SECRETION AND ANCHORING OF PROTEINS IN LACTOBACILLUS PLANTARUM: STUDIES OF A DENDRITIC CELL-TARGETED MYCOBACTERIUM TUBERCULOSIS ANTIGEN

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Abstract

The work described in this thesis is part of a larger project where the goal is to develop oral vaccines based on lactic acid bacteria (LAB). LAB have been used in food for centuries, are a natural inhabitant of the gastrointestinal (GI) tract of humans and are generally recognized as safe (GRAS). These characteristics make LAB attractive candidates for use as delivery vectors of therapeutic proteins to mucosal sites. The ability of *Lactobacillus plantarum* to persist in the GI tract of humans for up to a week together with its resistance to bile and low pH makes it well suited as an oral delivery vector. Dendritic cells (DC) are major contributors to the initiation of an immune response and it has previously been shown that targeting oral vaccines to DCs may enhance the subsequent immune response. This thesis describes studies on the use of *L. plantarum* as a live delivery vector for a *Mycobacterium tuberculosis* antigen fused with a DC binding peptide (DC-pep).

Vectors for secretion, cell wall anchoring and membrane anchoring of Ag85B-ESAT6 fused with a DC-pep were constructed. Secretion and surface display of this antigen in *L. plantarum* was successfully accomplished with the efficiency seemingly unaffected by the DC-pep fusion. However, correct anchoring of the antigen could not be determined with certainty since the secreted version of antigen was also detected on the surface of the cells. Experiments with DCs showed that their internalization of *L. plantarum* was higher for strains producing antigen with DC-pep than for the corresponding strains without the DC-pep. Further experiments showed that all antigen producing *L. plantarum* strains as well as the strain harbouring the empty vector (pEV) were able to induce expression of the maturation marker CD83. Mice were immunized with *L. plantarum* strains producing DC-pep fused Ag85B-ESAT6 and further challenged with BCG. Upon stimulation with antigen, peripheral blood mononuclear cells (PBMCs) isolated from these mice showed elevated production of the cytokines IFN-γ and IL-17A, both of which are pro-inflammatory cytokines; an up-regulation indicates initiation of a correct immune response. In mice which were immunized with strains producing Ag85B-ESAT6 without DC-pep, the PBMCs showed no elevation in the production of these cytokines. This experiment strongly indicates that the DC-pep stimulates the immune response.

In conclusion, the work described in this thesis shows that *L. plantarum* is able to secrete and possibly anchor DC-pep fused Ag85B-ESAT6. The presence of the DC-pep enhanced the immune response indicating that further development of the constructs described in this study is a promising strategy for developing novel vaccines against *M. tuberculosis.*

Sammendrag

Arbeidet beskrevet i denne oppgaven er en del av et større prosjekt hvor målet er å utvikle orale vaksiner med melkesyrebakterier som vektor. Melkesyrebakterier har vært brukt i mat i århundrer, de finnes naturlig i mage-tarmkanalen til mennesker og de er generelt betraktet som trygge. Disse egenskapene gjør melkesyrebakterier til attraktive kandidater som leveringsvektorer for terapeutiske proteiner til spesifikke områder i slimhinnen. *Lactobacillus plantarum* sin evne til å vedvare i menneskers mage-tarmkanal i opp til en uke, i tillegg til dens motstandsdyktighet mot galle og lav pH, gjør den egnet som en oral leveringsvektor. Dendrittiske celler (DC) er viktige bidragsytere til initieringen av en immunrespons og det har tidligere blitt vist at orale vaksiner rettet mot DCer kan øke den påfølgende immunresponsen. Denne masteroppgaven beskriver studier av bruken av *L. plantarum* som en levende leveringsvektor av et *Mycobacterium tuberculosis*-antigen som er fusert med et DC-bindende peptid (DC-pep).

Vektorer for sekresjon, celleveggforankring og membranforankring av Ag85B-ESAT6 fusert med et DC-pep ble konstruert. Sekresjon og overflateuttrykk av dette antigenet i *L. plantarum* ble oppnådd og effektiviteten av disse prosessene så ikke ut til å bli påvirket av fusjonen med DC-pep. Riktig forankring av antigen kunne imidlertid ikke fullstendig bekreftes, da sekretert antigen også ble påvist på overflaten av cellene. Forsøk med DCer viste at opptak av *L. plantarum* i DCer var høyest for stammene som produserte antigen med DC-pep, sammenlignet med stammene uten DC-pep. Videre forsøk viste at alle de antigenproduserende *L. plantarum*-stammene i tillegg til stammen med tom vektor (pEV) kunne indusere ekspresjon av modningsmarkøren CD83. Mus ble immunisert med *L. plantarum*-stammer som produserte DC-pep-fusert Ag85B-ESAT6 og videre utsatt for BCG. Perifere mononukleære blodceller (PBMC) isolert fra disse musene viste økt produksjon av cyokinene IFN-γ og IL-17A etter stimulering med antigen. IFN-γ og IL-17A er begge proinflammatoriske cytokiner og en oppregulering indikerer at en korrekt immunrespons har blitt initiert. I mus som ble immunisert med stammer som produserte Ag85B-ESAT6 uten DC-pep, viste ikke PBMCene økt produksjon av disse cytokinene. Dette eksperimentet gir en sterk indikasjon på at DC-pep kan stimulere immunresponsen.

Avslutningsvis viser arbeidet som er beskrevet i denne masteroppgaven at *L. plantarum* er i stand til å sekretere og muligens også forankre DC-pep-fusert Ag85B-ESAT6. Tilstedeværelsen av DC-pep forsterket immunresponsen, noe som indikerer at videre utvikling av konstruktene beskrevet i denne oppgaven er en lovende strategi for utviklingen av nye vaksiner mot *M. tuberculosis*.

Abbreviations

APC	Antigen presenting cell
ATP	Adenosin triphosphate
BCG	Bacillus Calmette-Guérin
bp	Base pair
BSA	Bovine Serum Albumin
CFU	Colony forming units
DC	Dendritic cell
DC-pep	Dendritic cell binding peptide
dNTP	Deoxyribonucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
GIT	Gastrointestinal tract
GM-CSF	Granulocyte-machrophage colony-stimulating factor
GRAS	Generally Recognized as Safe
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Inducer peptide
LAB	Lactic acid bacteria
M cell	Microfold cell
MHC	Major Histocompatibility Complex
OD	Optical density
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sIgA	secreted immunoglobulin A
SP	Signal peptide
SPase	Signal peptidase
Th	T helper
TNF	Tumour necrosis factor

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APPENDIX i

1. INTRODUCTION

The use of lactic acid bacteria (LAB) in food both as a preservative and for flavour goes far back in history. Several species of LAB are a part of the normal bacterial flora of the human body, where they inhabit the gastro-intestinal tract, and some are thought to have health beneficial effects (Herich & Levkut 2002). The tolerance for the acidic environment in the gut and their GRAS (Generally Recognized as Safe) status makes LAB ideal candidates for development as live delivery vectors of vaccines. *Mycobacterium tuberculosis* is the causative agent of tuberculosis and is the second most common cause of death from an infectious disease worldwide, following HIV/AIDS. The BCG vaccine is the only vaccine against *M. tuberculosis* on the market today. In several countries this vaccine has little to no effect. Furthermore, there is an increase in multi drug resistant tuberculosis (Russell et al. 2010). Therefore, a new and more effective vaccine is urgently needed. This thesis describes studies on the use of *Lactobacillus plantarum* as a live delivery vector for a *M. tuberculosis* antigen fused with a dendritic cell binding peptide.

