high temperature between 90°C and 100°C. The high temperature breaks hydrogen bonds between the DNA strands.
2. Hybridization steps by cooling to between 30°C and 65°C to let primers anneal to their complementary sequence of the now, single stranded DNA. The higher the temperature, the higher stringency of hybridization between primer DNA template.
3. Elongation steps by heating to between 60°C and 75°C to let DNA polymerase synthesize new DNA strands from the 3´OH-ends of the primers and using the single stranded DNA as template.

In principal, the amount of target DNA sequence doubles after each cycle of PCR.

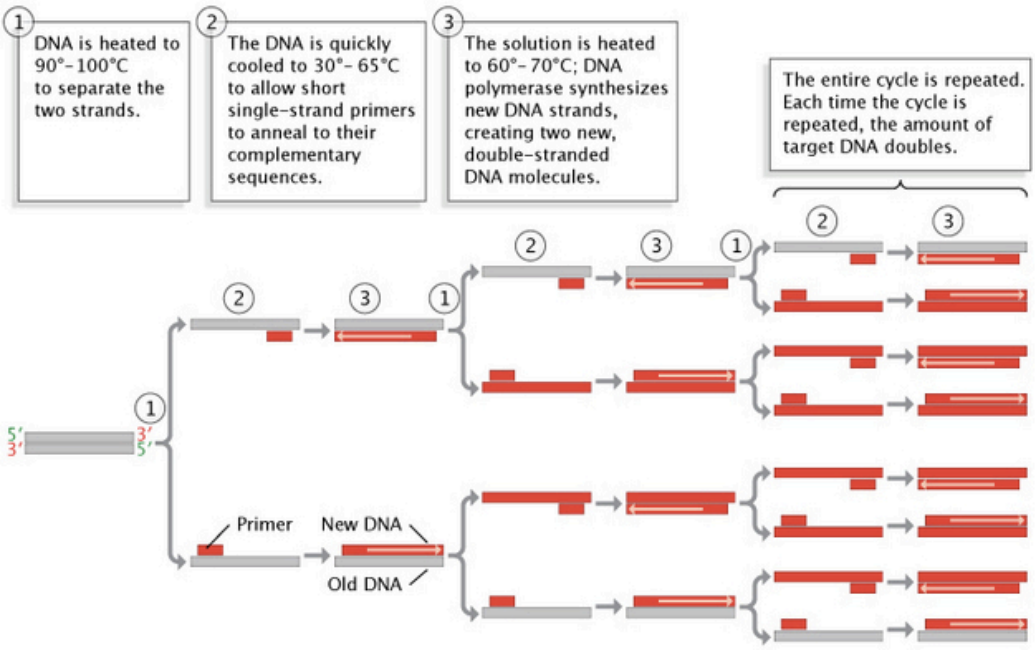


Figure 3.1 Polymerase chain reaction (PCR) (This figure is from Scitable by Nature Education. © 2014 Nature Education. All rights reserved.)

See section 2.2 for overview of the primers used in this study.

Q5® High-Fidelity 2X Master Mix (New England Biolabs)

The Q5® High-Fidelity 2X Master Mix was used for PCR-amplification of fragments used for cloning with the In-Fusion® HD Cloning Kit (see section 3.2.8). Primers used for the In-Fusion® HD Cloning Kit are designed to produce inserts with a 15bp extension, including the restriction sites, that overlap with the 5' overhang of the linearized plasmid vector. Vectors used in this study contain restriction sites that give sticky ends, thus bases complementary to 5' overhangs are included in the primer sequence and bases in the 3' overhangs are not.

Materials:

Q5 High-Fidelity 2X Master Mix (Q5 MaMix)

10 µM Forward primer

10 µM Reverse primer

Plasmid DNA (template DNA source)

dH_2O

6X Loading Dye

PCR tubes

PCR machine (thermocycler)

Procedure:

1. Use NEB Tm Calculator (http://tmcalculator.neb.com/#!/) to calculate the annealing temperature for the each specific primer pair.
2. Make the reaction mix in a PCR tube on ice:

	25 µl mix	**50 µl mix**
Q5 MaMix	12.5 (1X finale concentration)	25 (1X finale concentration)
10 µM Forward primer	1.25	2.5
10 µM Reverse primer	1.25	2.5
Plasmid DNA	variable	variable (1 pg- 1 ng)
dH_2O	to 25 µl	to 50 µl

3. Mix gently and collect all liquid at the bottom of the PCR tube by spinning shortly.
4. Transfer the PCR tubes with reaction mix to a PCR machine and perform thermocycling using the following settings:

Step	Temperature	Time
Initial denaturation	98°C	30 seconds
25 cycles (may increase up to 35 cycles)	98°C	10 seconds
	50-72°C (Annealing temperature as recommended by NEB Tm Calculator)	20 seconds
	72°C	30 seconds
Final extension	72°C	2 minutes
Hold	10°C	

5. Store the PCR reaction mix in the refrigerator (for experiments the next day) or at -20°C if gel electrophoresis is not going to be performed right after thermocycling.
6. For gel electrophoresis: Add 5 µl or 10 µl of 6X Loading Dye to 25 µl or 50 µl PCR reaction mix, respectively (1X final concentration of loading dye).
7. Load the sample(s) onto an agarose gel for gel electrophoresis (see section 3.2.6) to check for PCR product, and if applicable, excise and isolate the PCR product.

(Adapted from the Q5® High-Fidelity 2X Master Mix manual from 2014)

VWR Red Taq DNA Polymerase Master Mix (VWR)

2x Master Mix Kit (1.5 mM MgCl2)

VWR Red Taq DNA Polymerase Master Mix was used to perform colony-PCR as a preliminary control of transformants (see section 3.2.9). PCR-products were loaded onto an agarose gel for gel electrophoresis. If target bands were observed, it strongly indicated successful transformations. An inert red dye is presented in the master mix to allow for direct loading of sample onto agarose gel for electrophoresis analysis (VWR International, 2013).

Materials:

Taq 2x Master Mix (Taq 2xMaMix)

10 µM Forward primer

10 µM Reverse primer

Transformant colony (template DNA source)

dH_2O

PCR tubes

PCR machine (thermocycler)

Procedure:

1. Use NEB Tm Calculator (http://tmcalculator.neb.com/#!/) to calculate the annealing temperature for the each specific primer pair.
2. Make the reaction mix in a PCR tube on ice:

	50 µl mix
Taq 2xMaMix (spin briefly before use)	25 (1X final concentration)
10 µM Forward primer	1
10 µM Reverse primer	1
Transformant colony	Pick a sample with a sterile toothpick and smear in the bottom of the PCR tube
dH_2O	23

3. Mix gently and collect all liquid to the bottom of the PCR tube by spinning shortly.
4. Transfer PCR tubes with reaction mix to a PCR machine and perform thermocycling using the following settings:

Step	Temperature	Time
Initial denaturation	95°C	2 minutes
30-35 cycles	95°C	20 seconds
	50-65°C (annealing temperature as recommended by NEB Tm Calculator)	25 seconds
	72°C	30 seconds
Final extension	72°C	5 minutes
Hold	10°C	

5. Store PCR reaction mix in the refrigerator (for experiments the next day) or at -20°C if gel electrophoresis is not going to be performed right after thermocycling.
6. For gel electrophoresis: Load 10 - 30 % (5 – 15 µl) of the reaction mix volume directly onto an agarose gel and run gel electrophoresis analysis (see section 3.2.6) and check for PCR products with the expected size band.

(Adapted from the VWR Red Taq DNA Polymerase Master Mix manual from 2013)

3.3.6 DNA separation and analysis by agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse and purify digested plasmid DNA and PCR products. The nucleic acid molecules are loaded into wells in cast agarose gel placed in a container filled with buffer. When an electric current is applied in the field, negatively charged nucleic acids migrate toward the anode (positive) pole (Yilmaz, Ozic, & Gök, 2012). As nucleic acids migrate through network of pores in the gel, they are separated by size differences, where smaller molecules migrate faster. The migration is visualized under UV light using a fluorescent DNA binding dye and appears as size dependent bands along the migration path. The desired "band", meaning the desired DNA fragment, can be excised and the DNA fragment can purified (see section 3.2.7) for further experiments like transformation. A DNA fragment size standard is used to estimate the size of the DNA fragments.

Materials (agarose gel stock solution):

Agarose (SeaKem® LE Agarose from Lonza, molecular biology grade)

1X TAE buffer

Procedure (agarose gel stock solution):

1. Add 500 ml 1X TAE buffer to 6 g agarose (1.2 % v/w) and stir with a magnetic stirrer. Remove the magnet before autoclaving.
2. Autoclave the agarose solution for 15 minutes at 121°C.
3. Store in 50°C incubator.

Materials (gel electrophoresis):

1.2 % Agarose solution

peqGREEN DNA and RNA dye (PEQLAB, VWR)

Nucleic sample (section 3.2.4 and 3.2.5)

6X DNA Loading Dye (Thermo Scientific)

GeneRuler™ 1kb DNA Ladder (Thermo Scientific)

Quick-Load® 1kb DNA Ladder (New England Biolabs)

Gel caster

Gel tray

Comb, 8 wells

Gel tank (Mini-Sub cell GT cell)

PowerPac™ Basic Power Supply

Gel Doc™ EZ system

UV Sample Tray

(Gel casting set-up and imaging device are from Bio-Rad)

Procedure (gel electrophoresis):

1. Prepare for gel casting by placing the gel tray on the gel caster. Place the comb at one end of the gel tray. Make sure that the gel caster is flat so that agarose solution can distribute evenly for even thickness of the gel.

2. Add 2.5 µl peqGREEN to 60 ml of agarose solution. Swirl gently to mix solution before pouring into the gel tray. Use a pipette tip to remove bubbles if there are any. Allow the gel to polymerize for at least 15-20 minutes at room temperature. After polymerization, transfer gel to a gel tank and fill tank with 1X TAE buffer until the mark indicating maximum volume.
3. Add5 µl 6X DNA Loading Dye to 25 µl sample (1X final concentration).
    a. PCR-products that contain VWR Red Taq DNA Polymerase Master Mix are already loaded and dyed.
4. Pipette samples and molecular marker into the wells:
    - 10 µl GeneRuler™ or Quick-Load® and
    - 30 µl loaded Q5® PCR-product (section 3.2.5, first part)
    - 15 µl VWR Red Taq PCR-product (section 3.2.5, second part)
    - Whole volume of digested pDNA (section 3.2.4)
5. Close the lid of the gel tank and connect to the PowerPac™ Basic Power Supply. Run electrophoresis at 70 volt for 45 – 60 minutes.
6. Visualize nucleic acids with Gel Doc™ EZ system on an UV sample tray.
7. Excise desired DNA fragments for further analysis if necessary.

3.3.7 DNA fragment purification from agarose gel

To purify DNA fragments from an excised agarose gel, the gel was mixed with binding buffer and dissolved by heating. Chaotropic salt in binding buffer make it possible for DNA to bind to the silica in the purification column, and a pH indicator in the buffer ensure pH for optimal binding (colour of the dissolved agarose gel should be yellowish) of DNA (“NucleoSpin® Gel and PCR Clean-up Kit,” 2013).

Material:

NucleoSpin® Gel and PCR Clean-up Kit

- Buffer NTI
- Buffer NT3
- Buffer NE
- NucleoSpin® Gel and PCR Clean-up Columns (yellow rings)
- Collection tubes

dH_2O

Water bath, 50°C

Procedure:

1. For each 100 mg of agarose gel, add 200 µl of Buffer NTI (binding buffer) and incubate sample for 5-10 minutes at 50°C water bath. Vortex briefly for every 2-3 minutes until the gel is completely dissolved.
2. Prepare a NucleoSpin® Gel and PCR Clean-up Column and collection tube for each sample. Load a maximum of 750 µl sample onto the column and centrifuge at 11,000 x g for 1 minute. Repeat if there is more sample left. Discard flow-through.
3. Wash plasmid DNA, bound to the silica in the column, with 700 µl Buffer NT3 and centrifuge at 11,000 x g for 1 minute. Discard flow-through.
4. After washing step, dry the silica membrane with centrifugation at 11,000 x g for 2 minutes and optionally air-dry for 2 minutes.
5. After drying step, transfer the NucleoSpin® Gel and PCR Clean-up Column to a 1.5 ml microtube for collection of DNA. Elute plasmid DNA by adding 15 µl of preheated Buffer NE or dH20 to the column and incubate for 3 minutes at 50°C before centrifugation at 11,000 x g for 1 minute. Reload the NucleoSpin® Gel and PCR Clean-up Column with the eluted volume of plasmid DNA (15 µl) and repeat the elution step.
6. Measure DNA concentration (section 3.2.3) and proceed to further experiments or store at -20°C.

(Adapted from the NucleoSpin® Gel and PCR Clean-up Kit manual from 2013)

3.3.8 Recombining linearized plasmid and DNA insert

As mentioned, two different methods were used to recombine plasmid vector. One of the method was to digest plasmids using FastDigest® restriction enzymes and recombine by ligation using DNA ligase. The other method was to PCR-amplify an insert that consisted the insert gene and a 5'-end and a 3'-end extension that overlap with the plasmid vector, and recombining the linearized vector and insert with In-Fusion® Cloning Kit.

In-Fusion® HD Cloning Kit

In-Fusion® HD Cloning Kit was used for recombining linearized vector plasmid (section 3.2.4) and PCR amplified insert (section 3.2.5).

Materials:

Vector (linearized plasmid)

Insert (purified PCR product)

5X In-Fusion HD Enzyme Premix

dH_2O

Water bath, 50°C

Procedure:

1. Make the reaction mix in a 1.5 ml microtube:

	10 µl mix
5X In-Fusion HD Enzyme Premix	2
Linearized vector plasmid *	50-200 ng
Purified PCR insert *	10-200 ng
dH_2O	To 10 µl

   In general, good efficiency is achieved with 50-200 ng of vector and inserts, respectively.

   * If the volume of vector and inserts excess 7 µl, double the amount of 5X In-Fusion HD Enzyme Premix and add dH_2O to a total volume of 20 µl.

2. Incubate reaction mix for 15 minutes at 50°C in a water bath.
3. Chill on ice after incubation and proceed to transformation or store at -20°C until needed for transformation.

(Adapted from the In-Fusion® HD Cloning Kit manual from 2011)

Quick Ligation™ Kit

Quick Ligation™ Kit was used to recombine digested plasmid of vector and insert.

Materials:

Linearized vector plasmid

Linearized insert plasmid

2X Quick Ligation Buffer

Quick Ligase

dH_2O

Procedure:

1. In general, mix 50 ng of vector and 3-fold molar excess of insert. Use NEBioCalculator (http://nebiocalculator.neb.com/#!/) to calculate the vector-insert ratio.
2. Adjust volume to 10 µl with dH_2O.
3. Add 10 µl 2X Quick Ligation Buffer and mix.
4. Add 1 µl Quick Ligase and mix thoroughly.
5. Spin briefly and incubate for 5 minutes at room temperature.
6. Chill on ice after incubation, then transform or store at -20°C until needed for transformation.

   Do not heat inactivate, because this will dramatically reduce transformation efficiency.

(Adapted from the Quick Ligation™ Kit manual, accessed from New England Biolabs website in 2014)

3.3.9 Transformation of *E. coli*

Recombinant plasmid DNA was introduced into competent *E. coli* host cell by transformation, see section 2.1 for an overview of the combinations of strains and plasmid, and for an overview of the antibiotic used. Chemically competent cells were used for transformation. These cells are transformable because they have been treated with buffer

that contain $CaCl_2$ and other salts that partially disrupt the cell membrane, making it possible for plasmids to pass into the cell ("Competent Cell Compendium: Tools and Tips for Successful Transformations," 2006). After transformation, the cells are diluted in rich media to recover before cultivation on selective media.

Materials:

Ligation mix

Competent *E.coli*

S.O.C. medium

Medium plates with antibiotics

Water bath, 42°C

Procedure (New England Biolabs):

1. Thaw competent cells on ice for at least 5-10 minutes.
2. Add 5 ng (or 2 µl) of ligation mix to 50 µl of competent cells in a 1.5 ml microtubes or a falcon tube. Mix by pipetting up and down or flicking the tube 4-5 times. Do not vortex.
3. Place the mixture on ice for 30 minutes without mixing.
4. Heat shock at 42°C for 30 seconds.

   NB! Duration and temperature may be adjusted according to recommendation provided by the manufacture of the competent cells.
5. Add 950 µl S.O.C. medium and incubate for 60 minutes at 37°C in a shaking incubator.

   NB! The type of medium and amount may be adjusted according to recommendation provided by the manufacture of the competent cells.
6. Pre-warm selection plates to 37°C.
7. Spread 50 and 100 µl of the transformation mix onto plates and incubate overnight at 37°C.

(Adapted from New England Biolabs' manual for transformation, accessed from New England Biolabs website in 2014)

3.3.10 Confirmation of successful transformation into host cell

To confirm successful transformation, transformants were checked preliminary by colony-PCR (section 3.2.5) and sent to GATC Biotech for LIGHTrun™ Sequencing for a final confirmation. Each transformant colony tested was subjected to colony-PCR and overnight cultivation (section 3.2.1 for cultivation). Plasmid DNA isolated from overnight culture was sent to GATC Biotech LIGHTrun™ Sequencing for DNA sequencing and results were analysed using CLC Genomics Workbench 5.

Materials for LIGHTrun™ Sequencing:

Plasmid DNA isolated from overnight culture (section 3.2.2)

Sequencing forward and reverse primers, 10 µM (section 2.2)

dH_2O

Barcode from GATC Biotech (for identification of samples)

Procedure for LIGHTrun™ Sequencing:

1. Mark one 1.5 ml microtube with barcode. One barcode for each primer.
2. Make the reaction mix in the barcode-marked 1.5 ml microtube:

Plasmid DNA	400 - 500 ng
Primer	2.5 µl
dH2O	to 11 µl

3. Read results using CLC Genomics Workbench.

3.3.11 Electroporation with electrocompetent *L. plantarum* WFCS1

To express fusion proteins used in this study in *L. plantarum* WCFS1 purified plasmid DNA was transformed to the microbe by electroporation. In an electroporation device mixture of cells and plasmids is subjected to a pulse of electricity. The pulse of electricity temporarily disrupts the cell membranes and for a short moment, allow plasmids to pass through ("Competent Cell Compendium: Tools and Tips for Successful Transformations," 2006). *L. plantarum WCFS1* was made electrocompetent prior to electroporation according to (Aukrust, Brurberg, & Nes, 1995)

Preparing Electrocompetent *L. plantarum* WCFS1

Materials:

2X MRS (section 3.2.1, double amount of MRS)

MRS

20 % glycine (w/v)

30 % PEG-1500

$MgCl_2$

TEN buffer

L. plantarum WCFS1 overnight culture

Chilled Corex tube

Ice

Procedure:

1. Dilute *L. plantarum* WCFS1 overnight culture with MRS to absorbance at OD_{600} (optical density at 600 nm) = 0.5-0.7.
2. Add 5-10 ml of the diluted culture to a 50 ml Nunc tube containing 20 ml 2X MRS, 5 ml 20% glycine and 5-10 ml dH_2O (total volume should be 40 ml).
    - Make a negative sample by replacing the culture with MRS.
3. Measure the absorbance at OD_{600} and incubate at 30°C until about 2.5 generation has passed (absorbance of about 1.0).
    - Glycine inhibits formation of crosslinkage in the cell wall and enhances transformability.
4. After incubation, chill the sample on ice and harvest by centrifugation at 5000 x g for 5 minutes at 4°C. It is important to keep cells cool to maximise transformation efficiency. Discard supernatant.
    - Chill a Corex tube and 10 1.5 ml microtubes on ice for later.
5. Wash cell pellet with 10 ml chilled TEN buffer. Centrifuge at 5000 x g for 5 minutes at 4°C. Discard supernatant.
6. Wash with 40 ml chilled 1mM $MgCl_2$ and centrifuge at 5000 x g for 5 minutes at 4°C. Discard supernatant.

7. Resuspend cell pellet in 5 ml 30% PEG-1500 and transfer to a chilled Corex tube. Centrifuge at 5000 x g for 10 minutes at 4°C. Discard supernatant.
8. Resuspend cell pellet in 400 µl 30% PEG-1500 and distribute into 40 µl aliquots in chilled 1.5 ml microtubes. Store at -80°C.

Electroporation of *L. plantarum* WCFS1

Materials:

Electrocompetent *L. plantarum* WCFS1

MRSSM medium

MRS plate with erythromycin

Electroporation cuvette

Electroporator

Ice

Procedure:

1. Chill MRSSM and cuvette on ice.
2. Mix 40 µl electrocompetent *L. plantarum* WCFS1 and 5 µl plasmid DNA and immediately transfer to the chilled cuvette. Tap gently to mix and avoid air bubbles.
3. Adjust the Electroporator to the following parameter:
   - Tension 1.5 kV
   - Capacitance 25 mF
   - Resistance 400 W
4. Place cuvette in the electroporation device and deliver the tension pulse.
5. Add 950 µl chilled MRSSM to the cuvette and mix by pipetting up and down.
6. Transfer the mixture to a 1.5 ml microtube and incubate at 30°C for at least 2 hours and overnight at most.
7. Streak 50 and 100 µl of the cell suspension on MRS plate with erythromycin and incubate at 30°C overnight.

(Adapted from Aukrust et al., 1995)

3.4 Protein production

3.4.1 Intracellular protein production and extraction of protein in *E. coli*

pET-16b constructs made in this study were all made for intracellular expression. To purify fusion proteins, overproducing *E. coli* cells were lysed by sonication and the soluble fraction was used for protein purification with immobilized metal ion affinity chromatography (IMAC). The pET-16b vector include a coding sequence for a N-terminal His-tag that enable production of His-tagged fusion proteins that can be purified by IMAC by employing the interaction between histidine residues and the immobilized metal ion (more detail in section 3.3.4).

In the pET-16b expression vector the gene of interest is under the control of bacteriophage T7 transcription signal and expression is induced in host cells that provide T7 RNA polymerase (Novagen, 2011). pET-16b vectors were transferred to *E. coli* that contains the T7 RNA polymerase gene under the lacUV5 promoter. Expression of the T7 RNA polymerase was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG), a molecular mimic of allolactose. In general a final concentration of 0.4 mM IPTG was used. In this study an induction experiment was performed testing three different concentrations of IPTG (0.04 mM, 0.1 mM and 0.4 mM) and induction at 10°C and 18°C. See results for more details.

Materials:

Day 1: 3 ml overnight LB culture of *E. coli* carrying plasmid
400 ml LB broth with ampicillin (200 μg/ml)

Day 2: 0.4 mM IPTG

Day 3: Lysis buffer
0.22 μm syringe filter
50 mM PMSF

Procedure:

1. Day 1: Transfer 3 ml overnight LB culture of *E. coli* to 400 ml LB broth with ampicillin and incubate overnight at 18°C in shaking incubator.

2. Day 2: Induce expression with IPTG at a final concentration of 0.4 mM when absorbance at OD_{600} is between 0.5 – 1.5. Incubate overnight at 18°C in shaking incubator.

   Day 3:
3. Harvest the cells by centrifugation at 5000 x g for 10 minutes at 4°C. Discard supernatant.
4. Resuspend the cell pellet in 16 ml lysis buffer and incubate at room temperature for 20 minutes with occasional shaking. For convenience, transfer cell suspension to a 50 ml Nunc tube.
5. Place the tube with cell suspension in ice to cool down suspension during sonication. Submerge the sonication probe approximately 1 cm into the cell suspension and make sure it does not touch the wall of the tube. Sonicate with these parameters:
   - 20 % amplitude
   - 5 seconds ON/OFF
   - 5 minutes (total of ON time)
6. Spin down cell debris at 10,000 x g for 10 minutes at 4°C. Transfer supernatant to a clean container. Sterile filtrate the supernatant with 0.22 µm syringe filters and add PMSF (2 µl per 1 ml).
7. Store in refrigerator or proceed directly to further experiment and analysis.

3.4.2 Intracellular protein production and extraction of fusion protein in *L. plantarum* WCFS1

Intracellular protein production in *L. plantarum* WCFS1 was done using a derivative of the pSIP400-series (termed pLp hereafter) that contains the promoter for sakacin P (Sørvig et al., 2003). Expression was induced by addition of the peptide pheromone SppIP. SppIP leads to phosphorylation of a membrane located histidine kinase, which then activates a response regulator that binds to, in this case, the regulated sakacin P promoter and thus induce expression of the target gene that is under the control of the sakacin P promoter.

To purify fusion proteins from *L. plantarum* WCFS1, cells were lysed by sonication and the soluble fraction was used for protein purification with IMAC. pLp vectors do not

contain coding sequence for N-terminal His-tag, thus a construction of insert gene that contain coding sequence for a N-terminal His_6-tag was constructed in this study to produce fusion protein with His-tag that could be purified by IMAC (more detail in section 3.3.4).

Materials:

Overnight culture *L. plantarum* WCFS1 carrying plasmid
500 ml MRS broth with erythromycin (200 µg/ml)
Inducer peptide SppIP, 100 µg/ml stock solution
Lysis buffer
0.22 µm syringe filter
50 mM PMSF

Procedure:

1. Dilute the overnight culture to an absorbance at OD_{600} = 0.1 500 ml MRS with erythromycin.
2. Incubate the diluted culture at 30°C in an incubator without shaking. Add 25 ng/ml SppIP when OD_{600} = 0.3. Incubate the culture for 5 hours.
3. Harvest the cells by centrifugation at 5000 x g for 10 minutes at 4°C. Discard supernatant.
4. Resuspend the cell pellet in 16 ml lysis buffer and incubate at room temperature for 20 minutes with occasional shaking. For convenience, transfer cell suspension to a 50 ml Nunc tube.
5. Place the tube with cell suspension in ice to cool down suspension during sonication. Submerge the sonication probe approximately 1 cm into the cell suspension and make sure it does not touch the wall of the tube. Sonicate with these parameters:
   - 30 % amplitude
   - 5 seconds ON/OFF
   - 15 minutes (total of ON time)
6. Spin down cell debris at 10,000 x g for 10 minutes at 4°C. Transfer supernatant to a clean container. Sterile filtrate the supernatant with 0.22 µm syringe filters and add PMSF (2 µl per 1 ml).

7. Store in refrigerator or proceed directly to further experiment and analysis.

3.4.3 Displaying fusion proteins on cell surface of *L. plantarum* WCFS1

Production of fusions proteins with surface anchors for direct display on cell surface of *L. plantarum* WCFS1 was done using the pLp expression vector (see above for more detail about the pSIP system).

For analysis of protein production, induced *L. plantarum* WCFS1 carrying plasmid was analysed with flow cytometry or Western blot. For Western blot analysis, cells were lysed with FastPrep®-24 homogenizer and the cell-free extract was analysed.

Materials:

Overnight culture *L. plantarum* WCFS1 with recombinant plasmid
50 ml MRS broth with erythromycin (200 μg/ml)
Inducer peptide SppIP, 100 μg/ml stock solution
1X PBS
Glass beads, acid washed <106 μm
FastPrep®-24 homogenizer and tubes

Procedure:

1. Dilute the overnight culture to an absorbance at OD_{600} = 0.1 in a Nunc tube with 50 ml MRS with erythromycin.
2. Incubate the diluted culture at 30°C in an incubator without shaking. Add 25 ng/ml SppIP when OD_{600} = 0.3. Incubate the culture for 2-5 hours.
3. Harvest cells by centrifugation at 3000 x g for 5 minutes at 4°C. Discard supernatant.
4. Wash cell pellet 2 times with 10 ml 1X PBS and use the same centrifugation parameters as in step 3.

   Cell disruption with acid washed glass beads for Western blot analysis:
5. Store cell pellet at -20°C or resuspend cells in 1 ml 1X PBS.
6. Weigh in 0.5 – 0.6 g glass beads in a FastPrep®-24 tube and transfer the cell suspension from step 5 over to the tube.

7. Disrupt cells with the FastPrep®-24 homogenizer using the following parameters: 6.5 m/s and 45 seconds
8. Centrifuge disrupted cells at 16,000 x g for 5 minutes at 4°. Transfer supernatant to a 1.5 ml microtube.
9. Repeat the centrifugation at step 8 to remove residues of glass beads. Transfer the supernatant to a new 1.5 ml microtube.
10. Store the cell-free extract at -20°C or proceed to further experiment and analysis.

3.4.4 Protein purification by immobilized metal ion affinity chromatography (IMAC)

Immobilized metal ion affinity chromatography (IMAC) was performed to purify polyhistidine tagged fusion protein from the soluble fraction of cell-free protein extract.

This procedure is based on interaction between the immobilized Ni^{2+} ions and the polyhistidine tag in the fusion proteins. Protino® Ni-NTA Agarose beads consists of nitrilotriacetic acid (NTA), a tetradentate chelator, that is immobilized on 6 % agarose beads ("Purification of His-tag proteins - User manual Protino® Ni-NTA Agarose, Protino® Ni-NTA Columns 1 mL, Protino® Ni-NTA Columns 5 mL," 2013). The chelator occupies four of six of the binding sites of Ni^{2+}. The two binding sites left are usually occupied with water molecules that are exchangeable with histidine residues in fusion protein (figure 3.2). A buffer with a competing agent, imidazole in this case, can then elute fusion protein with histidine tag.

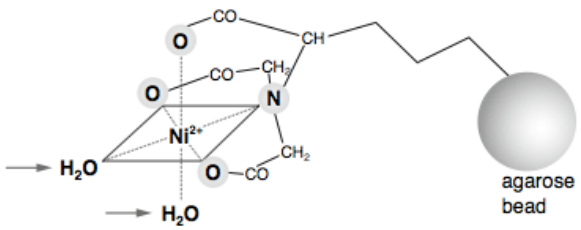


Figure 3.2 NTA in complex with Ni^{2+} (This figure was taken from "Purification of His-tag proteins - User manual Protino® Ni-NTA Agarose, Protino® Ni-NTA Columns 1 mL, Protino® Ni-NTA Columns 5 mL" 2013)

Materials:

Soluble fraction of cell-free protein extract (keep on ice throughout purification)

Buffer A

Buffer B

20 % Ethanol

Protino® Ni-NTA Agarose

BioLogic™ LP System

Ni-NTA Column

Procedure:

1. Pack a column with approximately 5 ml Protino® Ni-NTA Agarose and allow the beads to sink for 15-30 minutes. Avoid air bubbles.
2. Run Buffer A through the system with 1.5 mg/ml flow rate for about 10 minutes. This is the baseline (background signal). Zero the baseline by pressing the "UV" button on the instrument and choose "Zero".
3. Stop the flow and move the aspiration tube to the sample tube. Run the sample through the system. The curve in the chromatogram will begin to climb upward which indicates detection of the flow-through of waste products. Collect the flow-through for later analysis.
4. When the entire sample has passed the system, run Buffer A through the system again. The curve in the chromatogram will start to climb downward and let Buffer A flow through the system till the chromatogram has been showing a flat line for 5 minutes.
5. Move the aspiration tube to Buffer B to elute fusion protein and start collecting the eluate in a clean tube. Base line will usually be higher because of higher imidazole concentration, and the eluate-peak will usually be smaller than the flow-through.
6. After collecting the eluate, move aspiration tube back to 20 % ethanol and direct the flow back to the waste tank. Rinse by purging the system with 20 % ethanol.
7. Protino® Ni-NTA Agarose, now in 20 % ethanol, can be transferred to a 15 ml Nunc tube and store at 4°C to be reused later.

3.5 Evaluative analysis of protein production

Protein production was analysed and checked with protein gel electrophoresis, Western blot or flow cytometry. To measure protein concentration, the Bradford method was used.

3.5.1 Measuring protein concentration with the Bradford protein assay

The method of Bradford was used to measure protein concentration in solution. This method is a dye-binding assay in which Coomassie blue, an acidic dye, undergoes a colour change upon binding to protein ("Bio-Rad Protein Assay - LIT33 Rev C," n.d.). The colour change is then measured at 595 nm and concentration can be calculated using a standard curve. The dye binds primarily to basic and aromatic amino acid residues, especially arginine.

Materials:

1X Dye Reagent Concentrate (room temperate)
Buffer A
Protein sample (set up parallels)
BioPhotometer and cuvettes

Procedure:

1. Cool 1X Dye Reagent Concentrate to room temperature.
2. Blank sample: 800 µl Buffer A
3. Protein sample: 10 µl sample + 790 µl Buffer A
4. With about 30 seconds in between each sample, add 200 µl 1X Dye Reagent Concentrate to the blank sample and the protein samples. Pipette or vortex to mix samples thoroughly.
5. Incubate at room temperature for at least 5 minutes and maximum for 60 minutes.
6. Use the "Bradford micro" program and reset BioPhotometer with the blank sample. All samples, including blank, has to be incubated with dye reagent for the same amount of time. Thus it is important to measure following samples at the exact time difference between the additions of dye reagent to samples.

(Adapted from the Bio-Rad Protein Assay – LIT33 Rev C manual)

3.5.2 Sodium dodecyl sulphate – polyacrylamide gel electrophoresis

To examine the efficiency of protein production, protein content of harvested samples were separated by Sodium dodecyl sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE). In SDS-PAGE the anionic detergent SDS binds to proteins, which mask the proteins' intrinsic charge, resulting in a net negative charge (Garca-Descalzo, Garca-Lpez, Alczar, Baquero, & Ci, 2012). Complexes of SDS-protein thus migrate through the gel with a speed that is highly related to the protein's molecular weight. In this study, lithium dodecyl sulphate (LDS) was used instead of SDS. Samples for SDS-PAGE were mixed with sample buffer that contain LDS and denatured by heating in water bath.