1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are Gram positive bacteria that are characterized by their production of lactic acid from fermentation of hexose sugars. LAB produce lactic acid by two main pathways, one is by fermentation of hexose sugars yielding lactic acid as the main product, the other is by heterofermentation of hexose sugars yielding lactic acid, CO_2, acetic acid and/or ethanol as the products (Makarova et al. 2006). Lactic acid bacteria have a limited metabolism and depend on living in an environment with easy access to sugars, vitamins and amino acids. They are naturally found in vegetables, cereal grains and milk, and they inhabit the gastrointestinal tracts (GIT) of animals and humans (Todar 2008). LAB have for a long time been used for preservation of food because of their production of lactic acid, which prevents the growth of other microorganisms. In addition, the consumption of lactic acid bacteria is assumed to have health beneficial effects in the GIT, by positive effects on the overall microflora (e.g. through competition with pathogens) and by stimulating the immune system (Herich & Levkut 2002).

LAB belonging to the genus *Lactobacillus* are good candidates as oral delivery vectors of antigens, because of their ability to tolerate acidic environments and, thus, the transit through the stomach. They are also able to interact with the mucosal surface of the intestinal tract, which is a prerequisite for mucosal delivery vectors. (Mohamadzadeh et al. 2009). Microbial pathogens such as *Mycobacterium, Salmonella* and *Bordetella,* are also adapted to interact with the mucosal surface of the intestine and weakened variants of these bacteria have therefore been popular as live delivery vectors. However, due to the possibility of regaining pathogenicity, these weakened pathogens are not safe for use in humans. The GRAS status of lactobacilli makes them better candidates as live delivery vectors. While using lactobacilli avoids the problem of pathogenicity, it also means forfeiting any advantages for mucosal interaction gained when using pathogens (Bermudez-Humaran et al. 2011).

1.1.1 *Lactobacillus plantarum*

L. plantarum is a versatile lactic acid bacterium that is found in a variety of food and plant fermentations. *L. plantarum* is one of the few species in the genus *Lactobacillus* that are both involved in industrial fermentations of food and a natural inhabitant of the GIT of humans. Although there is little support from clinical trials, a variety of *L. plantarum* strains are marketed as probiotics that supposedly give beneficial health effects (de Vries et al. 2006; Kleerebezem et al. 2003). In the genus *Lactobacillus*, *L. plantarum* is the species which has one of the largest genomes. The large genome size may explain its ability to inhabit a variety of environmental niches. The complete genome sequence of *L. plantarum* WCFS1was determined by Kleerebezem et al (2003) and a large number of genes involved in sugar uptake and utilization was annotated. The genome also encodes many secreted proteins, of which again many are cell wall or membrane anchored proteins, most of them having a single transmembrane anchor. Of the 25 proteins that were predicted to contain the cell wall anchoring LPxTG motif (see below for more details), 22 had a conserved distinct sequence motif, LPQTxE. The functions of all the secreted and anchored proteins have not been determined, but many of these proteins show similarities to proteins involved in mucus binding, fibronectin-binding and intercellular adhesion, confirming the potential of *L. plantarum* to interact with various surfaces (Kleerebezem et al. 2003). A study by Vesa et al. (2000) assessed the influence of the human GIT on three lactic acid bacteria, namely *L. plantarum* NCIBM 8826*, L. fermentum* KLD and *Lactococcus lactis* MG 1363. They found that the survival of bacterial strains in the ileum after ingestion was significantly higher for

the *L. plantarum* strain compared to the two other strains. In addition they found that *L. plantarum* was able to persist in the gut for up to a week.

1.2 Inducible gene expression in LAB

Production of desired proteins in LAB requires a suitable gene expression system. The expression system should be able to overproduce protein when induced and have a very low basal production of the protein when not induced. A system with very low basal production of protein makes it possible to produce proteins that are harmful or even lethal to the bacteria. Several systems for regulated gene expression in LAB exists (Kuipers et al. 1997) with the best known being the nisin controlled expression (NICE) system in *Lactococcus lactis*, which is activated by the addition of the lantibiotic nisin (De Ruyter et al. 1996). Originally, a two-plasmid nisin-inducible system was developed to function in other LAB species (Kleerebezem et al. 1997) and later a single plasmid system was also developed (Pavan et al. 2000). However, both Pavan et al. and, later, Sørvig et al. (2003) found that the NICE system has a considerable amount of basal activity in *L. plantarum.*

The pSIP vector expression system, a one plasmid expression system, was constructed by Sørvig et al. 2003 (Fig. 1.1). This expression system is based on the regulatory genes and promoters involved in the production of the bacteriocins sakacin A and sakacin P. The regulatory genes of this system are the histidin kinase (HK) gene and the response regulator (RR) gene, which are activated by a pheromone induction peptide (IP) encoded by a gene preceding the HK- and RR- encoding genes (in the same operon). In the pSIP vectors the HK- and RR- encoding genes are present while the IP-encoding gene has been deleted. This allows strict regulation of the production of the gene of interest by externally added IP (Sørvig et al. 2003). Expression of the gene of interest is controlled by the sakacin P promoter (P_{SppA}). When the IP is externally added it binds to membrane-located HK, which then leads to activation of the RR through a series of phosphorylations. The phosphorylated RR activates the inducible promoters, P_{SppA} and P_{SppIP}. Thus, expression of the protein of interest (cloned downstream of the P_{sppA}) is initiated, while the strength of the induction signal is multiplied by increased transcription of the HK- and RR- encoding genes driven by activated P_{SppIP}. (Diep et al. 2009; Nes et al. 1996).

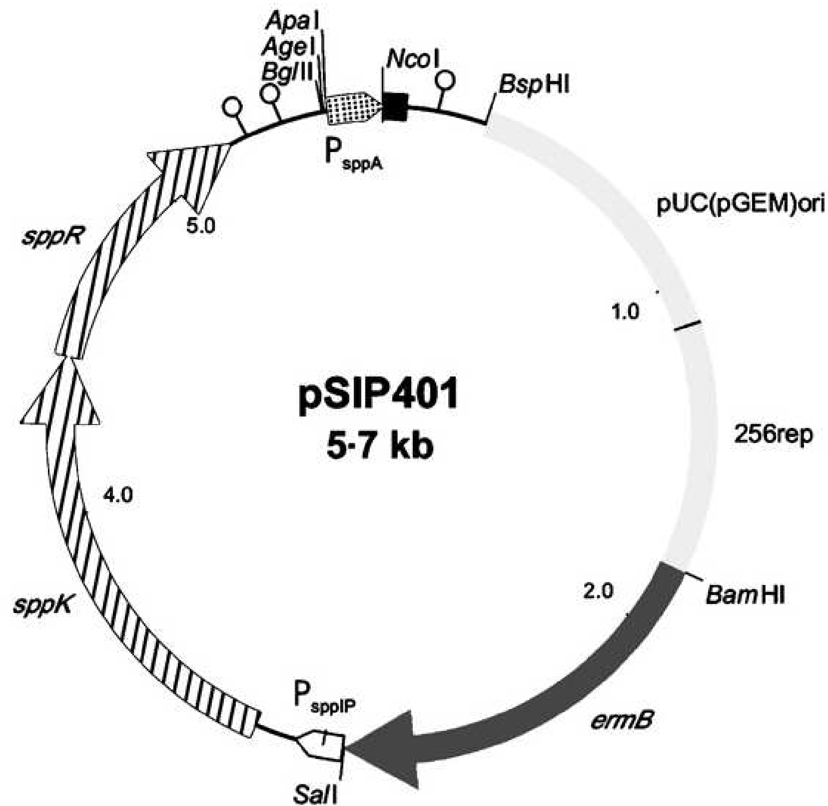


Figure 1.1. Graphic representation of pSIP401. Light gray regions, replicons pUC8pGEMori from *E.coli* and 256rep from *L. plantarum*; dark gray region, the erythromycin resistance gene; white region, inducible P_{sppIP} promoter; vertically hatched region, histidin protein kinase and response regulator genes; dotted region, inducible P_{sppA} promoter; black box, multiple cloning site. Target genes will be cloned downstream of the P_{sppA} promoter, possibly ending up in a translational fusion if the NcoI site is used. The figure is taken from Sørvig et al. 2005.