Materials:

10% Mini-PROTEAN® TGX Stain-Free™ Precast Gels, 10 or 15 wells
BenchMark™ Unstained Protein Ladder
MagicMark™ XP Western Protein Standard
NuPAGE® LDS Sample Buffer (4X)
NuPAGE® Sample Reducing Agent (10X)
Gel Doc™ EZ imaging systems
1X TGS Buffer
Mini-PROTEAN Tetra Cell System, 4 gels
PowerPac™ 300
Water bath, 100°C

Procedure:

1. Make a 10 ml stock solution of 2X sample buffer and store at room temperature:
   - 5 ml 4X LDS Sample Buffer
   - 2 ml Reducing Agent
   - 3 ml dH_2O
2. Make SDS sample by mixing 2X sample buffer and protein sample in 1:1 ratio. Denature samples in 100°C water bath for 5 minutes.
3. Prepare precast gels in Mini-PROTEAN Tetra Cell System and fill the electrophoresis chamber with 1X TGS buffer until the indicated mark.

4. Add 10 µl BenchMark™ ladder in one well.

   NB! Add or replace with 5 µl MagicMark™ ladder if the gel is going to be further analysed with Western blot. The amount MagicMark™ might have to be adjusted to optimal signal.
5. Add 12 µl or 20 µl of sample per well for 15-wells and 10-wells gel, respectively.
6. Run electrophoresis at 280 V for 18 minutes or expand run time till the dye band reaches the black line at the bottom of the gel.
7. Place the gel on a stain-free tray and analyse with Gel Doc™ EZ imaging systems.
8. If the gel is being further analysed with Western blot, wash gel in dH_2O for 5 minutes before blotting (see section 3.4.2).

3.5.3 Western blot analysis

Western blot (immunoblotting) was used to detect the target protein. This is a method of immunodetection where proteins on a membrane surface (usually of nitrocellulose or polyvinylidene difluoride) can be detected through specific antigen-antibody interactions. In this study antibody is conjugated with an enzyme label (horseradish peroxidase) that can oxidize a substrate to produce a detectable light-emission signal (chemiluminescence). To prevent unspecific binding of antibodies to unbounded sites, the blotted membrane needs to be blocked prior to incubation with antibodies. The following was done in this study:

- SDS-PAGE was electrophoretically transferred to a nitrocellulose membrane.
- The membrane was blocked with bovine serum albumin (BSA).
- Indirect detection (figure 3.3) was performed, meaning that the enzyme label is attached to a secondary antibody that binds to the primary antibody.
- For signal detection, the method of chemiluminescence with the substrate luminol and capture of light emission on film was employed (figure 3.3).

Most of the Western blot analysis was performed with the SNAP i.d.® 2.0 instrument because Western blotting with this instrument is less time consuming. But in cases where higher specificity was desired, traditional Western blotting was performed.

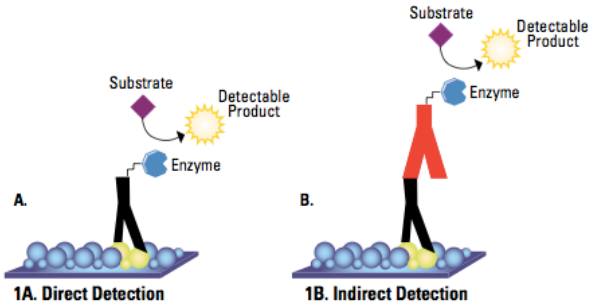


Figure 3.3 Direct and indirect detection in Western blot (This figure was taken from "Western Blotting Handbook and Troubleshooting Tools, Version 3" 2010)

Blotting with the iBlot® Dry Blotting System

Materials:

iBlot® Gel Transfer Device

iBlot® Transfer Stacks Nitrocellulose Mini

- iBlot® Cathode Stack, top
- iBlot® Anode Stack, bottom
- iBlot® Disposable sponge
- iBlot® Filter paper

Roller

Procedure:

1. Wash SDS-PAGE gel in dH_2O for 5 minutes.
2. Place the iBlot® Anode stack on the transfer device and place the gel upon the membrane. Roll out air bubbles between membrane and gel. Place a wet filter paper atop the gel and roll out air bubbles again.
3. Place iBlot® Cathode Stack atop with the copper electrode side up. Roll one more time over the area.
4. Place the disposable sponge as indicated inside the lid of the transfer device and close the lid.
5. Perform blotting by applying 20 V for 7 minutes (program 3). Proceed to immunoblotting.

Western blot with the SNAP i.d.® 2.0 Protein Detection System

Materials:

Bovine serum albumin (BSA), powder

10X TBS or 10X PBS

Tween 20 %

Primary antibody

Secondary antibody

iBlot® Dry Blotting System

SNAP i.d.® Protein Detection System

Roller

Table 3.1 Antibody dilutions used for the SNAP i.d.® 2.0 Protein Detection System

	Dilution	**Incubation time**
Primary: Mouse-α-HspX 0.1 mg/ml Mouse-α-His 0.1 mg/ml	 1 : 2500 or 1 : 3000 1 : 3000	10 minutes
Secondary: Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP 1.3 mg/ml	 1 : 5000	10 minutes

Procedure:

1. To make 300 ml 0.1 % Tween-TBS or 1% Tween-PBS wash buffer:
   - 300 µl Tween 20%
   - 30 ml 10X TBS or 10X PBS
   - add dH_2O to 300 ml
2. To make antibody solution:
   - Transfer 40 ml wash buffer to a 50 Nunc tube and dissolve 0.2 g BSA in the wash buffer (= 0.5 % solution).
   - In two 15 ml Nunc tubes, transfer 3 ml of 0.5 % BSA/wash buffer to each. One is for the primary antibody, and the other is for the secondary antibody.
3. To make 1% BSA blocking buffer: Pour off the 0.5 % BSA/wash buffer till there are 30 ml left and dissolve 0.15 g BSA in the remaining 0.5% BSA/wash buffer.

4. Wet the membrane layer (white side) of the blot holder and place the membrane in the middle of the blot holder with the protein side down. Roll out air bubbles gently and close the blot holder, then roll one more time.
5. Place the blot holder with the protein side up in the blot holder frame. Close and lock the frame on the SNAP i.d. 2.0 instrument.
6. Add 30 ml blocking buffer and press down the frame and apply vacuum. Turn off the vacuum when the frame is completely empty.
7. Add antibody solution and incubate for 10 minutes. Apply vacuum for 5-8 seconds after incubation and leave the vacuum on for washing. Wash three times with 10 ml wash buffer after each incubations. Add antibody almost right before incubation.
8. Remove membrane from the blot holder and incubate with detection reagent.

(Adapted from the SNAP i.d.® 2.0 Quick Start Guide 00114816 Rev. C manual)

Traditional Western Blotting

Materials:

5 % skimmed milk/TBS or 5 % skimmed milk/PBS

10X TBS or 10X PBS

Tween 20 %

Primary antibody

Secondary antibody

Table 3.2 Antibody dilutions and incubation time used for traditional Western blotting

	Dilution	**Incubation time**
Primary: Mouse-α-HspX 0.1 mg/ml Mouse-α-His 0.1 mg/ml	 1 : 2500 or 1 : 3000 1 : 3000	60 minutes
Secondary: Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP 1.3 mg/ml	1 : 5000	60 minutes

Procedure:

1. To make 300 ml 0.1 % Tween-TBS or 1% Tween-PBS wash buffer:
   - 300 µl Tween 20%
   - 30 ml 10X TBS or 10X PBS
   - add dH_2O to 300 ml
2. To make blocking buffer:
   Transfer 40 ml wash buffer to a 50 Nunc tube and dissolve 2 g skimmed milk powder in the wash buffer to make a 5 % skimmed milk blocking buffer.
3. To make antibody solution:
   In two 15 ml Nunc tubes, transfer 10 ml of the 5 % skimmed milk blocking buffer to each one. One is for the primary antibody, and the other is for the secondary antibody.

On a benchtop shaker:

4. Block membrane in 20 ml of the 5 % skimmed milk blocking buffer for 60 minutes on a benchtop shaker.
5. Incubate with 10 ml primary antibody solution and incubate for 60 minutes at room temperature. Wash three times with 20 ml washing buffer.
6. Incubate with 10 ml primary antibody solution and incubate for 60 minutes at room temperature. Wash three times with 20 ml washing buffer.
7. Proceed to incubation with detection reagent.

Detection with chemiluminescent luminol

Materials:

SuperSignal® West Pico Chemiluminescent Substrate

Luminol/enhancer Solution

Stable Peroxide Solution

CL-Xposure™ Film

Developer

Fixative

Procedure:

1. Mix 5 ml of Luminol/enhancer Solution and 5 ml of Stable Peroxide Solution (1:1 working solution) and incubate with antibody treated membrane for 5 minutes. Make sure that working solution covers the whole area of the membrane.
2. After incubation, cover the membrane with plastic foil and avoid air bubbles.
3. Place the membrane in a film cassette with the protein side facing up.
4. Prepare developer and fixative in a dark room.
5. Place a CL-Xposure™ Film on the membrane and close the film cassette. Expose for about 1 minute (up to 10 minutes depending on signal strength).
6. Develop the exposed film for about 1-2 minutes.
7. Fixate the developed film for at least 1 minute.
8. Wash the film in dH_2O and let it air-dry.

(Adapted from the SuperSignal® West Pico Chemiluminescent Substrate manual from 2014)

Stripping and reprobing of Western blots

Materials:

Restore™ Plus Western Blot Stripping Buffer

1X TBS or 1X PBS wash buffer

Procedure:

1. Cover the membrane in a plastic foil or air-dry and stored at 4°C until stripping can be performed.
2. Wash the membrane 2x5 minutes with wash buffer.
3. Incubate the membrane in stripping buffer for 5-15 minutes at room temperature.
4. Repeat step 2.

(Adapted from the Restore™ Plus Western Blot Stripping Buffer instructions printed on bottle)

3.5.4 Flow cytometry analysis and immunofluorescent detection of fusion proteins on cell surface

In this study, a secondary antibody coupled to fluorescein isothiocyanate (FITC) was used to detect a primary antibody bound to the target protein on the cell surface of *L. plantarum* WCFS. Fluorescent signals were then analysed with flow cytometry. In flow cytometry single cell particle can be detected. This is achieved by injecting sample into a core that is surrounded by an outer liquid sheath, and through hydrodynamic focusing, single particles in the core can pass through the detection system one by one (Rahman, 2014). When a particle pass through the detection system, the surface properties of the particle can then be analysed through detection of light scattering and fluorescence emission.

Materials:

1X PBS

2% BSA/PBS (w/v)

Primary antibody: mouse-anti-HspX

Anti-mouse IgG FITC-antibody

Overnight culture of *L. plantarum* WFCS1 carrying plasmid

Procedure:

1. See section 3.2.1 for cultivation and section 3.3.1-3.3.3 for protein expression.
2. Dilute the overnight culture of *L. plantarum* WCFS1 to OD=0.5 (approximately)
3. Harvest 1 ml the diluted culture by centrifugation at 5000 x g for 3 minutes. Discard supernatant.
4. Wash the cell pellet two times with 1 ml PBS and recollect cells by centrifugation at 5000 x g for 2 minute. Aspirate supernatant after the last wash.
5. Resuspend cell pellet in 50 µl 2 % BSA/PBS + 0.4 µl primary antibody and incubate for 60 minutes at room temperature.
6. After incubation, harvest the cells by centrifugation as in step 3.
7. Wash the cell pellet three times with 1 ml 2 % BSA/PBS at 5000 x g for 2 minutes. Aspirate supernatant after the last wash.

8. Resuspend the cell pellet in 50 µl 2 % BSA/PBS + 0.2 µl secondary antibody and incubate for 30 minutes at room temperature in a dark environment.
9. Repeat wash as in step 7.
10. Store samples on ice and in a dark environment until analysis with a flow cytometer.
11. Perform flow cytometry analysis with MacsQuant® Analyzer and MacsQuantify™ Software. Use the appropriate setting for detection of FITC signals on bacteria cells.

3.7 Binding assay

The binding capacity of fusion proteins that contain LysM domain to *Lactobacillus* spp was investigated by incubating bacteria cells with cell-free protein extracts from the soluble fraction. After incubation, cells were washed and the presence of cell wall bound fusion proteins of interest was analysed with Western blot (section 3.4.3) or flow cytometry (section 3.4.4).

Materials:

Cell-free protein extract from soluble fraction

Overnight culture or culture with cells harvested during the exponential phase:

L. plantarum WCFS1

L. rhamnosus GG

L. sakei Lb790

1X PBS

Procedure:

1. Harvest cell by centrifugation at 5000 x g for 3 minutes. Discard supernatant.
2. Wash the cell pellet two times with 1 ml PBS and recollect cells by centrifugation at 5000 x g for 2 minute. Aspirate supernatant after the last wash.
3. Resuspend the cells in PBS and dilute suspension to OD=0.5 (approximately) with PBS.
4. Harvest cells from 1 ml of the cell suspension from step 3 by centrifugation at 5000 x g for 3 minutes. Discard supernatant.
5. Resuspend and incubate cells with 1 ml cell-free protein extract (approx. 2 mg/ml). Incubate for 1 – 2 hours in room temperature with occasional mixing.
6. After incubation, harvest the cell by centrifuge at 5000 x g for 3 minutes. Discard supernatant.
7. Wash the cell pellet three times with 1 ml PBS and recollect cells by centrifugation at 5000 x g for 2 minute. Aspirate supernatant after the last wash.
8. Proceed to Western blot analysis (section 3.4.3) or flow cytometry (section 3.4.4) for detection protein.

4 Results

The *M. tuberculosis* fusion antigens used in this study were TB10.4 and HspX (TH). It had been shown that this combination is able to generate humoral and cell-mediated immune responses and has the potential as a multistage tuberculosis vaccine that provides protection against both active and latent infections (Niu et al., 2011). To promote targeting of dendritic cells, TH was coupled to a dendritic cell binding peptide (DC-seq), FYPSYHSTPQRP (Curiel et al., 2004), which was attached at the TH's C-terminus.

This study had two different non-GM approaches to produce and attach *M. tuberculosis* fusion antigens to *Lactobacilli* spp. In one of the non-GM approach, peptidoglycan binding LysM-anchors were N-terminally coupled to TH_DC-seq and the fusion proteins were produced intracellularly in *E. coli*. The binding ability of these LysM-coupled fusion proteins to *Lactobacillus* spp was not tested due to challenges encountered during protein extraction. In the other non-GM approach, LysM_TH_DC-seq was produced intracellularly in and extracted from recombinant *L. plantarum* WCFS1, and the peptidoglycan binding ability to *Lactobacillus* spp was tested.

In an alternative GM-approach, attempts were made to engineer recombinant *L. plantarum* WCFS1 to produce and display TH_DC-seq itself. In this GM-approach, three different anchors, a LysM-anchor, a lipobox-anchor and a LPxTG-anchor, were coupled to TH_DC-seq to enable anchoring.

4.1 Intracellular production of LysM-coupled TH_DC-seq in *E.coli*

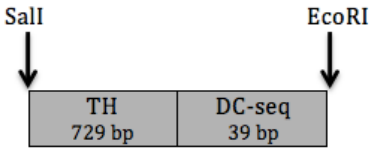
4.1.1 Cloning of LysM constructs into pET-16b expression vector for expression in *E. coli*

The LysM domains used for intracellular production in *E.coli*, were derived from the genes *lp_3014* and *lp_2162* from *L. plantarum* WCFS1 (Zhou et al., 2010). *Lp_3014* is a putative extracellular transglycosylase containing one LysM-domain at the N-terminus. *Lp_2162* is a putative gamma-*D*-glutamate-meso-diaminoimelate muropeptidase with two LysM-domains at the N-terminus. These LysM-anchors were taken from existing pLp constructs (Table 2.4) and as outlined below. The genes of interest were cloned into pET-

16b expression vector (Novagen, 2011) and transferred to chemically competent *E.coli* (section 3.2). Signal peptides were predicted with SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and were excluded from the coding sequence to secure intracellular protein production. Note that pET-16b expression vectors produce a N-terminal His-tag for protein purification with immobilized ion metal affinity chromatography (section 3.3.4), but this is not reflected in the plasmid names used in this study.