1.3 Secretion of proteins in Gram positive bacteria

The cytoplasmic membrane of Gram positive bacteria is covered with a thick layer of peptidoglycan. In Gram negative bacteria the layer of peptidoglycan is thin and it is covered with an additional outer membrane. Protein synthesis is carried out at the ribosomes in the cytosol and a protein that has a function outside of the cytosol needs to be transported across the cytoplasmic membrane. The secretion of proteins in Gram positive bacteria is easier than in Gram negative bacteria because the proteins only have to cross one membrane. This makes Gram positive bacteria more promising candidates for the production of secreted proteins. In Gram positive bacteria there are seven known protein secretion pathways: the secretion (sec), twin-arginine translocation (Tat), flagella export apparatus (FEA), fimbrillin-protein exporter (FPE), holin (pore forming), peptide-efflux ABC and the WXG100 secretion system (Wss) pathways (Kleerebezem et al. 2010). Most of the secreted proteins use the Sec pathway; an illustration of the Sec pathway and its components is shown in Fig. 1.2. Proteins that are

going to be secreted by the Sec system are synthesized with an N-terminal signal sequence which is recognized by the chaperone SecB (Fig 1.2 a). For the protein to cross the membrane it needs to be unfolded; SecB stabilizes the unfolded protein and transports it to SecYEG-bound SecA. SecA is an ATP-driven motor protein which drives the translocation of protein through the SecYEG pore. After translocation, the signal sequence is removed by a membrane bound signal peptidase (SPase) (Driessen & Nouwen 2008; Kleerebezem et al. 2010; Tjalsma et al. 2004).

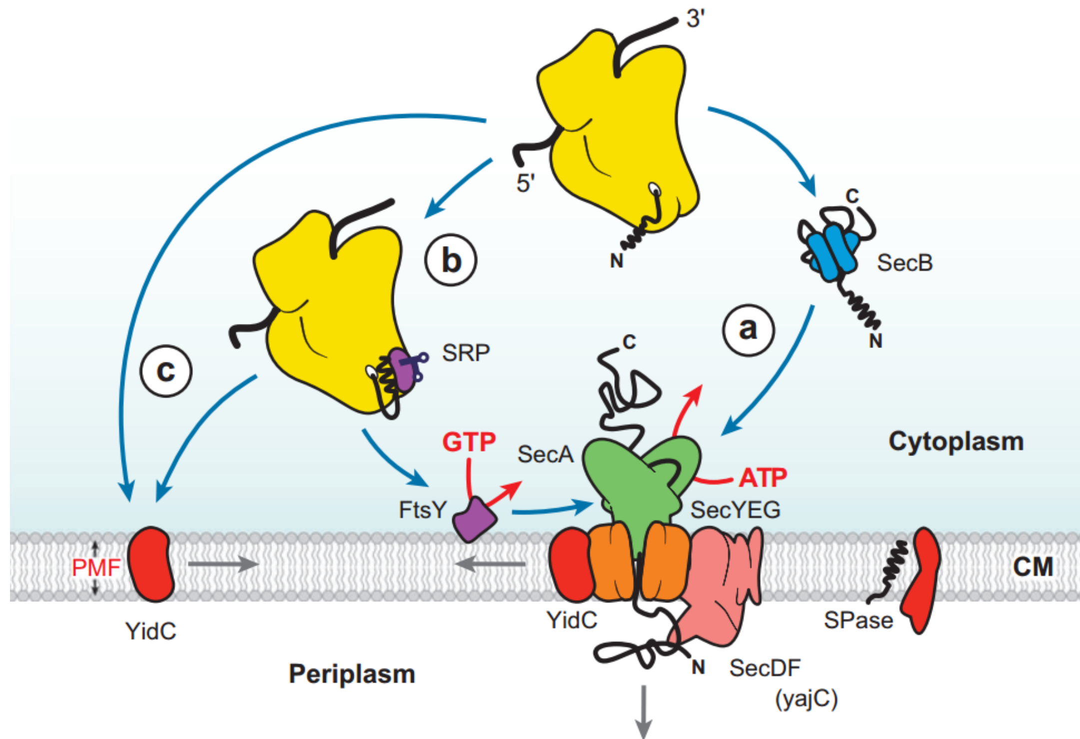


Figure 1.2. Illustration of the Sec protein secretion system in bacteria. The Sec translocase complex sits in the cytoplasmic membrane (CM) and consists of the motor protein SecA (green), the protein conducting channel SecYEG (orange) and the accessesory proteins SecDF (pink) and YidC (red). **(a)** Proteins synthesized at the ribosomes (yellow) are transported to the Sec translocase by the molecular chaperone SecB (blue). **(b)** In the case of membrane proteins the signal sequence or hydrophobic transmembrane segment is recognized by the signal recognition particle (SRP) (purple) and the ribosome nascent chain (RNC) is targeted to the SecYEG by the membrane bound FtsY (purple) at the expense of GTP. After translocation the SPase (red) cleaves off the signal sequence. **(c)** Some membrane proteins are inserted into the membrane by YidC. See text for more details. The figure is taken from (Driessen & Nouwen 2008).

Membrane proteins are targeted to the membrane by the binding of a signal recognition particle (SRP) to the signal sequence or, for integral membrane proteins that lack a signal sequence, to a hydrophobic transmembrane segment (TMS) of the ribosome-nascent chain (RNC) (Fig 1.2 b). The RNC bound SRP interacts with the membrane bound FtsY and upon hydrolysis of GTP the RNC is transferred to the SecYEG which then binds the ribosome. In this case, the elongation of the polypeptide chain by the ribosome provides the energy for

insertion of the protein into SecYEG. A second method for insertion of proteins into the membrane is by the help of YidC (Fig 1.2 c). YidC either inserts the proteins in the membrane on its own or in cooperation with the Sec system (Driessen & Nouwen 2008).

1.4 Anchoring of proteins in Gram positive bacteria

Proteins that are targeted to the SecYEG translocase are either secreted out of the cell and into the extracellular environment or they are anchored to the membrane or the cell wall of the bacteria. Secreted proteins that are anchored to the cell membrane or cell wall in Gram-positive bacteria can be divided into four main groups of proteins (Fig. 1.3):

1. Transmembrane proteins
2. Lipoproteins
3. LPxTG-anchored proteins
4. Noncovalent cell-wall-binding proteins

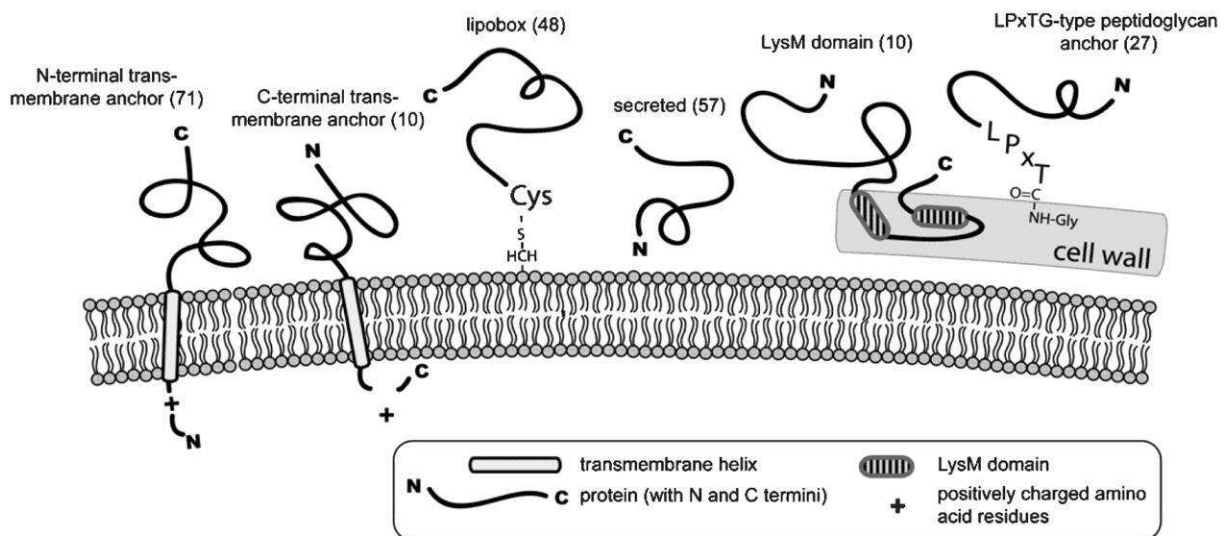


Figure 1.3 Illustration of different types of protein anchors. Two types of transmembrane anchors are shown, the N-terminal and the C-terminal anchor, of which the N-terminal anchor (i.e. a non-cleaved signal peptide) is the most common. Lipoproteins are anchored to the membrane by a lipobox motif (third from the left). Cell wall bound proteins can be noncovalently bound through domains with cell wall affinity such as a LysM domain, or they can be covalently anchored to the cell wall by a LPxTG peptidoglycan anchor. Numbers in parenthesis represent the predicted number of proteins of different kinds in *L. plantrarum* WCFS1. See text for more details. The figure is taken from (Boekhorst et al. 2006).

Proteins are directed to their final destinations by their signal peptides. The signal peptide that directs the translocation consists of three parts. The N-terminal region contains one to three positively charged amino acid residues and is called the N domain. The middle part, called the H domain, consists of 10-15 hydrophobic residues. The third part is the C domain, consisting of more polar residues and, if present, the SPase cleavage site (Driessen & Nouwen 2008).

1.4.1 Transmembrane proteins

Many proteins with N-terminal signal peptides do not have a cleavage site in the C domain of the signal peptide. In such proteins, the signal peptide is not cleaved and the protein will remain N-terminally anchored to the cell membrane (Fig. 1.3). N-terminally anchored transmembrane proteins are the largest fraction of membrane-anchored proteins in *Lactobacillus* and they are mainly involved in extracellular processes (Kleerebezem et al. 2010). Some transmembrane proteins are C-terminally anchored to the membrane. In this case, the signal peptide is cleaved off by SPase I following translocation and a hydrophobic C-terminal anchor inserts into the membrane (Kleerebezem et al. 2010).

1.4.2 Lipoproteins

Lipoproteins are a diverse class of peripheral of membrane proteins (Hutchings et al. 2009) and are predicted to be the second most common class of membrane associated proteins in lactobacilli (Kleerebezem et al. 2010). The lipoproteins contain the lipobox motif [L-(A/S)-(A/G)-C] in the C domain of their signal peptides. After translocation, a lipoprotein diacylglyceryl transferase covalently binds the protein to the membrane by transferring a diacylglyceryl moiety to the Cys-residue in the lipobox motif. The signal peptide is then cleaved N-terminally of the modified cysteine by SPase II. Lipoproteins are involved in different tasks like binding of substrates and antibiotic resistance, as well as protein secretion, folding and translocation (Hutchings et al. 2009; Kleerebezem et al. 2010).

1.4.3 LPxTG-like proteins

LPxTG-like proteins are named after a conserved sequence motif in the C-terminal regions of these proteins (Navarre & Schneewind 1999). After translocation of protein through the Sec system the signal peptide is cleaved by a SPase I. The LPxTG motif is followed by a stretch of hydrophobic amino acids that retains the protein in the membrane. A sortase (SrtA) enzyme recognizes the LPxTG motif and cleaves it between the T and G residues. The sortase then attaches the threonine carboxyl group covalently to the peptidoglycan (Kleerebezem et al. 2010). Notably, the proteins are thus anchored at their C-terminus, while their N-termini may be protruding into the environment.

1.4.4 Noncovalent cell wall binding proteins

Proteins can be noncovalently anchored to the cell wall by various cell wall binding domains, some of which are: LysM domains, cholin-binding domains, SLH domains and SH3 domains (Kleerebezem et al. 2010). The LysM domain is a widely distributed anchor domain that is found in both prokaryotes and eukaryotes (Buist et al. 2008). In *Lactobacillus* almost all proteins with a LysM domain are predicted to be involved in cell wall metabolism (Kleerebezem et al. 2010).

1.5 The human mucosal immune system

The mucosal membranes in the intestine separate the internal sterile environment from the external environment which is rich in microbes. These membranes represent the first line of defence against pathogens and parasites (Montilla et al. 2004). The GIT has to manage the tasks of taking up nutrients and distinguishing between harmless antigens in food, our own intestinal flora, and antigens from pathogens. To have an effective uptake of nutrients, the surface area of the intestine has to be large; in adult humans the surface area of the intestine is $400m^2$ (MacDonald 2003). The mucosal membrane consists of only a single layer of cells which are renewed every 2-3 days. This thin layer needs to be protected from harmful microorganisms and in addition to being covered with mucus it is heavily guarded by immune cells from the inside. The number of antibody producing cells in the intestine is larger than the sum of all other antibody producing cells in the body (Perdigón et al. 2001). Fig. 1.4 provides an illustration of the intestinal mucosa.

Antigens from the intestinal content may reach the immune cells below the mucosa by entering through microfold cells (M-cells) which are incorporated in the epithelial cell layer. M-cells are connected to Peyer's patches which are lymph nodes with large clusters of T-cells, B-cells and dendritic cells (MacDonald 2003). M-cells transport the antigens to dendritic cells (DC) and other antigen presenting cells which in turn present the antigen to naïve T-cells. The DCs can also get in direct contact with the antigen in the gut lumen by extending dendrites through the epithelial cell layer (O'Hara & Shanahan 2006) as shown in Fig. 1.4, step 3.