TH_DC-seq was constructed, with the restriction sites SalI and EcoRI, in a commercial pUC plasmid from Genscript (Figure 4.1a). To couple TH_DC-seq with the full-length 3014-LysM from gene *lp_3014* (see above and description in Table 2.3), TH_DC-seq was first subclone into the pre-existing pLp_3014_Inv (Fredriksen et al., 2012) as described in Figure 4.1b, resulting in pLp_3014_TH_DC-seq (all constructs constructed in this study are listed in Table 2.3).

a



b

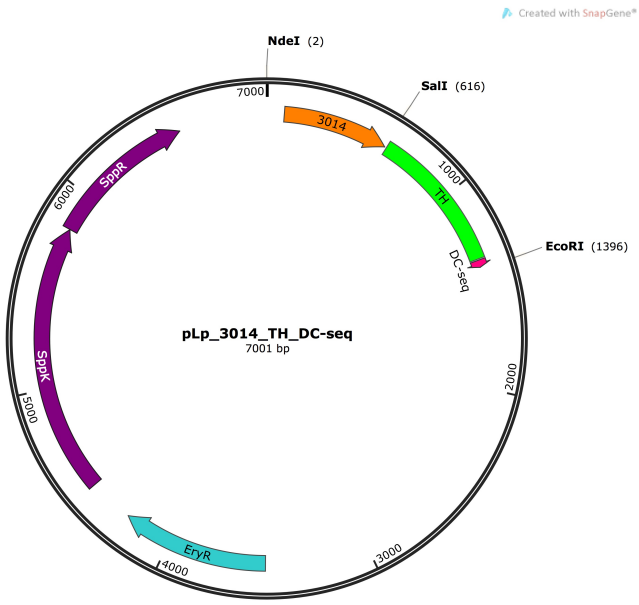


Figure 4.1 The TH_DC-seq insert from pUC and plasmid map of pLp_3014_TH_DC-seq (a) TH-DC-seq insert from pUC with the restriction sites SalI and EcoRI at the 5'-end and 3'-end of TH_DC-seq, respectively **(b)** Plasmid map of pLp_3014_TH_DC-seq. Inv was excised from pLp_3014_Inv with SalI and EcoRI and TH_DC-seq was then subcloned into the pLp vector resulting in pLp_3014_TH_DC-seq.

The insert, 3014_TH_DC-seq, was then subcloned into pET-16b with In-Fusion® cloning (section 3.2.5 and 3.2.8), resulting in pET16b_3014_TH_DC-seq (**Figure 4.2**a). To do this, fragment of the 3014_TH_DC-seq, with flanking NdeI and XhoI sites, were generated by PCR. The PCR-fragments were constructed to generate an overlap at their ends with the pET-16b plasmid vector. The forward and reverse primers used were pET 3014 F and pET Mtb.HspX_DC R, respectively (all primers used in this study are listed in Table 2.5 and Table 2.6). NdeI/XhoI-digested pET-16b plasmid vector and PCR-amplified insert gene of 3014_TH_DC-seq was then recombined with the In-Fusion® Cloning Kit. To make pET16b_3014S_TH_DC-seq and pET16b_2162S_TH_DC-seq (**Figure 4.2** b and c, Table 2.3), 3014S and 2162S were excised from NdeI/SalI-digested pET_3014S_AgESAT and pET_2162S_AgESAT (provided by Katarzyna Kuczkowska from the PEP research group at NMBU), respectively. The 3014S and 2162S are not the full-length sequence of *lp_3014* and *lp_2162*, and only encodes for the one and two LysM-domains derived from these genes (see above).

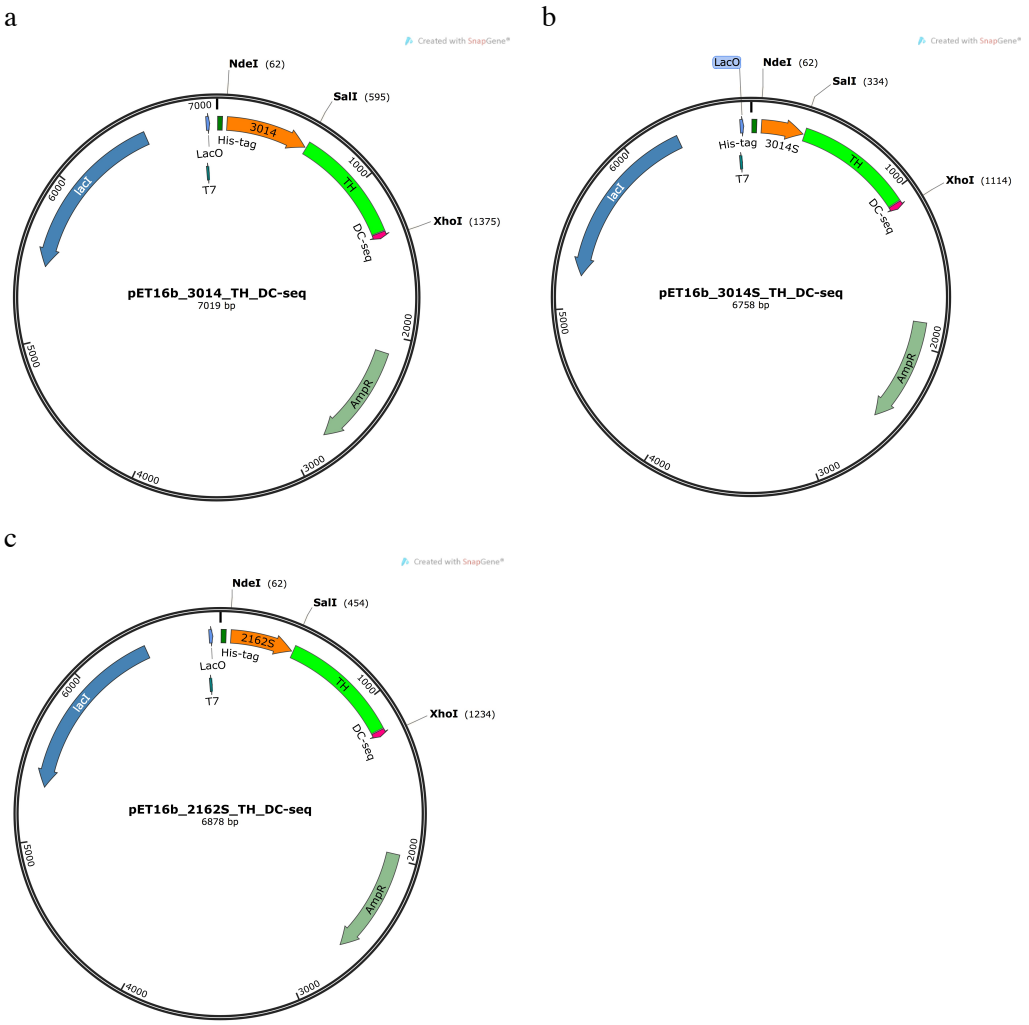


Figure 4.2 Plasmid maps of pET-16b derivatives. **(a)** pET16b_3014_TH_DC-seq **(b)** pET16b_3014S_TH_DC-seq **(c)** pET16b_2162S_TH_DC-seq

4.1.2 Making control constructs with the pET-16b expression vector

Of the three constructs made, pET16b_3014S_TH_DC-seq and pET16b_2162S_TH_DC-seq, encoding proteins with only the LysM domain(s), were targeted for further work and controls were constructed with that in mind. Control plasmids were generated using In-Fusion® Cloning Kit and PCR amplification with overlap extension, basically utilizing the same principal as in constructing pE16b_3014_TH_DC-

seq described in section 4.1.1. In designing PCR-generated inserts, sequences encoding part of the fusion protein (e.g. LysM-anchors or DC-seq), were excluded. A total of four pET-16b-based control constructs were made as listed below:

Control constructs (description in Table 2.3)	Primers (sequence and description in Table 2.5 and Table 2.6)
pET16b_2162S_TH	F: pET 2162 F R: pET Mtb.HspX R
pET16b_3014S_TH	F: pET 3014 F R: pET Mtb.HspX R
pET16b_TH_DC-seq	F: pET Mtb.HspX F R: pET Mtb.HspX_DC R
pET16b_TH	F: pET Mtb.HspX F R: pET Mtb.HspX R

4.1.3 Intracellular production of 2162S_TH_DC-seq in *E. coli* and purification of the protein

Intracellular protein production in *E.coli* using pET-16b plasmids was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) (section 3.3.1). Bacterial cultures were lysed by sonication for 5 minutes with the amplitude of the probe set at 20%. Soluble and insoluble fraction of lysate was separated by centrifugation, and the soluble fraction was used in purification experiments with immobilized metal ion chromatography (IMAC) using Ni-NTA agarose beads (section 3.3.4). All the pET-16b plasmids carry a N-terminal His-tag as mentioned in section 4.1.1, and His-tagged proteins are expected to bind reversibly to immobilized Ni^{2+} ions on the Ni-NTA agarose beads packed in the column. Specific elution of His-tagged proteins can then be achieved by introducing a competing agent, in this case, a high concentration of imidazole. Flow-through of waste products was also collected for analysis.

2162S_TH_DC-seq (44.90 kDa) was produced in *E. coli* harbouring the pET16b_2162S_TH_DC-seq plasmid by inducing with 0.4 mM IPTG and incubated at 37°C for four hours. Analysis of the soluble and insoluble fraction of the cell lysate showed that production was strong, but all the protein appeared in the insoluble fraction, indicating that it was precipitated as insoluble inclusion bodies (Figure 4.3, lane 1 and 2). The soluble

fraction was used for protein purification by IMAC, which did not yield detectable amounts of protein (Figure 4.3, lane 3 and 4; the chromatogram gave no indication of purified proteins, data not shown). There were no bands that clearly indicated presence of target protein in the SDS-PAGE gel, and the protein was also not detectable by Western blot analysis of the eluate (data not shown). The buffers used in this attempt contained 20 mM imidazole in running buffer (Buffer A) and 100 mM imidazole in elution buffer (Buffer B).

Based on this result, expressions of remaining pET-16b plasmids were set on hold and optimization experiments for production and purification of 2162S_TH_DC-seq was conducted (section 4.4.2).

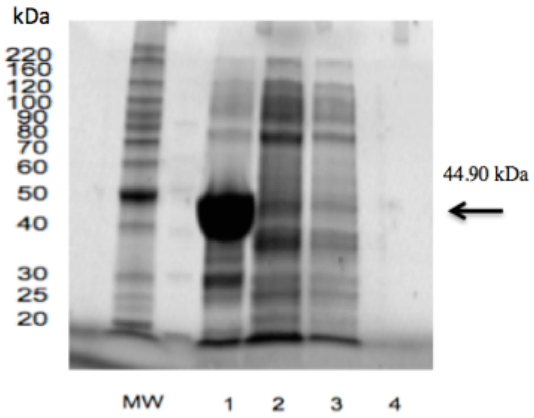


Figure 4.3 SDS-PAGE gel of protein extracts obtained from protein production and purification of 2162S_TH_DC-seq (44.90 kDa) *E. coli* carrying pET16b_2162S_TH_DC-seq was induced with 0.4 mM IPTG and incubated at 37°C for four hours. Soluble fraction of protein extract was purified with IMAC. Lane 1 = Insoluble fraction of protein extract. Lane 2 = Soluble fraction of protein extract. Lane 3 = Flow-through from IMAC. Lane 4 = Eluate from IMAC. MW = molecular weight

4.1.4 Optimization experiments for the production and purification of 2162S_TH_DC-seq

In attempt to reduce formation of inclusion bodies in the production of 2162S_TH_DC-seq, inductions at lower temperatures were tested. This strategy was inspired by Song et al. (2012) where the research team managed to overcome the insolubility problem of fusion proteins in *E.coli* by lowering the incubation temperature (Song, An, Kang, Lee, & Cha, 2012). Two incubation temperatures were tried: 10°C for 4-

7 days; and 18°C overnight. The concentration of IPTG was also varied, where cultures were induced with 0.04 mM, 0.1 mM and 0.4 mM IPTG.

Growing the cultures at 10°C seemed to increase the solubility at bit, but not substantially as the presence of the protein in the soluble fraction had to be confirmed with Western blot analysis (Figure 4.4 and Figure 4.5). Judging from the Western blot analysis, incubation at 10°C for more than 4 days seemed to increase production of soluble protein, but more than 5 days did not seem to make a substantial difference (Figure 4.5).

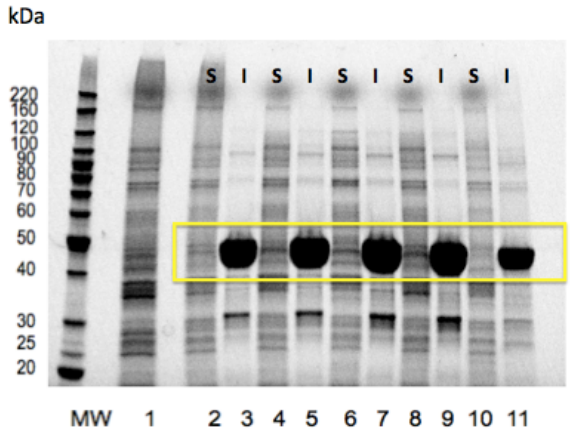


Figure 4.4 SDS-PAGE gel of protein extracts obtained during optimization experiments for the production of 2162S_TH_DC-seq (44.90 kDa) In all the cases except for lane 1, cells were induced with 0.4 mM IPTG. Lane 1 = soluble fraction obtained from uninduced culture grown at 10°C for 7 days. Lane 2&3 = 10°C for 4 days. Lane 4&5 = 10°C for 5 days. Lane 6&7 = 10°C for 6 days. Lane 8&9 = 10°C for 7 days. Lane 10&11 = 18°C overnight. MW = molecular weight. S = soluble fraction, I = insoluble fraction.

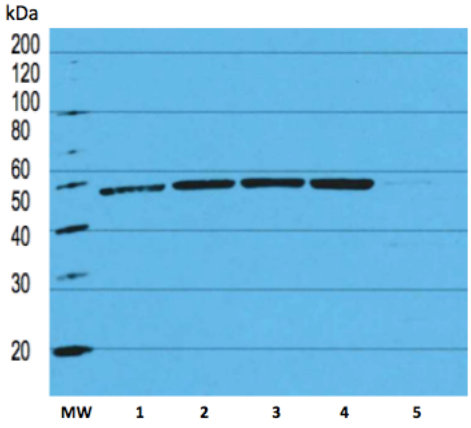


Figure 4.5 Western blot analysis of the soluble fraction of protein extracts obtained from optimization experiments for production of 2162S_TH_DC-seq (44.90 kDa) In all the cases, cells were induced with 0.4 mM IPTG. Mouse-α-His was used as the primary antibody. Lane 1 = 10°C for 4 days. Lane 2 = 10°C for 5 days. Lane 3 = 10°C for 6 days. Lane 4 = 10°C for 7 days. Lane 5 = 18°C overnight. MW = Molecular weight.

The IPTG concentrations that yielded most soluble protein for incubation at 10°C and 18°C seemed to be 0.4 mM and 0.1 mM IPTG, respectively (Figure 4.6), but there was not a clear concentration-dependent pattern since it seemed that the base expression without inducer at 10°C was relatively strong as well (Figure 4.6, lane 4).

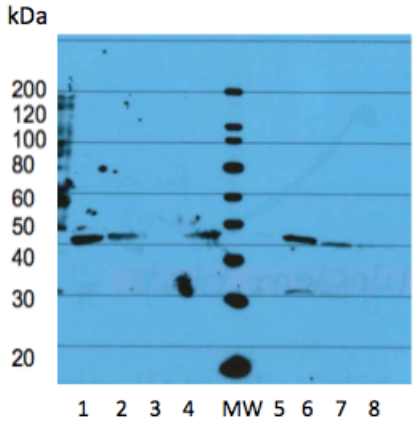


Figure 4.6 Western blot analysis comparing IPTG concentrations and solubility of 2162S_TH_DC-seq (44.90 kDa) Samples are the soluble fraction of protein extracts obtained from optimization experiments for production of 2162S_TH_DC-seq (44.90 kDa). Mouse-α-His was used as the primary antibody. Cultures grown at 10°C were incubated for 5 days (lane 1-4) and cultures grown at 18°C were incubated overnight (lane 5-8)were compared. Lane 1 = 0.4 mM. Lane 2 = 0.1 mM. Lane 3 = 0.04 mM. Lane 4 = uninduced. Lane 5 = 0.4 mM. Lane 6 = 0.1 mM. Lane 7 = 0.04 mM. Lane 8 = uninduced. MW=molecular weight.

Soluble fraction of protein extracts obtained from cultures grown at 10°C for 5 days and 18°C overnight, were purified. Purification was tried with lowering the imidazole concentration in running buffer to 0.5 mM imidazole and increasing the concentration to 300 mM imidazole in elution buffer. Purification yield of 2162S_TH_DC-seq was still low. Band indicating the target protein in eluate was not seen in SDS-PAGE gel and only possibly detectable by Western blot analysis (Figure 4.7, lane 6 and 7).

a b

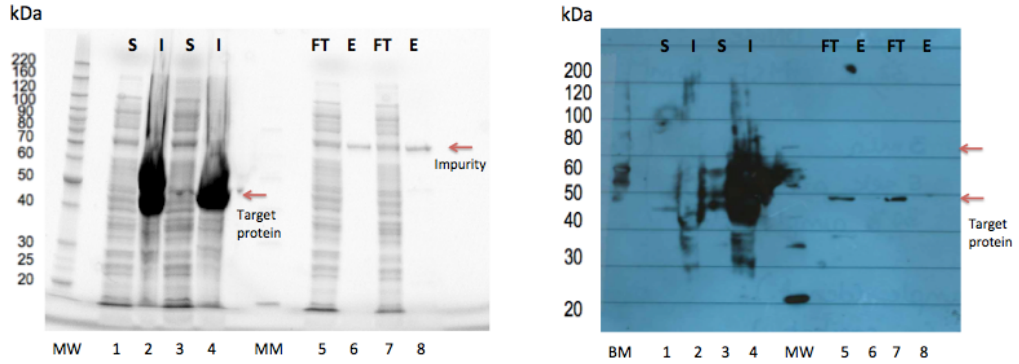


Figure 4.7 SDS-PAGE gel and Western blot analysis of protein extract obtained from optimization experiments for purification of 2162S_TH_DC-seq (44.90 kDa) (a) Cultures grown at 10°C were incubated for 5 days and induced with 0.4 mM IPTG. Samples from soluble (lane 1) and insoluble fraction (lane 2) of protein extract were collected. Soluble fraction of protein extract was purified with IMAC and samples of flow-through (lane 5) and eluate (lane 6) were collected. Cultures grown at 18°C were incubated overnight and induced with 0.1 mM IPTG. Samples from soluble (lane 3) and insoluble fraction (lane 4) of protein extract were collected. Soluble fraction of protein extract was purified with IMAC and samples of flow-through (lane 7) and eluate (lane 8) were collected. **(b)** Western blot analysis of the same SDS-PAGE-gel. Mouse-α-His was used as the primary antibody MW = molecular weight. MM = Magic Mark MW standard for Western blot. S = soluble fraction, I = insoluble fraction. FT = flow through of IMAC purification. E = eluate of IMAC purification.

There was an impurity in protein purification of 2162S_TH_DC-seq with a molecular weight of 70-80 kDa (Figure 4.7a, lane 6&8). Another member of the PEP group who used the same pET-16b system encountered a similar problem (similar non-expected band in protein electrophoresis), and mass-spectrometry analysis showed that this protein is catalase from *E. coli*.

Due to no substantial increase of solubility, a tight time schedule and the impurity in purifications, the optimization experiment was terminated. The alternatives to produce proteins in *L.* plantarum WCFS1 (non-GM and GM approach) were prioritized. The remaining two pET-16b constructs for expression of LysM-tagged fusion antigens were not analysed further.

4.2 Intracellular production of LysM-coupled TH_DC-seq in *L. plantarum* WCFS1

4.2.1 Cloning of LysM constructs into pLp expression vector for expression in *L. plantarum* WCFS1

The full-length sequence of the gene *lp_3014* was used for intracellular production in *L. plantarum* WCFS1 (see section 4.1.1 for more detail, and all constructs made in this study are listed in Table 2.3). The plasmid was first made in *E.*coli (section 3.2.9) and plasmid DNA derived from *E. coli* was then isolated and transferred to *L. plantarum* WCFS1 by electroporation (section 3.2.11). The gene of interest were subcloned into a pLp expression vector, a derivative of the pSIP-400 series (Sørvig et al., 2003). The signal peptides was predicted with SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and excluded from the coding sequence to secure intracellular protein production. pLp expression vectors do not have a sequence that encodes for a N-terminal His-tag for protein purification with IMAC (section 3.3.4). Thus, in this study, a N-terminal His_6-tag was constructed for the pLp plasmid used for intracellular protein production in *L. plantarum* WCFS1. By utilizing the same principal as in constructing pE16b_3014_TH_DC-seq described in section 4.1.1, PCR amplification with vector-overlap extension and recombination with Fusion® Cloning Kit was done to construct pLp_His3014_TH_DC-seq (Figure 4.8). For this construct, the fragment encoding His3014_TH_DC-seq was generated by PCR-amplification. The PCR-fragment includes a self-made N-terminal His_6-tag and overlap extension with the pLp vector, which includes the restriction sites NdeI and EcoRI. The forward and reverse primers used were pLp His3014uSP F and pLp TbH_DC R, respectively (table 2.5). NdeI/EcoRI-digested pLp_3014_TH_DC-seq (section 4.1.1 and Figure 4.1) was used as the vector.

Attempts to construct a control, a pLp_HisTH_DC-seq plasmid that did not have the 3014-LysM-anchor, was not successfully cloned to *E. coli*. Both In-Fusion® Cloning Kit and Quick Ligation™ Kit were tried and recombination with different ratio of vector and insert DNA was also varied, but none of the attempts were successful.

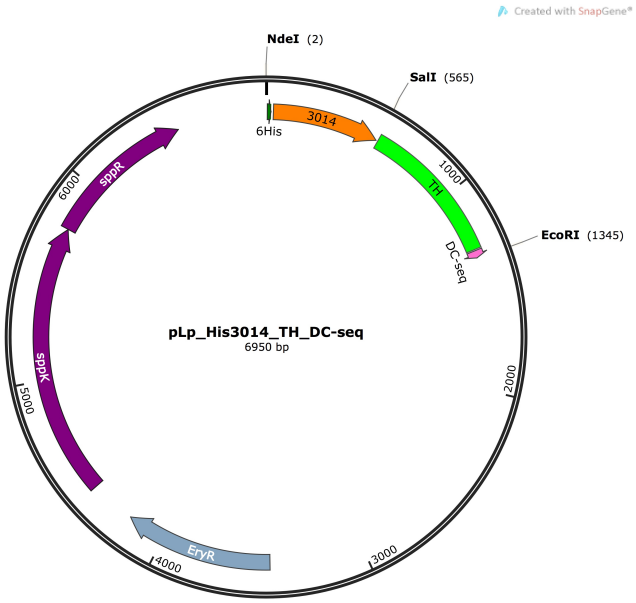


Figure 4.8 Plasmid mad of pLp_His3014_TH_DC-seq

4.2.2 Intracellular production of His3014_TH_DC-seq in *L. plantarum WCFS1*

Expression of pLp_His3014_TH_DC-seq in *L. plantarum* WCFS1 was induced with 25 ng/ml peptide pheromone SppIP (section 3.3.2). Bacterial culture was incubated at 30°C for five hours and lysed by sonication for 15 minutes with the amplitude of the probe set at 30%.

His3014_TH_DC-seq (48.51 kDa) was produced and the presence of the protein in the soluble fraction of protein extract could be detected by Western blot analysis with antibodies specific to HspX (α-HspX) and histidine (α-His) (Figure 4.9). Western blot showed that production of His3014_TH_DC-seq gave a band with bigger molecular weight (ca. 60 kDa) than what was predicted (48.51 kDa) in-silico (Figure 4.9). This size discrepancy was also observed in production of 3014_TH_DC-seq (section 4.4.2; the GM-approach). A band in SDS-PAGE gel of the insoluble fraction of protein extract indicated that some of the protein was precipitated, but this was not confirmed with Western blot (Figure 4.10). Comparing the insoluble fractions of protein extract in Figure 4.10 and Figure 4.1, expression level of pLp_His3014_TH_DC-seq obtained upon intracellular

expression in *L. plantarum* WCFS1, was substantially lower than that of intracellular expression of pET16b_2162S_TH_DC-seq in *E. coli*.

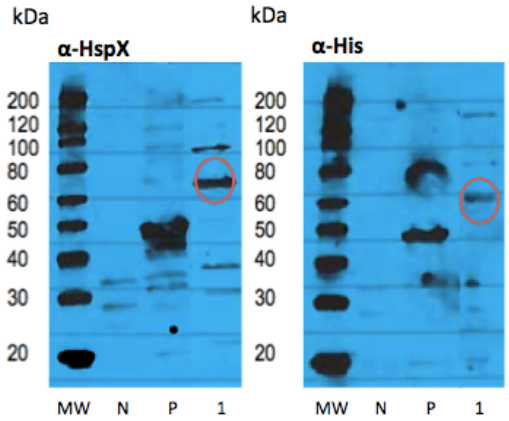


Figure 4.9 Western blot analysis of the soluble fraction of protein extract obtained from production of His3014_TH_DC-seq (48.51 kDa) with pLp vector in *L. plantarum* WCFS1 Expression of His3014_TH_DC-seq with pLp plasmid in *L. plantarum* WCFS1 gave size discrepancy (ca. 60 kDa). MW = Molecular weight. N = negative control. P = positive control (44.90 kDa). Lane 1 = His3014_TH_DC-seq.

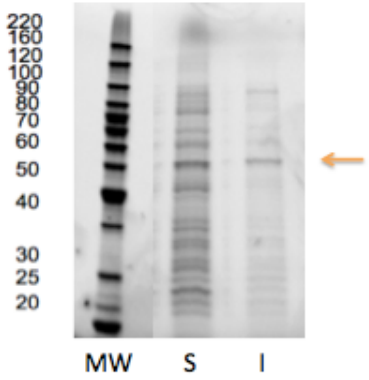


Figure 4.10 SDS-PAGE gel of the soluble and insoluble fraction of protein extract obtained from production His3014_TH_DC-seq with pLp plasmid in *L. plantarum* WCFS1 Expression of His3014_TH_DC-seq with pLp plasmid in *L. plantarum* WCFS1 gave size discrepancy (ca. 60 kDa). MW = molecular weight. S = soluble fraction. I = insoluble fraction

Longer induction times seemed to increase the yield, but the differences were not substantial (Figure 4.11). The Western blot analyses were carried out with both α-HspX and α-His, and the same pattern was seen. Cells in Figure 4.11 were in this case treated with glass beads and lysed with FastPrep®-24 homogenizer and not by sonication.

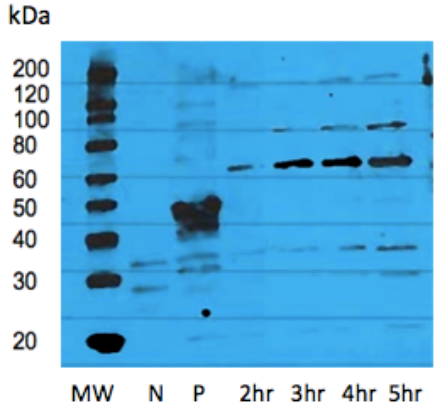


Figure 4.11 Western blot analysis comparing induction times of intracellular production of His3014_TH_DC-seq (48.51 kDa) in *L. plantarum* pLp_His3014_TH_DC-seq was expressed in *L. plantarum* WCFS1 induced with 25 ng/ml SppIP and incubated at 30°C for 2-5 hours. Samples are the soluble fractions of protein extract from cells lysed with glass beads treatment, using a FastPrep®-24 homogenizer. Mouse-α-HspX was used as the primary antibody. Expression of His3014_TH_DC-seq with pLp plasmid in *L. plantarum* WCFS1 gave size discrepancy (ca. 60 kDa). MW = molecular weight. N = negative control, P = positive control (44.90 kDa).

4.2.3 Purification of His3014_TH_DC-seq from the soluble fraction of protein extract

His3014_TH_DC-seq was produced in *L. plantarum* WCFS1 carrying the plasmid pLp_His3014_TH_DC-seq as described in section 4.2.2 and the soluble fraction of protein extract was purified with IMAC (section 3.3.4). The imidazole concentrations used in running buffer and elution buffer were 0.5 mM and 300 mM, respectively. As mentioned in section 4.2.1, pLp vectors do not contain a sequence that encodes for a N-terminal His-tag. Thus, to purify His3014_TH_DC-seq with IMAC, this protein contains an in-house constructed N-terminal His_6-tag (theory explained in section 3.3.4 and section 4.1.3). Flow-through of waste products was also collected for analysis.

Purification of His3014_TH_DC-seq did not yield detectable amounts of protein. The chromatogram gave no indication of purified proteins (data not shown). No band indicating the presence of the target protein in SDS-PAGE gel and Western blot analysis of the eluate was observed (Figure 4.12; note that the Western blot analysis was not blotted from the same SDS-PAGE gel from Figure 4.12, but the same samples were used for both the SDS-PAGE gel and the Western blot analysis).

Due to little time left for this study, further purification experiments were not conducted. Instead, His3014_TH_DC-seq from the soluble fraction of protein extract was used to test the fusion protein's binding ability to *L. plantarum* WCFS1.

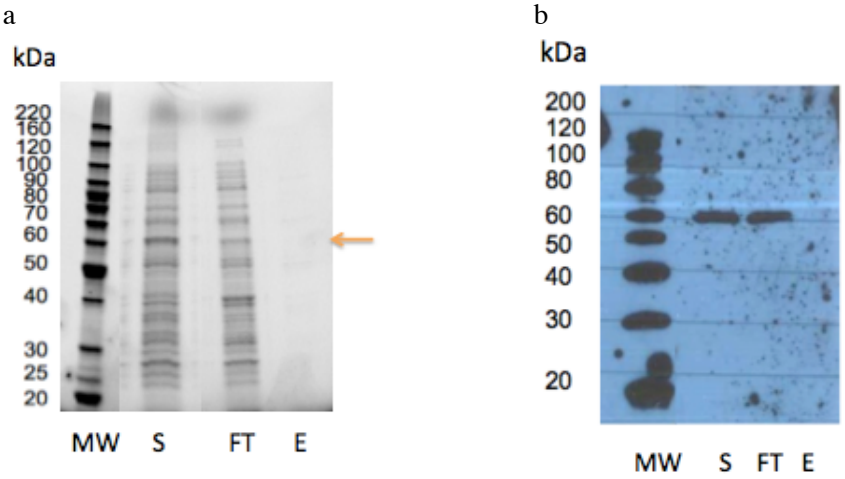


Figure 4.12 Purification of His3014_TH_DC-seq (48.51 kDa) from soluble fraction of protein extract (a) SDS-PAGE gel showing protein contents from soluble fraction of protein extract, flow-through and eluate from IMAC.**(b)** Western blot analysis of purification from soluble fraction of protein extract. Mouse-α-HspX was used as the primary antibody. Expression of His3014_TH_DC-seq with pLp plasmid in *L. plantarum* WCFS1 gave size discrepancy (ca. 60 kDa).

4.3 Testing the binding ability of His3014_TH_DC-seq to the surface of *Lactobacillus* spp

The soluble fraction of protein extract obtained by sonication cell lysis of *L. plantarum* WCFS1 cells, and that contained the fusion protein His3014_TH_DC-seq was used to test the binding ability of the protein to the cell surface of *L. plantarum* WCFS1, *Lactobacillus sakei* Lb790 and *Lactobacillus* rhamnosus GG. *L. plantarum* WCFS1, *L. sakei* Lb790 and *L. rhamnosus* GG was cultivated overnight in 10 ml MRS medium at 30°C. Bacterial cultures were diluted with wash buffer (PBS) to approximately OD = 0.5 and cells from the diluted suspension were harvested and incubated with the soluble fraction of protein extract (≈2 mg/ml) containing His3014_TH_DC-seq, for one hour (section 3.5). In this set up, a control extract of a fusion protein that lacks the LysM-anchor, was not included.

After incubation, cells were washed with PBS for excess unbound proteins and analysed with Western blot using α-HspX to analyse for the presence of binding of His3014_TH_DC-seq to *Lactobacillus* spp. The Western blot showed the target band indicating the presence of His3014_TH_DC-seq (Figure 4.13).

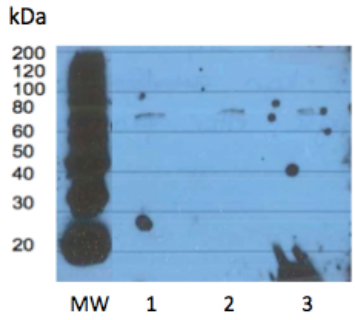


Figure 4.13 Western blot analysis analysing the binding of His3014_TH_DC-seq to the cell surface of *Lactobacillus* spp The soluble fraction of protein extract containing His3014_TH_DC-seq was obtained form lysis of recombinant *L. plantarum* WCFS1 and incubated with *L. plantarum* WCFS1 (lane 1), *L. rhamnosus* GG (lane 2) and *L. sakei* Lb790 (lane 3) for one hour. Mouse-α-HspX was used as the primary antibody. Expression of His3014_TH_DC-seq with pLp plasmid in *L. plantarum* WCFS1 gave size discrepancy (ca. 60 kDa).MW = molecular weight.

To analyse the presence of binding of His3014_TH_DC-seq to *Lactobacillus* spp without lysing the cells, flow cytometry analysis (section 3.4.4) was done to detect surface display of His3014_TH_DC-seq on live *Lactobacillus* spp. The primary and secondary antibody used was α-HspX and anti-mouse IgG FITC, respectively. The soluble fraction of protein extract obtained from expression of a pLp construct that did not have any insert gene (pEV), was also incubated with *Lactobacillus* spp and used as a negative control for α-HspX's specificity in flow cytometry, which would be the background signal. Cells obtained from expression of pLp_3014_TH_DC-seq which directly displays 3014_TH_DC-seq on the cell surface of recombinant *L. plantarum* WCFS1 (section 4.4.2; the GM-approach), was tested at the same time. This was due to lack of a positive control for flow cytometry analysis using α-HspX in the laboratory for PEP research group. Since this construct constitutively displays the 3014_TH_DC-seq on the cell surface, it would in theory, give signals that are markedly above the background signal in flow cytometry analysis with α-HspX. No positive signals could be detected with flow cytometry, with signals from *L. sakei* Lb790 cells incubated with protein extract containing

His3014_TH_DC-seq or the negative control being unusually low (Figure 4.14, note that signals of the theoretically positive control in histogram for *L. sakei* Lb790 is the same as in the histograms for *L. plantarum* WCFS1 and *L. rhamnosus* GG).