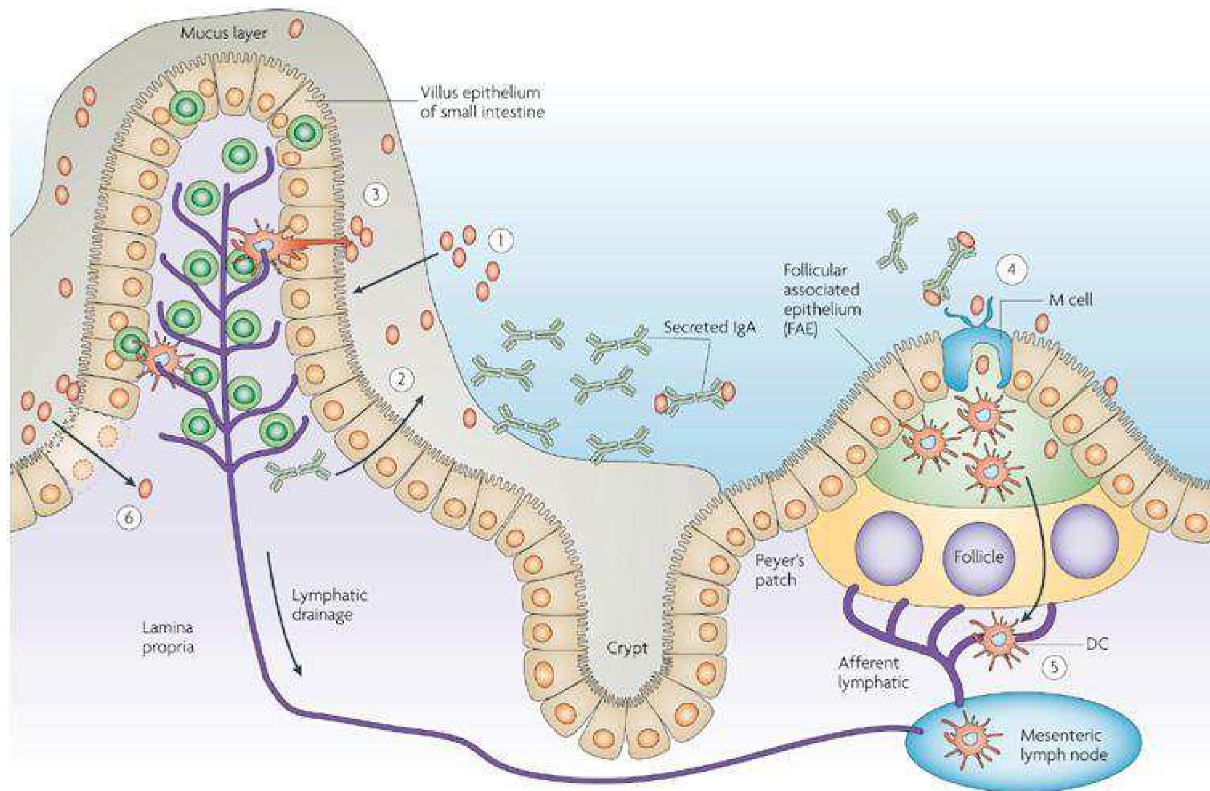


Figure 1.4. Fate of bacteria at the mucosal surface of the intestinal tract. Most of the bacteria (red) that survive the journey through the degrading environment in the stomach end up in the lumen or are trapped in the mucus layer. Bacteria and their secreted proteins could come in contact with the epithelium (1) where they can be sampled by DCs (red with blue nucleus) that extend their dendrites through the epithelial cell layer (3) or they can be sampled by M cells (blue) (4) and transported to the underlying immune cells in the Peyer's patch (PP) (5). IgA is secreted by mature plasma cells (green) into the gut lumen and could limit bacterial uptake and persistence (2). Damage of the epithelial cell layer, as a result of for example intestinal inflammation, could lead to bacteria crossing the epithelia and being found in the mucosal tissue (6). The figure is taken from Wells & Mercenier (2008).

As the mucosa is the major entry point for pathogens, it is advantageous to deliver a vaccine directly to this point of infection. A mucosal vaccine should ideally provide both humoral and cell mediated protection at the site of delivery and throughout the rest of the body. Several routes can be used for the delivery of a mucosal vaccine, those being oral, nasal, rectal or vaginal. So far, the nasal route has proven to be the most promising; in humans, monkeys and mice this route of delivery has been shown to induce IgA antibody responses in the salivary glands, upper and lower respiratory tract, the small and large intestine as well as the male and female genital tracts (Holmgren & Czerkinsky 2005; Neutra & Kozlowski 2006). There are, however, several challenges connected to using the mucosal route for vaccine delivery. At the mucosal surface the antigen faces attack by proteases and nucleases. Furthermore, the antigen

may become buried in mucus gel or diluted by mucosal secretion. It has been pointed out that a soluble non-adherent antigen is unlikely to be taken up in the intestine, and would generally induce immune tolerance (Neutra & Kozlowski 2006). Therefore, the general idea is that one needs to use some sort of delivery and adjuvant system to ensure sufficient uptake of the antigen and optimal activation of a mucosal immune response. Different delivery systems exist for ensuring uptake of the antigen. Examples of such systems are microparticles of poly lactide-co-glycolide (PLG) with enclosed antigen, liposomes formulated with antigen, chitosan formulated with antigen, and the use of live or attenuated microorganisms to deliver the antigen (Ryan et al. 2001).

1.6 Dendritic cells and priming of T-cells

The stimulation of an immune response depends on the uptake of the antigen, its subsequent presentation on the surface of an antigen presenting cell (APC), and the presentation of the antigen to T-cells. Macrophages, B-cells and DCs are three types of APC and among these it is the DCs that are able to efficiently stimulate an immune response and further lead to immunological memory (Guermonprez et al. 2002). In most tissues DCs are in an immature state and in this state there is a high representation of antigen-capturing receptors on the surface of the DCs. The DCs can capture antigens in three different ways; they can use phagocytosis to take up microbes and particles, they are able to take up extracellular fluid by invagination of the membrane (called macropinocytosis), and they can take up antigens that have bound to receptors on their surface (Banchereau & Steinman 1998). Upon antigen uptake the DC goes into a maturing phase and its ability to take up antigens is down-regulated. The internalized antigens are loaded onto major histocompatibility complex (MHC) class II molecules which present the antigen on the surface of the cell. Maturation of DCs enhances the expression of adhesion and costimulatory molecules (necessary for the priming of T-cells) such as B7-1 (CD80) and B7-2 (CD86) on the surface and leads to secretion of different cytokines. CD80 and CD86 bind to specific molecules on the surface of T-cells, which facilitates T-cell priming (Guermonprez et al. 2002). Antigens presented by MHC class II are recognized by $CD4^+$ T-cells which are further divided into the subtypes T helper 1 (Th1) and T helper 2 (Th2) based on their secretion of cytokines. Th1 cells secrete interferon γ (IFN-γ) and tumor necrosis factor β (TNFβ) and mediate cellular immunity against intracellular bacteria and small parasites. Th2 cells secrete the cytokines IL-4, IL-5, IL-10 and IL-13 and mediate immunity to extracellular bacteria and parasites. Th2 cells also induce the

production of antibodies against bacterial toxins by B-cells. The regulation of Th1 and Th2 cells is governed by the cytokines they secrete and the innate immune system (Ryan et al. 2001).

The type of immune response generated depends on several factors like the nature of the antigen or microorganism involved and the type of APC involved (Holmgren & Czerkinsky 2005).The pre-requisite for vaccines to be effective is the uptake of antigen and its presentation by an APC. It has been pointed out, and shown by experiments (Mohamadzadeh et al. 2009; Mohamadzadeh et al. 2010) that the unique role of DCs in antigen uptake and presentation should be exploited in vaccine development (Foged et al. 2002). A vaccine delivery system with the aim of targeting DCs is expected to improve efficiency, reduce dosages and side effects, and better control the type of immune response (Foged et al. 2002). In a landmark study, Mohamadzadeh et al. (2009) managed to enhance the immune response in mice towards *Bacillus anthracis* antigens by fusing the antigens with a DC targeting peptide (DC-pep). The antigens were delivered orally by the use of *Lactobacillus acidophilus* as a live delivery vector. The survival rate in mice challenged with *B. anthracis* was 75 % for the group given DC-pep fused antigen, compared to 25 % for the group given antigen fused with a control peptide.

1.7 Bacteria as live vectors

As previously mentioned the immunogenicity of soluble antigens that are administered orally or intranasally is low, but may be enhanced if a delivery vector is used. The delivery system should prevent degradation of the antigen and promote uptake by immune cells in the GIT. The advantages of utilizing the mucosal route for vaccination relate to both economy and safety. There is no need for needles or syringes for delivery thus eliminating the use for trained personnel and the cost of sterile equipment. Another advantage of a mucosal vaccine is its ability to induce both a systemic and a mucosal immune response, with fewer side effects than systemic vaccines. (Bermudez-Humaran et al. 2011). Administration of vaccines to the mucosa could elicit the secretion of IgA at several mucosal sites. IgA antibodies can neutralize viruses and toxins and prevent colonization by enteric microbes (Holmgren & Czerkinsky 2005; Neutra & Kozlowski 2006; Wells 2011).