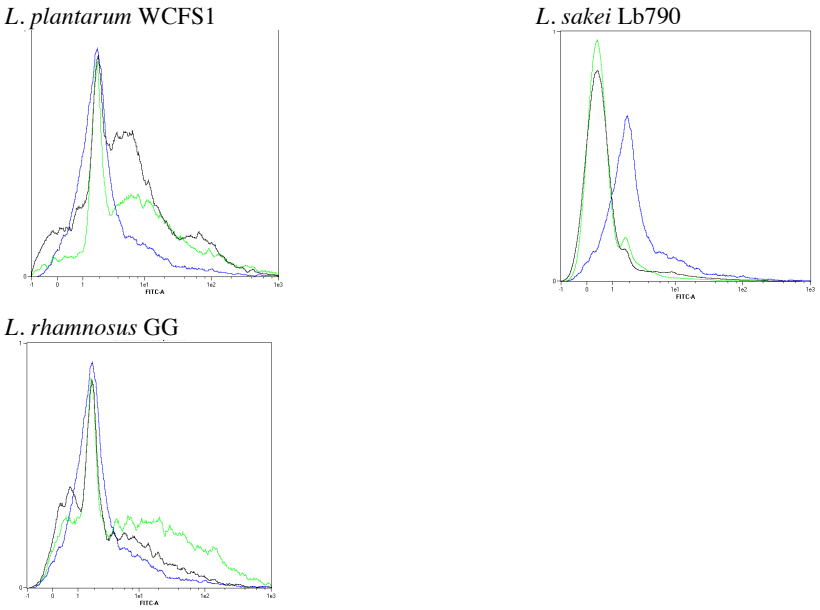


Figure 4.14 Histograms of flow cytometry analysis of the binding of His3014_TH_DC-seq to *Lactobacillus* spp. Cells (*L. plantarum* WCFS1, *L. sakei* Lb790 and *L. rhamnosus* GG) were incubated with soluble fraction of protein extract containing His3014_TH_DC-seq (green) or that did not have any insert gene (pEV, black). Cells constitutively displaying 3014_TH_DC-seq (blue) were also tested as a positive control. Mouse-α-HspX was used as the primary antibody. **NB!** Note that signals from positive control in the histogram for *L. sakei* Lb790 is the same as in the other histograms.

4.3.1 Further analysis of His3014_TH_DC-seq's binding ability to L. plantarum WCFS1 using Western blot analysis

Further experiments to test the binding ability of His3014_TH_DC-seq to the cell surface of *L. plantarum* WCFS1 was done. The efficacy of binding at stationary and exponential phase was tested, and the efficacy of binding with incubation for one and two hours was also tested. Two independent cultivations of *L. plantarum* WCFS1 were done in parallel to repeat the experiment. *L. plantarum* WCFS1 cells were harvested as described in section 4.3 and incubated with soluble fraction of protein extract (≈2 mg/ml) containing His3014_TH_DC-seq.

Comparison of the binding ability of fusion proteins with and without the 3014-LysM-anchor to the cell surface of *L. plantarum* WCFS1 was also experimented. Since attempts made to construct the control plasmid pLp_HisTH_DC-seq was not successful as described in section 4.2.1, soluble fraction of protein extract containing TH_DC-seq (30.99 kDa) obtained from expression of pET_TH_DC-seq (Table 2.3 and section 4.1.2) in *E.coli* was used. pET_TH_DC-seq was expressed in *E. coli*, induced with 0.1 mM IPTG and incubated overnight at 37°C. *L. plantarum* WCFS1 cells were harvested as described in section 4.3 and incubated with soluble fraction of protein extract (≈3.6 mg/ml) containing TH_DC-seq. After incubation with *L. plantarum* WCFS1, the cells were washed with PBS for excess unbound proteins and analysed with Western blot using α-HspX to analyse for the presence of binding of His3014_TH_DC-seq and TH_DC-seq to *L. plantarum* WCFS1.

Western blot analysis showed target band in both incubation with His3014_TH_DC-seq and TH_DC-seq (Figure 4.15). Binding was indicated in both stationary and exponential phase, and after both one and two hours of incubation. Due to little time left in this study, these experiments were not tested with *L. sakei* Lb790 and *L. rhamnosus* GG.

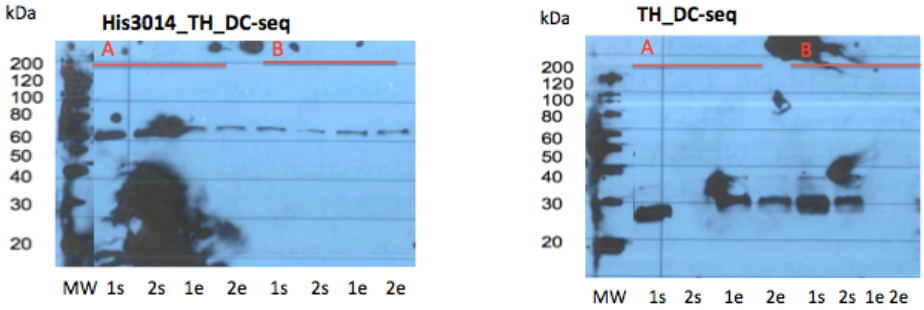


Figure 4.15 Western blot analysis of the binding ability of His3014_TH_DC-seq and TH_DC-seq to *L. plantarum* WCFS1 *L. plantarum* WCFS1 cells were incubated with His3014_TH_DC-seq (48.51 kDa) or TH_DC-seq (30.99 kDa) obtained from the soluble fraction of protein extract from production in *L. plantarum* WCFS1 and *E. coli*, respectively. Incubation for one (1) and two (2) hours was done with cells from stationary (s) and exponential (e) phase. Two independent cultivations (A and B) were done in parallel to repeat the experiment. Mouse- α -HspX was used as the primary antibody. Expression of His3014_TH_DC-seq with pLp plasmid in L. plantarum WCFS1 gave size discrepancy (ca. 60 kDa). MW = molecular weight.

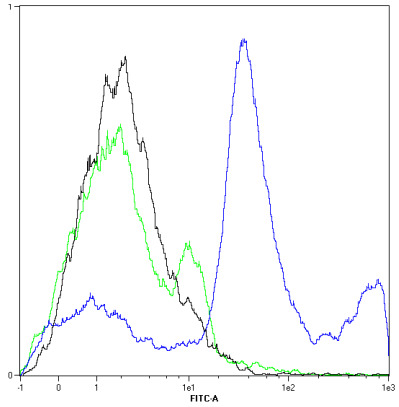
4.3.2 Further analysis of His3014_TH_DC-seq's binding ability to L. plantarum WCFS1 using flow cytometry

The binding ability of His3014_TH_DC-seq and TH_DC-seq to *L. plantarum* WCFS1 was also analysed with flow cytometry using α-HspX and anti-mouse IgG FITC as the primary and secondary antibody, respectively. In these experiments, set-ups only included cells from stationary phase that were incubated for one hour and two independent cultivations of *L. plantarum* WCFS1 were done in parallel to repeat the experiment. The concentrations used of the soluble fraction of the protein extracts are as described in section 4.3.1. The soluble fraction of protein extract obtained from expression of a pLp construct that did not have any insert gene (pEV), was also incubated with *L. plantarum* WCFS1 and used as a negative control for α-HspX's specificity in flow cytometry, which would be the background signal. Because of difficulty obtaining positive signals, different dilutions of primary (α-HspX) and secondary antibody (anti-mouse IgG FITC) were tested as listed below:

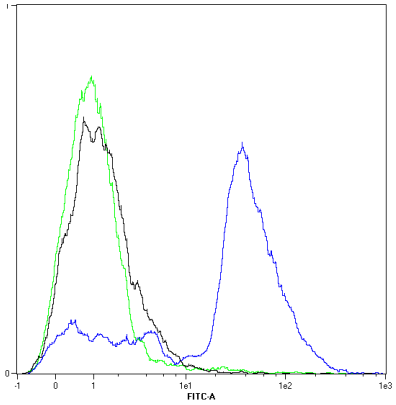
Set-up	**α-HspX**	**anti-mouse IgG FITC**
F1	1:125	1:125
F2	1:125	1:250
F3	1:50	1:250

Surprisingly, only incubation with TH_DC-seq gave positive signals and at all the three different antibody set-ups (Figure 4.16).

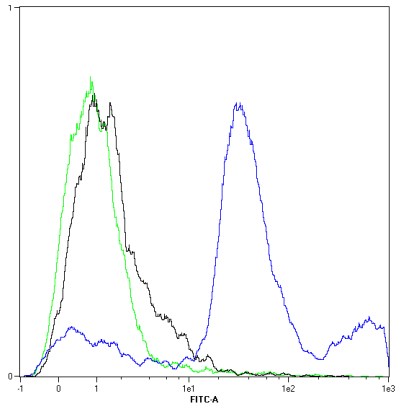
F1



F2



F3



	α-HspX	**anti-mouse IgG FITC**
F1	1:125	1:125
F2	1:125	1:250
F3	1:50	1:250

Figure 4.16 Histograms of flow cytometry analyses of the binding ability of His3014_TH_DC-seq and TH_DC-seq to *L. plantarum* WCFS1 *L. plantarum* WCFS1 cells were incubated with the soluble fraction of protein extract containing His3014_TH_DC-seq (green), TH_DC-seq (blue) or that did not have any insert gene (pEV, black). . Mouse-α-HspX was used as the primary antibody. The table shows the different dilutions of antibodies used in the set-ups F1, F2 and F3.

4.5 Direct display of TH_DC-seq on the cell surface of recombinant *L. plantarum* WCFS1

4.5.1 Cloning constructs into pLp expression vector for expression in *L. plantarum* WCFS1

Due to problems with protein purification, an alternative GM-approach was commenced. In this GM-approach (as explained in introduction of section 4), TH_DC-seq was coupled to three different anchors derived from the genes of *L. plantarum* WCFS1: a N-terminal LysM-anchor derived from *lp_3014* encoding for a putative extracellular transglycosylase; a N-terminal lipobox-anchor derived from *lp_1261*encoding for a substrate binding oligopeptide ABC transporter; and a C-terminal LPxTG-anchor derived from *lp_2578* encoding for a collagen-binding adherence protein (Zhou et al., 2010). The GM-approach was to produce TH_DC-seq using pLp expression vector and display TH_DC-seq directly on the cell surface of recombinant *L. plantarum* WCFS1. As proceeded in section 4.2, constructs were first made in *E.*coli (section 3.2.9) and plasmid DNA derived from *E. coli* were then isolated and transferred to *L. plantarum* WCFS1 by electroporation (section 3.2.11).

The construct pLp_3014_TH_DC-seq, was already made as described in section 4.1.1 and Figure 4.1b (all constructs constructed in this study are listen in Table 2.3). The construct pLp_1261_TH_DC-seq (Figure 4.17a) was made by excising TH_DC-seq from pLp_3014_TH_DC-seq using SalI and EcoRI and subcloned into SalI/EcoRI-digested pLp_1261_Inv (Fredriksen et al., 2012). For the pLp_0373_TH_DC-seq_2578 construct (Figure 4.17b), In-Fusion® cloning (section 3.2.5 and 3.2.8) was done due to the lack of an MluI site, which is normally used for swapping C-terminal anchors in the pSIP400 vectors (in which pLp is a derivative of), in the TH_DC-seq sequence. A PCR-generated fragment encoding TH_DC-seq with flanking SalI and MluI sites was made and recombined with SalI/MluI-digested pLp_0373_OFA_cwa2 (Fredriksen et al., 2010). The forward and reverse primers used for generating the fragment were pLp 0373_TbH_DC F and pLp TH_DC_cwa2 R, respectively (table 2.5). 0373 refers to the N-terminal signal peptide used in this construct and is derived from gene *lp_0373* from L. plantarum WCFS1 that encodes

for a cell surface protein precursor and which also contain a LPxTG-anchor (Zhou et al., 2010).

a b

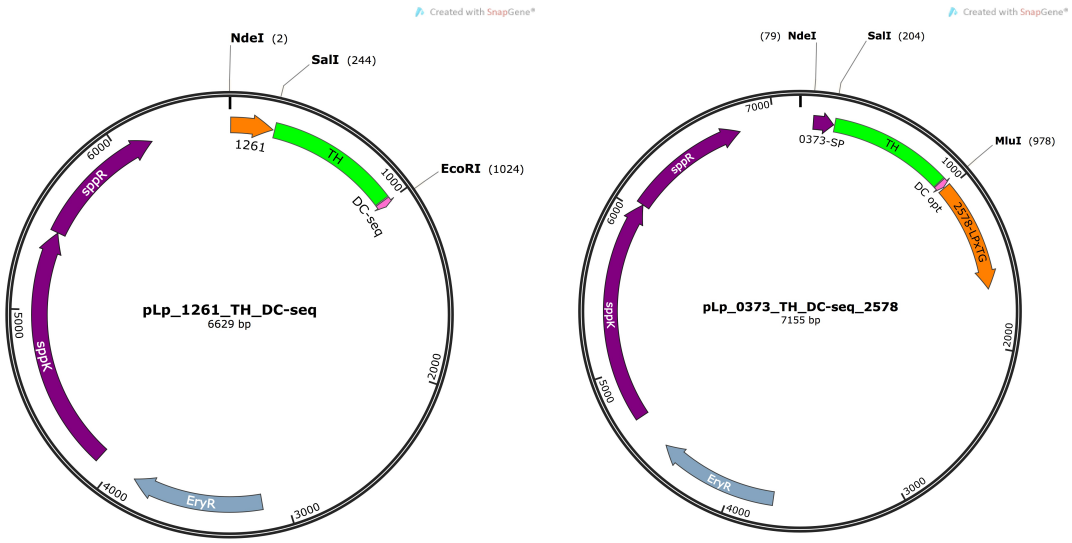


Figure 4.17 Plasmid maps of pLp constructs in the GM approach (a) pLp_1261_TH_DC-seq **(b)** pLp_0373_TH_DC-seq_2578.

4.5.2 Direct display of TH_DC-seq on cell surface of *L. plantarum* WCFS1

For this GM-approach, TH and DC-seq was fused with anchors, cloned into pLp expression vector and expressed directly on cell surface of *L. plantarum* WFCS1. TH_DC-seq was fused with three different anchors (see above in section 4.4.1) making the fusion proteins 3014_TH_DC-seq (LysM-anchor), 0373_TH_DC-seq_2578 (LPxTG-anchor) and 1261_TH_DC-seq (lipobox-anchor). Intracellular production of fusion proteins in *L. plantarum* WCFS1 using pLp expression vector was induced with 25 ng/ml peptide pheromone SppIP for 2-5 hours. Induced bacterial culture was then lysed with glass beads treatment and FastPrep®24 homogenizer.

No strongly indicative band of target proteins in SDS-PAGE gel was observed for all of the three fusion proteins (Figure 4.18). But production could be detected by Western blot analysis using α-HspX (Figure 4.19) with the exception of 0373_TH_DC-seq_2578 that

has the LPxTG-anchor. Western blot also showed that production of 3014_TH_DC-seq gave a band with bigger molecular weight (ca. 60kDa), than what was predicted (49.98 kDa) in-silico. This size discrepancy was also the case in intracellular production of His3014_TH_DC-seq (48.51 kDa) (section 4.5.1, non-GM approach using pLp vector and *L. plantarum* WCFS1).

Flow cytometry (section 3.4.4) was done to confirm cell-surface display of fusion proteins, but no positive signals were detected from any of the constructs (data not shown). α-HspX and anti-mouse IgG FITC were used as the primary and secondary antibody, respectively. And the dilution for both antibodies was 1:125. Due to lack of time, no further experiments were done for the alternative GM-strategy.

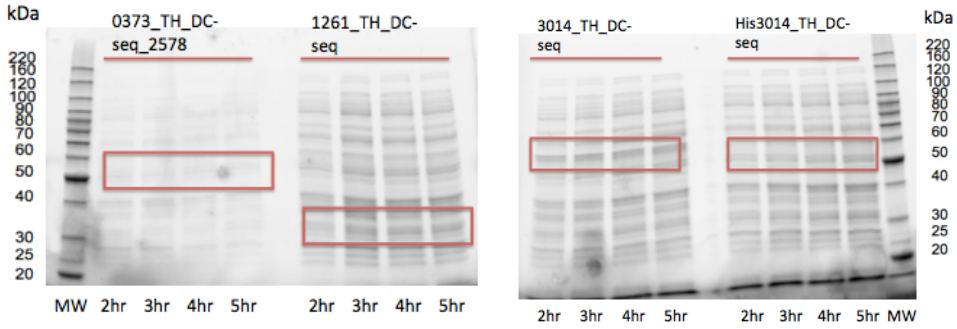


Figure 4.18 SDS-PAGE gel of soluble fraction of protein extract of fusion proteins obtained from production in *L. plantarum* WCFS1 SDS-PAGE analysis of protein production of 0373_TH_DC-seq_2578 (55.29 kDa, LPxTG-anchor), 1261_TH_DC-seq (36.82 kDa, lipobox-anchor), 3014_TH_DC-seq (49.98 kDa, LysM-anchor) and His3014_TH_DC-seq (48.51 kDa, LysM-anchor and with N-terminal His_6-tag). Incubation for 2-5 hours. Expression of His3014_TH_DC-seq with pLp plasmid in L. plantarum WCFS1 gave size discrepancy (ca. 60 kDa). MW = molecular weight.

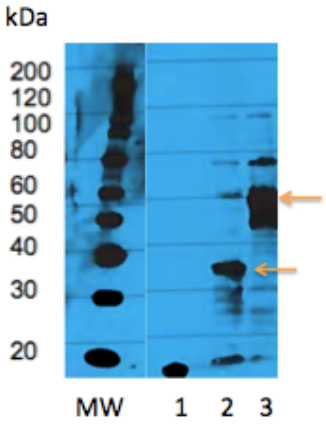


Figure 4.19 Western blot analysis of protein production in the *L. plantarum* WCFS1 for the GM-strategy TH_DC-seq was fused with three different anchors making **(1)** pLp_0373_TH_DC-seq_2578 (55.29 kDa, LPxTG-anchor), **(2)** pLp_1261_TH_DC-seq (36.82 kDa, lipobox-anchor) and **(3)** 3014_TH_DC-seq (49.98 kDa, LysM-anchor). Expression of His3014_TH_DC-seq with pLp plasmid in L. plantarum WCFS1 gave size discrepancy (ca. 60 kDa). MW = molecular weight.

5 Discussion

5.1 Intracellular production and purification of LysM-coupled fusion proteins

Overproduction of heterologous proteins in *E. coli* is one of the most commonly applied strategies. This is due to *E. coli*'s well studied genome, fast growth rate, high cell density production as well as the ease and low price of cultivation (Peternel, 2013). In this study it has been shown that it is possible to use the pET-16b expression vector for production of the *M. tuberculosis* fusion antigen TB10.4-HspX (TH). This was shown with the fusion protein 2162S_TH_DC-seq, where TH was coupled N-terminally to the 2162-LysM anchor that has two LysM-domains, and C-terminally to a dendritic cell binding peptide sequence (DC-seq). The 2162-LysM anchor is derived from the gene *lp_2162* from *L. plantarum* WCFS1 that codes for a putative gamma-D-glutamate-meso-diaminoimelate muropeptidase (section 1.5.1). The production of 2162S_TH_DC-seq was confirmed by a strong band in SDS-PAGE (Figure 4.3 and Figure 4.4) and immunodetection with Western blot analysis (Figure 4.7). However, the target protein was mostly found in the insoluble fraction of the protein extract, indicating that 2162S_TH_DC-seq aggregated in insoluble inclusion bodies (IBs). The high production level is often found to be stressful for the host and aggregates of target protein in IBs are common in overexpression of fusion proteins (Peternel, 2013). Reducing the expression rate could be useful, thus cultivations at lower temperatures and varying the inducer concentration was done in attempt to improve the solubility of 2162S_TH_DC-seq, but this only resulted in marginal improvement. Due to this negative result and considering other available options, the remaining LysM-containing pET-16b constructs were not used for further work in this study.

Previous studies have shown that it is possible to induce immune responses from antigens extracted from IBs that have been solubilized for purification and refolded before administrations to animal test model (Kesik, Saczyńska, Szewczyk, & Płucienniczak, 2004; Leal et al., 2006). Based on the results from purification of 2162S_TH_DC-seq from the soluble fraction done in this study, this alternative is already being explored in the PEP research group at NMBU.

Modifications of the construction of 2162S_TH_DC-seq is another strategy that could have been tested as small changes in the primary structure of the protein, such as insertion of linker peptide sequence in between genes/domains of interests, could be enough to shift the solubility of the protein (Peternel, 2013). This was not experimented in this study, but might be worth experimenting in future studies as successful shift towards better solubility of fusion proteins can avoid the burdensome procedures of solubilizing IBs for purification and the procedures of refolding of these solubilized aggregated proteins. It has in recent years been shown that a substantial amount of properly folded proteins can be found trapped inside IBs produced at lower growth temperatures, and that these proteins can easily be released from IBs using mild detergents under non-denaturing conditions (Peternel, Grdadolnik, Gaberc-Porekar, & Komel, 2008). This strategy could also be worth experimenting as this strategy too avoids the burdensome procedures of denaturing and refolding proteins.

As there was no substantial improvement of the solubility of 2162S_TH_DC-seq, the alternative strategy to use pLp expression vector in *L. plantarum* WCFS1 for another non-GM approach were prioritized. The pLp expression vector is a derivative of p400-series of the pSIP system (Sørvig et al., 2003) It has previously been shown that it is possible to produce heterologous proteins with over 50% dominance of the intracellular protein content using pLp expression vector in *L. plantarum* WCFS1 (Mathiesen et al., 2004; Nguyen et al., 2012). Thus, expression in *L. plantarum* WCFS1 using a pLp was explored in this other non-GM approach, using His3014_TH_DC-seq as the target protein. 3014 denotes the full-length peptide sequence derived from gene *lp_3014* that contains one N-terminal LysM-domain and includes the transglycosylase peptide sequence (section 1.5.1; the N-terminal signal peptide was excluded for intracellular production). A His_6-tag was fused N-terminally to enable purification with immobilized metal ion affinity chromatography (IMAC), as pLp vectors do not contain any polyhistidine coding sequence. The production yield of His3014_TH_DC-seq was low. The SDS-PAGE gel gave no indication of overproduction of the target protein (Figure 4.10). In general, protein production using pLp expression system in *L. plantarum* WCFS1 was low in this study, as the production of fusion proteins in the GM-approach was low as well (see further below). This indicates that the derivative pLp plasmid from the pSIP400-series is not suitable for

overexpression of TH_DC-seq, TH or DC-seq. Anchors used in this study were all derived from *L. plantarum* WCFS1 and have been used in successful expression before (Tjåland, 2011), so it seemed less possible that these anchors were causing the low production yields. However, the coupling of these anchors to TH_DC-seq, TH or DC-seq might have affected the production yield too.

In this study, the purification method used was to fuse N-terminal polyhistidine-tag to the fusion proteins and purify with IMAC (theory in section 3.3.4). Ni-NTA beads were preloaded onto column before loading of protein sample. The target proteins (2162S_TH_DC-seq and His3014_TH_DC-seq) could be detected by immunodetection with Western blot analysis. However, the target proteins were only detected in the soluble fraction of the protein extract and in the flow-trough of waste products from IMAC, but not in the purification eluates. This indicates inefficient binding between His-tag and Ni^{2+} ions and that the purification procedure might need to be optimized to extract the target protein from the soluble fraction. Incubating Ni-NTA beads with the protein sample before loading onto column, might result in more efficient binding of tagged protein to Ni^{2+} ions in Ni-NTA beads (Bornhorst & Falke, 2000). It should also be noted that the protein yield in the soluble fraction was low, making purification more challenging since it would require higher affinity of His-tagged proteins for Ni^{2+} ions to be sufficiently effective for purification. Concentration of protein in eluate might also result in detectable amount in SDS-PAGE gel and Western blot analysis.

A size discrepancy was observed in Western blot analysis of His3014_TH_DC-seq produced in *L. plantarum* WCFS1 using a pLp vector (section 4.2.2). The Western blot analysis showed a band with bigger molecular weight (ca. 60 kDa) than what was predicted (48.51 kDa) in silico. This may be caused by the combination of fusing 3014 with TH_DC-seq. The fact that production of the other fusion proteins did not showed this anomaly, except of the production of 3014_TH_DC-seq that also showed the same size discrepancy in Western blot analysis (section 4.4.1; the GM-strategy), support this hypothesis.

5.1.1 Binding of His3014_TH_DC-seq to *Lactobacillus* spp

In this study, the peptidoglycan binding ability of the 3014-LysM domain (section 1.5.1), coupled to the fusion protein TH_DC-seq, has been tested. Due to unsuccessful

purification with IMAC, purified eluate was not obtained in this study. Thus, the soluble fraction of protein extract containing the target protein was used for the binding assays. The plasmid pLp_His3014_TH_DC-seq was used for expression in *L. plantarum* WCFS1 and the soluble fraction of the protein extract containing His3014_TH_DC-seq was used for the binding assays. Cells of *L. plantarum* WCFS1, *L. sakei* Lb790 and *L. rhamnosus* GG were incubated, at room temperature, with protein extract containing His3014_TH_DC-seq. After incubation the cells were washed for excess unbound proteins and the cells were analysed with Western blot analysis for the presence of the proteins in which bindings seemed to had occurred (section 4.3 and Figure 4.13). Although binding seemed to have occurred between His3014_TH_DC-seq and the *Lactobacillus* spp, this was not confidentially confirmed. This is because a soluble fraction of protein extract that did not contain a TH_DC-seq that lacks the 3014-LysM anchor, was not included in this experiment. Thus, there were no controls that could support the indication of binding of His3014_TH_DC-seq to the cell surface of *L. plantarum* WCFS1, *L. sakei Lb790 and* L. rhamnosus GG. Without controls in the set-ups, it was not possible to verify that specific bands from Western blot analysis were actually due to binding between His3014_TH_DC-seq and the cell surface of *Lactobacillus* spp, and not due to technical issues such as insufficient washing of excess unbound proteins. Even if the observation of target bands were due to binding between the fusion protein and the cells, without a control that lacks the 3014-LysM, it was not possible to verify that it is actually the LysM that causes the binding. Attempts made to detect His3014_TH_DC-seq on the cell surface of live *L. plantarum* WCFS1, *L. sakei* Lb790 and *L. rhamnosus* GG by using flow cytometry analysis were not successful (section 4.3 and further discussed below).

The binding ability of His3014_TH_DC-seq was further investigated using only *L. plantarum* WCFS1. In these experiments, the soluble fraction of protein extract from *E. coli* containing TH_DC-seq (i.e. without the 3014-LysM) was also incubated with *L. plantarum* WCFS1. The fusion protein TH_DC-seq needed to be expressed from the plasmid pET16b-TH_DC-seq in *E.coli* because attempts made to construct a control with the pLp vector were unsuccessful (section 4.2.1). In the further experimentations, *L. plantarum* WCFS1 cells from stationary and exponential phase was incubated at room temperature, with the soluble fractions of protein extract containing His3014_TH_DC-seq and TH_DC-seq for

one and two hours. The results from Western blot analysis indicate that both His3014_TH_DC-seq and TH_DC-seq are able to bind to *L. plantarum* WCFS1 and that the binding efficacy is the same for cells in stationary and exponential phase (Figure 4.15). Incubation for one and two hours did not seem to result in any differences in binding efficacy for both His3014_TH_DC-seq and TH_DC-seq. These observations indicate that binding was stable with incubation at room temperature for up to two hours, but that two hours of incubation time did not seem to improve the binding efficacy. Other reasons such as saturated binding capacity due to insufficient amount of cells to bind to the proteins or vice versa, might also be the reason why a difference in binding capacity was not observed between one and two hours of incubation. This could have been investigated by varying the amount of cells or the soluble fractions of the protein extract used in incubation for one and two hours. The Western blot analysis of the binding ability of the fusion proteins to *L. plantarum* WCFS1 in Figure 4.15 gave stronger bands with cells incubated with TH_DC-seq than cells incubated with His3014_TH_DC-seq. This could be due to different reasons such as: that TH_DC-seq binds stronger than His3014_TH_DC-seq; binding is independent of 3014-LysM and that 3014-LysM hinder binding; abundance of TH_DC-seq was higher than His3014_TH_DC-seq in the soluble fraction of protein extract; and that the HspX-epitope in His3014_TH_DC-seq is less accessible for α-HspX.

To detect binding of His3014_TH_DC-seq and TH_DC-seq to the cell surface of live *L. plantarum* WCFS1, flow cytometry analysis was also carried out. Due to difficulties in obtaining positive signals, different dilutions of primary and secondary antibody were tested using three different set-ups (section 4.3.2). Surprisingly, only incubation with TH_DC-seq gave positive signals and at all the three different antibody set-ups (Figure 4.16). This observation could have the same causes as those listed above for the Western blot analysis. The amount of His3014_TH_DC-seq may have been too small and therefore under the detection limit of flow cytometry. Another possible explanation is that the HspX-epitope might not have been accessible for α-HspX when used in flow-cytometry since fusion proteins are detected in their native form in flow cytometry, in contrast to Western blot analysis where the fusion proteins are denatured for detection.

The results from assays of the binding ability of His3014_TH_DC-seq and TH_DC-seq to the cell surface of *Lactobacillus* spp, indicate binding of His3014_TH_DC-seq to *L.*

plantarum WCFS1, *L. sakei* Lb790 and *L. rhamnosus*. However, further experiments showed that TH_DC-seq is also able to bind to *L. plantarum* WCFS1, and might even have a stronger affinity for *L. plantarum* WCFS1 than His3014_TH_DC-seq. The data thus seem to suggest that LysM domain is not involve in binding of TH_DC-seq, but clearly, more work is needed to substantiate this indication. Ideally, purified proteins should be used. Specific affinity of the *M. tuberculosis* antigens TB10.4 and HspX (TH) or the dendritic cell binding peptide sequence (DC-seq) for peptidoglycan is not known. Further experiments should be carried out to investigate whether binding of TH_DC-seq to *L. plantarum* WCFS1 was due to affinity toward the peptidoglycan layer or was due to affinity toward other components on the cell wall of *L. plantarum* WCFS1. Gram-positive enhancer matrix (GEM) particles could be used to test the peptidoglycan binding ability of both His3014_TH_DC-seq and TH_DC-seq. These are cells of *Lactococcus lactis* that are subjected to treatment that removes the intracellular content and results in GEM particles composing of peptidoglycan and the shape of lactococcal cells (Berlec et al., 2012). GEM particles could therefore also be considered for use in this study for another non-GM approach by anchoring antigens to GEM particle that are free of genetic materials.

5.2 Direct display of *M. tuberculosis* antigens on the cell surface of *L. plantarum* WCFS1

In the GM-strategy, TH_DC-seq was coupled to three different anchors, which are derived from the genes of *L. plantarum* WCFS1 (section 1.5), and directly displayed on the cell surface of recombinant *L. plantarum* WCFS1. The three fusion proteins that were made in this study are 3014_TH_DC-seq (LysM-anchor), 1261_TH_DC-seq (lipobox-anchor) and 0373_TH_DC-seq_2578 (LPxTG-anchor) (see section 1.5, section 4.4.1 and Table 2.3 for more details). In this case, the proteins are produced with an N-terminal signal peptide for protein secretion. Production of the proteins was confirmed by Western blot analysis using α-HspX with the exception of 0373_TH_DC-seq_2578 (Figure 4.19). The growth curve of *L. plantarum* WCFS1 carrying the plasmid pLp_0373_TH_DC-seq_2578 showed that upon induction, growth rate decreased rapidly or that growth even stopped (Appendix B, Figure B.2). This indicates that expressing pLp_0373_TH_DC-seq_2578 is stressful for *L. plantarum* WCFS1 which leads to cell death and, presumably, degradation of proteins.

Attempts were made to detect anchor-coupled TH_DC-seq that was directly displayed on the cell surface of live recombinant *L. plantarum* WCFS1 using flow cytometry (data not shown). No positive signals were detected using the α-HspX as the primary antibody, thus successful cell surface display of TH_DC-seq through coupling to anchors was not confirmed. Flow cytometry analysis of TH_DC-seq (produced in *E.coli* from a pET-16b vector, section 4.3.1 and 5.1.1) gave positive signals using the same primary antibody and the same amount of cells. The lack of positive signals in flow cytometry analysis might therefore be due to expression with pLp vector and production in *L. plantarum* WCFS1, or that HspX-epitope seems not accessible for α-HspX when TH_DC-seq is coupled to an anchor. This might be due to steric hindrance caused by the folding of the proteins or that translocation of TH_DC-seq to the cell surface of *L. plantarum* WCFS1 was not complete.

Fluorescence microscopy is another method that can detect antigens on the cell surface. But due to little time left for this study, no further experiments were conducted for the GM-strategy.

6 Conclusions and future perspectives

In this thesis, the production of the *M. tuberculosis* fusion antigen TB10.4-HspX in *E. coli* using a pET-16b vector and in *L. plantarum* WCFS1 using a pLp vector (a derivative of the pSIP system), has been described. TH was coupled to the dendritic cell binding peptide, FYPSYHSTPQRP (DC-seq), to promote targeting of dendritic cells in future immunologic experiments. TH_DC-seq was fused to anchors derived from proteins encoded in genes of *L. plantarum* WCFS1. The anchors used in this study are N-terminal LysM anchors derived from the genes *lp_3014* and *lp_2162*, a N-terminal lipobox anchor derived from the gene *lp_1261*, and a C-terminal LPxTG anchor derived from the gene *lp_2578*.