Because of the degrading environment in the gut and intestine, it is preferable to use a delivery vector that tolerates this environment. Pathogenic bacteria such as *Mycobacterium, Salmonella* and *Bordetella* that initiate infections through mucosal surfaces are well adapted to interact with the mucosa. Attenuated strains of these bacteria could be effective delivery vectors. Unfortunately there is a risk of the bacteria recovering pathogenicity and they are therefore not safe for human use, especially in older people, children and immunosuppressed individuals (Bermúdez-Humarán 2009). Bacteria with the GRAS status are the other candidates for vaccine delivery, especially due to their well documented safe status. LAB have GRAS status and the ability to survive the harsh environment in the stomach and the intestine. The *Lactococcus* and *Lactobacillus* genera are the best studied genera of the LAB, also in terms of the production of therapeutic molecules (Daniel et al. 2011). The first constructed LAB-based vaccine was *Lactococcus lactis* producing a tetanus toxin fragment C antigen which was delivered orally to mice (Robinson et al. 1997). While *L. lactis* is considered the model LAB and there are well developed tools for production of heterologous proteins in this bacterium (Bermudez-Humaran et al. 2011), it does not persist in the intestinal tract like for example *Lactobacillus plantarum* (Vesa et al. 2000).

Antigens produced in LAB can end up at various locations: intracellular, extracellular or anchored to the cell wall/membrane. If produced intracellular, the antigen is well protected for degradation by external proteases, but for the antigen to get in contact with the immune cells cell lysis is required. Extracellular production of antigen promotes direct interactions between the antigen and the environment, but gives low protection from degradation by proteases. Anchored antigens have direct contact with the environment and may in addition be protected from proteolytic degradation, which explains why anchoring of antigens is the currently most popular strategy (Bermudez-Humaran et al. 2011; Daniel et al. 2011).

Cell wall anchoring may be achieved by genetic engineering to create antigens with the properties needed to achieve anchoring (section 1.4). A recent study by (Ribelles et al. 2013) describes a novel LAB-based delivery system that was employed for displaying the E7 protein from human papilloma-virus 16, which is the virus responsible for 90-99 % of cervical cancer incidents. The delivery system is based on non-genetically modified LAB which bind exogenously added antigen that is fused to a cell binding domain from the *Lactobacillus casei* A2 phage lysin, LysA2. The immune response was tested after intranasal immunisation of mice and this showed activation of an antigen-specific cytotoxic T-lymphocyte response. A

cellular and humoral immune response had previously been described for mice intranasally vaccinated with a recombinant *L. lactis* strain expressing surface anchored E7 antigen (Bermúdez-Humarán et al. 2005)

The use of live recombinant bacteria as vaccines raises some concerns. To be used as a vaccine in humans the bacteria need to be contained to the human host and transmission of bacteria to the environment needs to be prevented. Braat et al. (2006) developed a containment strategy for genetically modified *L. lactis* for treatment of Crohn's disease. The *L. lactis* gene encoding the thymidylate synthase was replaced with a sequence coding for human IL-10, making the bacteria depended on thymidine from an external source for growth while simultaneously making the bacteria produce IL-10 which may reduce inflammation of the GI tract. A phase II clinical trial was performed with this recombinant *L. lactis* strain and the results confirmed the suitability of the containment system, while significant clinical benefits were not observed. These studies show that genetically modified bacteria can be used for vaccine delivery in humans and be contained to the host (Daniel et al. 2011).

1.8 Tuberculosis

Tuberculosis is a disease caused by the bacterium *Mycobacterium tuberculosis*. This disease most commonly affects the lungs and the pathogen is transmitted through droplets from people with active respiratory disease. Each year nearly 2 million people die from a tuberculosis infection and approximately 9 million new cases are registered. Tuberculosis is treatable by antibiotic administration for six months, but for patients with drug resistant tuberculosis the treatment can take 20 months (World Health Organization 2012).

When a person is infected with tuberculosis, the bacterium is taken up by phagocytic cells. This may lead to destruction of the pathogen , but more commonly phagocytosis induces an inflammatory response, leading to recruitment of immune cells and, with time, to formation of granulomas, which are infected macrophages surrounded by other macrophages and lymphocytes (Russell et al. 2010). The bacterium can stay latent in granulomas for years. 95% of infected people develop this latent infection and it is estimated that approximately 2 billion people live with a latent tuberculosis infection (Dietrich & Doherty 2009). The infection can be contained at the granuloma stage; the granulomas will then shrink and disappear and T-cells will respond to *M. tuberculosis* antigens and further lead to elimination of the bacteria. If

the infection is not controlled by the immune system, the granuloma will grow and cell death in the granuloma leads to release of infectious *M. tuberculosis* that end up in the lungs and is spread by aerosols (Dietrich & Doherty 2009). The BCG vaccine is the only used vaccine against tuberculosis, but it is not effective in adults who live in areas where they are being exposed to environmental mycobacteria. The BCG vaccine is not recommended to HIV-infected children because of the risk of disseminated BCG disease which is a rare but severe side effect of the BCG vaccine. Tuberculosis is most widespread in poor countries and among people who are malnourished, immunocompromised, homeless or imprisoned, but it is not yet known why these factors lead to reduced resistance against tuberculosis. The best way to defeat tuberculosis is by vaccination and a new and more effective vaccine is urgently needed (Kaufmann et al. 2010).

1.9 *Mycobacterium tuberculosis* antigens used in this study

The antigen used in this study is a fusion protein of two *M. tuberculosis* antigens called 85B (or Ag85B; 30 kDa) and ESAT6 (6kDa). This fusion protein has proven more effective than the single antigens (Dietrich et al. 2006; Olsen et al. 2001).

The 30 kDa *M. tuberculosis* Ag85B is a part of a protein complex consisting of three related proteins, Ag85B, the 32 kDa Ag85A and the 32 kDa Ag85C. Ag85B is the most abundant protein produced by *M. tuberculosis* (Harth et al. 1996) and is known to induce protective immunity against pulmonary tuberculosis in guinea pigs (Horwitz et al. 1995). In another guinea pig model a recombinant BCG vaccine expressing Ag85B was compared to the parental BCG vaccine in an aerosol challenge experiment. The recombinant BCG vaccine showed better protective effects than the parental BCG vaccine (Horwitz et al. 2000).

Early secreted antigens and surface proteins are important targets for the immune system in the early phase of an infection with *M. tuberculosis* (Andersen et al. 1992) and therefore also promising as vaccine antigens (Sørensen et al. 1995). ESAT6 is a 6 kDa early secreted antigen from *M. tuberculosis* and was found to be the most immunogenic antigen among several lower molecular mass culture filtrate proteins from the culture supernatant of *in vitro* grown *M. tuberculosis.* In a study by Kamath et al. (1999) mice were immunized with different DNA vaccines encoding one of the secreted *M. tuberculosis* proteins MPT64, Ag85B or ESAT-6. This study showed that Ag85B gave the highest protection followed by ESAT6.