In the non-GM approaches, LysM coupled TB10.4-HspX_DC-seq was successfully produced intracellularly in *E. coli* and *L. plantarum* WCFS1, but the level of soluble proteins was low. There were also challenges met upon purification from extracts containing soluble proteins. Binding assays using the soluble fraction of protein extracts indicates that LysM-coupled TB10.4-HspX _DC-seq could bind to *L. plantarum* WCFS1, *L. sakei* Lb790 and *L. rhamnosus* GG. However, more detailed binding assays using *L. plantarum* WCFS1 only, also indicated that TB10.4-HspX _DC-seq without the LysM anchor also binds to *L. plantarum* WCFS1. Confirmation of the presence of TB10.4-HspX _DC-seq on the cell surface was not certain, as flow cytometry analysis was not successful. All in all, more experiments to investigate binding of TB10.4-HspX_DC-seq to the cell surface of *Lactobacillus* spp should be done to test the viability of the non-GM strategy.

In the GM-strategy, TB10.4-HspX _DC-seq was fused with three different anchors to be directly displayed on the cell surface of recombinant *L. plantarum* WCFS1. The anchors used were a N-terminal LysM anchor, a N-terminal lipobox anchor and a C-terminal LPxTG anchor, all of which are derived from proteins encoded by genes of *L. plantarum* WCFS1. Production of anchor-coupled TB10.4-HspX _DC-seq was confirmed using Western blot analysis with the exception of TB10.4-HspX _DC-seq coupled to the LPxTG-anchor. Confirmation of anchor-coupled TB10.4-HspX DC-seq displayed on the cell surface was not certain, as flow cytometry analysis was not successful. More experiments

to investigate the display of TB10.4-HspX_DC-seq on the cell surface should be done to test the viability of this GM-strategy.

The main long term objective of this work is to develop an oral tuberculosis vaccine using lactobacilli as the delivery vehicle, and preferably by using a non-GM system for delivery, e.g. by binding the fusion antigens of *M. tuberculosis* to wild type *L. plantarum* WCFS1 that is regarded as safe to consume. Work done in this study shows that it is possible to produce TB10.4-HspX _DC-seq coupled to a LysM anchor intracellularly in both *E. coli* and *L. plantarum* WCFS1, and that both TB10.4-HspX _DC-seq with and without fusing to a LysM anchor are able to bind to *L. plantarum* WCFS1. The main challenges met in this study were the production of sufficient amount of soluble proteins and the purification of these minor amounts from protein extracts. Both expression and purification methods should be further investigated, as the fusion antigen TB10.4-HspX is a promising candidate as a multistage vaccine against tuberculosis. Thus, obtaining larger amount of pure TB10.4-HspX coupled to LysM domain is of high interest for use in experiments, especially with immunological experiments in mind for future work.

7 References

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Appendix A Extended Material List

Software	Supplier
CLC Genomics Worckbench	CLC bio
Serial Cloner 2.6.1	SerialBasics
SnapGene® Viewer 2.7.1	GSL Biotech

Material List for Laboratory Equipments	Supplier
BioPhotometer	Eppendorf
Cell density meter, Ultrospec 10	Biochrom
Centrifuge; 5418R	Eppendorf
Centrifuge; Avanti™ J-25	Beckman Coulter
Corex tube	Corning Inc.
Cryogenic screwcap tubes 2ml	Sarstedt
Cuvette for spectrophotometry, 1.5 ml	Brand
Electroporation: - Gene Pulser®/Micropulser™ cuvettes, 0.2 cm - MicroPulser electroporator	Bio-Rad
Falcon 2059 Polypropylene Round-Bottom tubes	Bacton Dickinson
Magnetic stirrer	IKA
Millex-GP Syringe Filter Unit, 0.22 µm and 0.45 µm	Merck Millipore
pH meter	Metrohm
Syringe filter, 0.22 and 0.45 µm	Millex-GP, Merck Millipore
Vortex mixer, MS3 Basic	IKA

Material List for Chemicals and Solution	Supplier
Acetic acid	Merck KGaA
Boric acid	Merck KGaA
Calcium chloride dihydrate	Sigma-Aldrich
D(+)-Glucose anhydrous	VWR
di-Sodium hydrogen phosphate dihydrate	Merck KGaA
Ethanol 96%	Arcus
Glycerol solution 85%	Merck KGaA
Glycine	Merck KGaA
Magnesium chloride hexahydrate	Merck KGaA
Magnesium sulphate	Sigma-Aldrich
Polyethylen Glycol, PEG-1450	Sigma-Aldrich
Potassium chloride	Merck KGaA
Potassium phosphate monobasic	Sigma-Aldrich
Sodium chloride	Merck KGaA
Sodium hydroxide	Merck KGaA
Sucrose	Sigam-Aldrice
Tris-HCL	Sigma-Aldrich
Tritriplex® III (EDTA)	Merck KGaA
Trizma®Base (Tris Base)	Sigma-Aldrich
Tween® 20	Sigma-Aldrich

Material List for Agars, Media and Antibiotics	Supplier
Agar-agar	Merck KGaA
Ampicillin sodium salt	Sigma-Aldrich
Bacto™ Tryptone	BD
Bacto™ Yeast Extract	BD
Brain Heart Infusion broth	Oxoid™

Erythromycin	Sigma-Aldrich
MRS broth	Oxoid™

Material List for Molecular Biological Materials

Kits	Supplier
In-Fusion® HD Cloning Kit	Clontech Laboratories
JetStar™ 2.0 Plasmid Purification Kit	Genomed
NucleoSpin® Plasmid DNA Purification	Macherey-Nagel GmbH & Co. KG
NucleoSpin® Gel and PCR Clean-Up	Macherey-Nagel GmbH & Co. KG
Quick Ligation™ Kit	New England Biolabs
Mastercycler® gradient thermal cycler	Eppendorf AG
Qubit™ Fluorometer	Invitrogen™

Gel electrophoresis	Supplier
6X DNA Loading Dye	Thermo Scientific
Gel casting equipment - Gel caster - Gel tray	Bio-Rad
Comb, 8 wells	
GeneRuler™ 1kb DNA Ladder	Thermo Scientific
Mini-Sub cell GT cell	Bio-Rad
peqGREEN DNA and RNA Dye	PEQLAB, VWR
SeaKem® LE Agarose	Lonza
Supercoiled DNA Ladder	New England Biolabs
Quick-Load® 1kb DNA Ladder	New England Biolabs
Gel Doc™ EZ imaging system	Bio-Rad

PowerPac™ Basic Power Supply	Bio-Rad
UV Sample Tray	Bio-Rad

Material List for Protein Expression and Extraction

Protein expression	Supplier
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich
SppIP	Sigma-Aldrich

Cell disruption	Supplier
DNase	
FastPrep®-24	MP Biomedicals
Tubes and lids for FastPrep®-24	Fisher Scientific
Glass beads, acid washed, < 106 µm	Sigma-Aldrich
Lysozyme	Sigma-Aldrich
Imidazol 56748-250G	Sigma-Aldrich
PMSF (phenylmethylsulfonyl fluoride)	Sigma-Aldrich
Vibra-Cell VC750	Sonics & Materials

Affinity chromatography	Supplier
BioLogic™ Low-Pressure Chromatography Systems	Bio-Rad
Columns for Low-Pressure systems	Bio-Rad
Protino® Ni-NTA Agarose	Macherey-Nagel

Protein analysis	Supplier
10% Mini-PROTEAN® TGX Stain-Free™ Precast Gels, 10 wells 30 µl or 15 wells 15 µl	Bio-Rad

10X Tris/Glycine/SDS	Bio-Rad
BenchMark™ Unstained Protein Ladder	Novex®
Gel Doc™ EZ imaging system	Bio-Rad
NuPAGE® LDS Sample Buffer (4X)	Invitrogen™
NuPAGE® Sample Reducing Agent (10X)	Novex®
Mini-PROTEAN Tetra Cell System, 4 gels	Bio-Rad
PowerPac™ 300	Bio-Rad
Protein Assay 1X Dye Reagent Concentrate	Bio-Rad
Stain-Free Sample Tray	Bio-Rad

Western blot	Supplier
Bovine serum albumin (BSA)	VWR
CL-Xposure™ Film	Thermo Scientific
Developer and fixative solutions	Thermo Scientific
iBlot® Dry Blotting System:	
- iBlot® Gel Transfer Device	Life Technologies™
iBlot® Transfer Stacks Nitrocellulose Mini	Novex®
MagicMark™ XP Western Protein Standard	Novex®
Restore™ Plus Western Blot Stripping Buffer	Thermo Scientific
Skimmed Milk Powder	VWR
SuperSignal® West Pico Chemiluminescent Substrate	Thermo Scientific
SNAP i.d. 2.0 Protein Detection System - Mini	Merck Millipore

Flow cytometry	Supplier
MacsQuant® Analyzer	Miltenyi Biotec
MacsQuantify™ Software	Miltenyi Biotec

Appendix B Growth curves

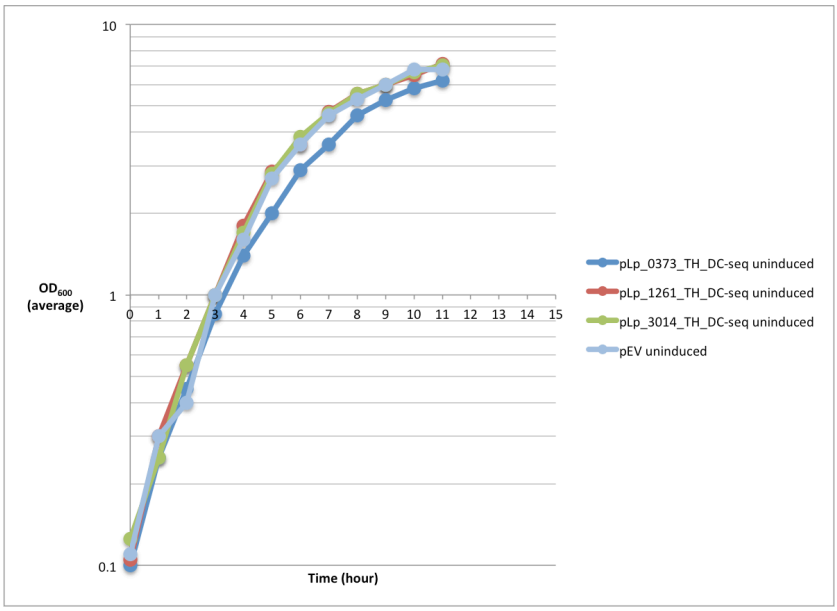

Figure B.1 Growth curve of uninduced *L. plantarum* WCFS1 carrying pLp plasmid for production of anchor-coupled TH_DC-seq at 30°C Overnight culture of all strains were diluted to OD_{600}=0.1 and growth was measured hourly.

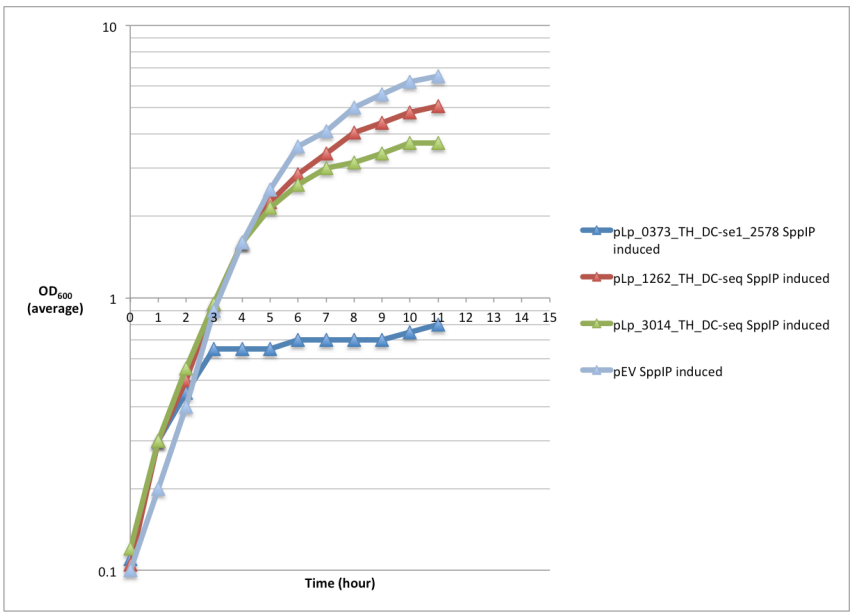

Figure B.2 Growth curve of induced *L. plantarum* WCFS1 carrying pLp plasmid for production of anchor-coupled TH_DC-seq at 30°C Overnight culture of all strains were diluted to OD_{600}=0.1 and growth was measured hourly. All strains were induced after two hours of incubation with 25 ng/ml SppIP.

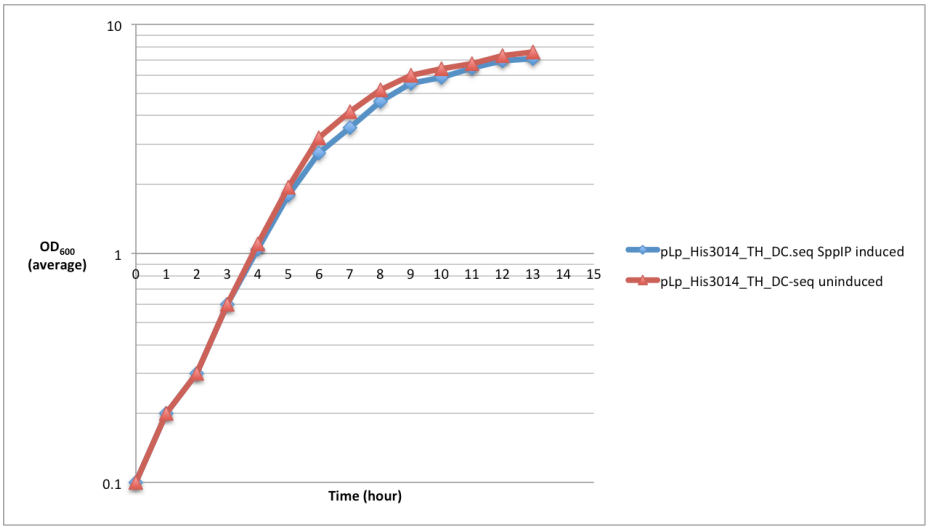

Figure B.3 Growth curve of induced and uninduced *L. plantarum* WCFS1 carrying pLp_His3014_TH_DC-seq at 30°C Overnight cultures of were diluted to OD_{600}=0.1 and growth was measured hourly. The induced culture was induced after two hours of incubation with 25 ng/ml SppIP.

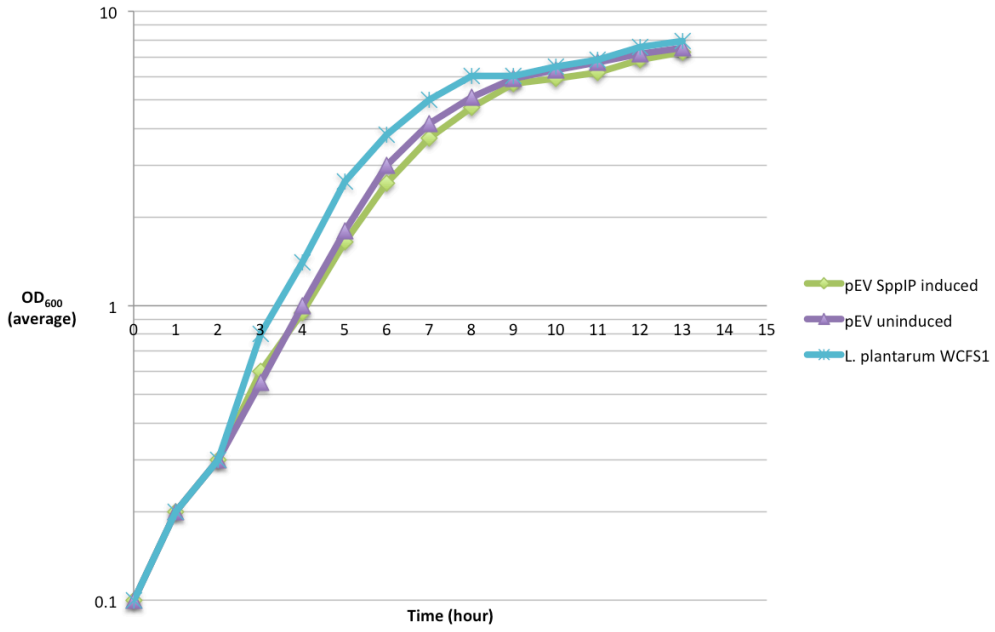

Figure B.4 Growth curve of wild type *L. plantarum* WCFS1 and induced and uninduced *L. plantarum* WCFS1 carrying pEV at 30°C Overnight cultures of were diluted to OD_{600}=0.1 and growth was measured hourly. The induced culture was induced after two hours of incubation with 25 ng/ml SppIP.

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