Several studies, using different animal models, have shown that a fusion protein of the two antigens Ag85B and ESAT6 can give protection against *M. tuberculosis* (Dietrich et al. 2006; Hall et al. 2009; Langermans et al. 2005; Olsen et al. 2004). Administration of this fusion protein with the adjuvant DDA/MPL to mice gave a stronger immune response than the individually administered antigens and induced the same level of protection as the BCG vaccine (Olsen et al. 2001). In another study, it was shown that intranasal administration of the Ag85B-ESAT6 fusion protein with the adjuvant LTK63, a heat labile enterotoxin mutant with no toxic activity but with adjuvant effect, led to long lasting immunisation against tuberculosis in mice. It was also found that the administration of these antigens to mice previously vaccinated with BCG would boost the BCG immunity (Dietrich et al. 2006). In an experiment by Hall et al. (2009), mice and guinea pigs were immunised with an attenuated strain of *Salmonella* expressing the fusion protein Ag85B-ESAT6 prior to infection with *M. tuberculosis.* This vaccination strategy, followed by boosting with antigen and an adjuvant, was just as effective as the BCG vaccine. The fusion protein Ag85B-ESAT6 has also shown protective immune response in cynomolgus monkeys (Langermans et al. 2005).

1.10 Goals of this study

The present study, which is part of a larger project where the goal is to produce oral LAB-based vaccines, had three main goals: (1) to translationally fuse the *Mycobacterium tuberculosis* Ag85B-ESAT6 hybrid antigen to a peptide sequence (FYPSYHSTPQRP) which has been shown to bind to DCs (Curiel et al. 2004), (2) to investigate the production and anchoring of this antigen in *Lactobacillus plantarum*, and (3) to compare various antigen-producing strains with respect to uptake by DCs as well as the maturation of the DCs. The idea behind this study was that targeting of DCs by the DC-binding peptide could enhance the immune response against the antigen. From previous work, it was known that the selected peptide indeed binds dendritic cells (Curiel et al. 2004) and that its addition to a *Bacillus anthracis* protective antigen drastically improved the immune response in mice induced by this antigen. (Mohamadzadeh et al. 2009). The starting point for vector constructions was a set of plasmids constructed by Tjåland (2011) that are based on the pSIP system for inducible production of proteins in lactobacilli (Sørvig et al. 2003). The ultimate goal of this research was to contribute to the development of a vaccine against *M. tuberculosis* which is more effective, cheaper and easier to administer than the BCG vaccine used today.

The experimental work of this study was carried out in the following steps:

- Construction of vectors for expression of Ag85B-ESAT6 fused to the DC-binding peptide.
- Investigation of production and anchoring of antigen in *L. plantarum* by the use of western blotting, flow cytometry and confocal laser scanning microscopy, with special focus on the effect of adding the DC-binding peptide to the antigen.
- Analysis of the uptake of *L. plantarum* by dendritic cells and analysis of the maturation of dendritic cells, following incubation with *L. plantarum* strains harbouring plasmids for production of Ag85B-ESAT6 variants with and without the DC-binding peptide.

2. MATERIALS

2.1 Laboratory equipment

Laboratory equipment	Supplier
CL-Xposure™ Film	Thermo Scientific
Cuvettes	
Disposable cuvettes, 1.5 ml	Brand
Electroporation cuvettes, Gene pulser®, 0.2 cm	Bio-Rad
Eppendorf tubes 1.5 ml	Axygen
Falcon 2059 Polypropylene Round Bottom tube 14 ml	Bacton Dickinson
FastPrep® tubes and lids	Fisher Scientific
Glass beads	Sigma
Macs CD14 MicroBeads (human)	Miltenyi Biotec
Macs Column	Miltenyi Biotec
Macs Separator	Miltenyi Biotec
MF-Millipore™ Membrane Filters, 0.025 µm pore size	Millipore
Nunc tubes, 15 ml and 50 ml	Nunc
PCR tubes 0.2 ml	Axygen
Sterile filters, 0.22 µm pore size	Millex GP
Syringes, 10-50 ml	Plastipak
Glass equipment	Labsystems
Waterbaths	

Instruments	Supplier
Centrifuges	
Eppendorf centrifuge 5415R	Eppendorf
Eppendorf centrifuge 5430R	Eppendorf
Table centrifuge	Biofuge Pico, Heraeus
Megafuge 1.0	Heraeus
Vacuum centrifuge	Maxi Dry Lyo, Heto

Coulter Counter® Z1	
Coulter® Particle Count Z1. Beckment Coulter™	Nerliens Meszansky
Electrophoresis electricity supplier	
MacsQuant®Analyser	Miltenyi Biotec
pH-meter	Metrohm
Photo equipment	
GelDoc machine	Bio-Rad
Qubit™ Fluorometer	Invitrogen
SNAP i.d. Protein Detection System	Millipore
Steri-Cycle CO_2 Incubator	Thermo-electron Corporation

Software	Supplier
MacsQuantify™ Software	Miltenyi Biotec
pDRAW32	www.acaclone.com

2.2 Chemicals

Chemicals	Supplier
Acetone	Merck
Agarose	
NuSieve®GTG®Agarose	Lonza
SeaKem® LE Agarose	Lonza
Calcium chloride, $CaCl_2$	Merck
Disodium hydrogen phosphate, Na_2HPO_4	Merck
EDTA, $C_{10}H_{16}N_2O_8$	Merck
Erythromycin, $C_{37}H_{67}NO_{13}$	Sigma-Aldrich
Ethanol, C_2H_5OH	Arcus
Ethidium Bromide, EtBr, $C_{21}H_{20}BrN_3$	Sigma
Gentamicin	Sigma
Glucose, $C_6H_{12}O_6$	Merck
Glycerol $C_3H_8O_3$	Merck
Glycine, $C_2H_5NO_2$	Merck
Kanamycin, $C_{18}H_{36}N_4O_{11}$	Sigma

Magnesium chloride, $MgCl_2$	Aldrich
Magnesium sulfate, $MgSO_4$	Sigma
Methanol, CH_3OH	Merck
Polyethylen glycol, PEG_{1450}	Aldrich
Potassium chloride, KCl	Merck
Potassium dihydrogen phosphate, KH_2PO_4	Merck
Skimmed milk powder	Difco
Sodium chloride, NaCl	Merck
Sodium Deoxycholate, $C_{24}H_{39}O_4Na$	Merck
Sodium hydroxide, NaOH	Merck
Sucrose, $C_{12}H_{22}O_{11}$	Sigma
Trichloroacetic acid (TCA), $C_2HCl_3O_2$	Sigma
Tris-base, $C_4H_{11}NO_3$	Sigma
Tris-HCl, $C_4H_{11}NO_3$ x HCl	Sigma
Tween-20	Sigma-Aldrich
Triton X-100	Sigma-Aldrich

2.3 Proteins and enzymes

Protein/enzyme	Supplier
Antibodies	
Anti-Mouse IgG FITC	Sigma
CD80 FITC Human cat. nr. 557226	BD Pharmingen™
CD83 Pe-cy™ Human cat. nr. 551058	BD Pharmingen™
CD86 Alexa Fluor Human cat. nr. 561124	BD Pharmingen™
ESAT6 Mouse mcAb (ab26246)	Abcam
HLA DR FITC Human cat. nr. 555558	BD Pharmingen™
HRP-Rabbit Anti-Mouse IgG	Invitrogen
Bovine Serum Albumin (BSA)	Sigma
Fetal Calf Serum (FCS)	PAA
GM-CSF	Immunotools
Inducer peptide (SppIP)	Sigma
Interleukin 4	Immunotools

Lysozyme	Sigma
Mutanolysin	Sigma
Trypsin	Fermentas
Trypsin EDTA, L11-004	PAA
Protein standards	
MagicMark™	Invitrogen
Phusion High Fidelity DNA polymerase	Finnzymes
Restriction enzymes and buffers	
BsaI	NEB
BsmI	NEB
HindIII	NEB
MluI	NEB
SalI	NEB
NEBuffer 2	NEB
NEBuffer 3	NEB
NEBuffer 4	NEB
T4 DNA ligase	NEB
VWR RED Taq DNA polymerase Master Mix	Finnzymes
Quick T4 DNA ligase (with 2x quick ligation buffer)	NEB

2.4 DNA

DNA	Supplier
dNTP-mix, 10mM	NEB
DNA standards	
GeneRuler™ 1kb DNA ladder	Fermentas

2.5 Primers

The sequences of primers used in this study are shown in Table 2.1, whereas Table 2.2 describes what the various primers were used for.

Table 2.1. Primer sequences

Name	Sequence	Restriction site in sequence
P1-DCF	CGCCACAACGGCCATTTAGTCGTCCAGGTTT	
P2-DCF	CCAAGTTATCATAGTACGCCACAACGGCCATTTAGT	
P3-DCF	GTCGACTTTTATCCAAGTTATCATAGTACGCCAC	SalI
AgE6PurR	CTGTAATTTGAAGCTTTTATGCAAACATGC	HindIII
AgMluR	CCTTAACGCGTTGCAAACATGCCGGT	MluI
E6-DCF	GTTACCGGCATGTTTGCATTTTATCCAAGTTATCATAGTACGCC	
Hind-DCR	TTGAAGCTTTTATGGCCGTTGTGGCGT	HindIII
DC-E6R	CTATGATAACTTGGATAAAATGCAAACATGCCGGTAAC	
pSekF	GGCTTTTATAATATGAGATAATGCCGAC	
SekR	CCTTATGGGATTTATCTTCCTTATTCTC	
Sh71F	ATAAGAATTCGGTACCCCGGGTT	
Sh71R	GCACTATCAACACACTCTTAAGTT	
pAcc65IR	TGGCTATCAATCAAAGCAACACGT	
Seq-Sh71R	AGCGCTACCGCTCGGCAAAAT	

Table 2.2. Primer description

Name	Description
P1-DCF	Forward primer for amplification of the DC-sequence*; overlapping with Ag85B-ESAT6 and P2-DCF
P2-DCF	Forward primer for amplification of DC-sequence*; overlapping with P1-DCF and P3-DCF
P3-DCF	Forward primer for amplification of DC-sequence*; overlapping with P2-DCF
AgE6PurR	Reverser primer for amplification of Ag85B-ESAT6 and DC-sequence*
AgMluR	Reverse primer for amplification of Ag85B-ESAT6 and DC-sequence*
E6-DCF	Forward primer for amplification of DC-sequence*; overlapping with the C-terminus of Ag85B-ESAT6
Hind-DCR	Reverse primer for amplification of DC-sequence, including a tail with the HindIII restriction site
DC-E6R	Reverse primer for amplification of Ag85B-ESAT6, including a tail with the N-terminus of the DC-sequence*
pSekF	Forward primer for amplification of lipo anchor sequence 1261 and Ag85B-ESAT6 Forward primer for sequencing of DC-sequence* and Ag85B-ESAT6
SekR	Reverse primer for sequencing of Ag85B-ESAT6
Sh71F	Forward primer for sequencing of the SH71 replicon
Sh71R	Reverse primer for sequencing of the SH71 replicon
pAcc65IR	Reverse primer for sequencing of Ag85B-ESAT6
Seq-Sh71R	Reverse primer for sequencing of the SH71 replicon

*The nucleotide sequence: ttttatccaagttatcatagtacgccacaacggcca, coding for the dendritic cell binding peptide (DC-pep), see below for details.

2.6 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Tables 2.3 and 2.4, respectively.

Table 2.3. Bacterial strains

Strain	Source
Escherichia coli TOP10	Invitrogen
Escherichia coli TG1	Lucigen
Lactobacillus plantarum WCFS1	Kleerebezem et al. (2003)
Lactococcus lactis MG1363	Wegmann et al. (2007)

Table 2.4. Plasmids

Plasmids	Description	Source
pEV	Empty vector (pSip401-derivative lacking any target gene)	L. Fredriksen (2010)
pLp_0373sh71-NucA	pSIP401-derivative for secretion of NucA with Lp_0373 as signal sequence; SH71 replicon	K. Maudal (2012)
pLp_1261-Ag85B-ESAT6	pSIP401-derivative for production of Ag85B-ESAT6 with the Lp_1261 lipo- anchor signal sequence	R. Tjåland (2011)
pLp_1261-Ag85B-E6-DC	pSIP401-derivative for production of Ag85B-ESAT6 with a C-terminally fused DC-binding peptide, with the Lp_1261 lipo- anchor signal sequence.	This work
pLp_1261-AG85B-E6-DC-sh71	pSIP401-derivative for production of Ag85B-ESAT6 with a C-terminally fused DC-binding peptide, with the Lp_1261 lipo- anchor signal sequence; SH71 replicon	This work
pLp_3050-Ag85B-ESAT6	pSIP401-derivative for production of Ag85B-ESAT6 with the Lp_3050 signal sequence	R. Tjåland (2011)
pLp_3050-Ag85B-ESAT6cwa2	pSIP401-derivative for production of Ag85B-ESAT6 with the Lp_3050 cell wall anchor signal sequence	R. Tjåland (2011)
pLp_3050-DC-Ag85B-E6	pSIP401-derivative for production of Ag85B-ESAT6 with a N-terminally fused DC-binding peptide, with the Lp_3050 signal sequence	This work
pLp_3050-DC-Ag85B-E6-cwa2	pSIP401-derivative for production of Ag85B-ESAT6 with a N-terminally fused DC-binding peptide, with the Lp_3050 cell wall anchor signal sequence	This work
pSIP411	pSIP401-derivtive with GusA as reporter gene; SH71 replicon	Sørvig et al. (2005)

2.7 Kits

Kits	Supplier
iBlot^{TM} Dry Blotting system	Invitrogen
Blotting roller	
iBlot^{TM} Gel Transfer Stack, Regular and Mini	
iBlot^{TM} Cathode Stack, top	
iBlot^{TM} Anode Stack, bottom	
iBlot^{TM} Disposable sponge	
iBlot^{TM} Filter paper	
iBlot^{TM} Gel Transfer Device	
Mini Trans-Blot® Electrophoretic Transfer Cell	Bio-Rad
2 gel holder cassettes	
4 fiber pads	
Modular electrode assembly	
Blue cooling unit	
Lower buffer chamber	
Lid with cables	
NucleoSpin® Plasmid miniprep Kit	Macherey-Nagel
NucleoSpin® Plasmid Columns	
Collection tubes 2ml	
Resuspension Buffer A1	
Lysis Buffer A2	
Wash Buffer AW	
Wash Buffer A4	
Elution Buffer AE	
RNase A	
NucleoSpin® Extract II	Macherey-Nagel
NucleoSpin® Extract II Columns	
Collection tubes (2ml)	
Binding Buffer NT	
Wash Buffer NT3	
Elution Buffer NE	
Novex® NuPAGE® SDS-PAGE Gel System	Invitrogen
NuPAGE® Bis- Tris Gels 10% 8 cm x 8 cm x 1 mm, 10 wells	

NuPAGE® LDS Sample Buffer (4x)
NuPAGE® Reducing Agent (10x)
NuPAGE® Mops Running Buffer

Pellet Paint® Co- Precipitant Merck
Pellet Paint® Co- Precipitant
3 M sodium acetate, pH 5.2

Qubit® dsDNA BR Assay Kit Invitrogen
Qubit™ Assay tubes
Qubit™ dsDNA BR buffer
Qubit™ dsDNA BR reagent
Qubit™ dsDNA BR standard 1 and 2
Qubit™ fluorometer

SNAP i.d.® Protein detection system Millipore
SNAP i.d. Single Well Blot Holder
SNAP i.d. Spacer
SNAP i.d. Blot Roller

SuperSignal® West Pico Chemiluminescent Substrate Pierce
Luminol/Enhancer Solution
Stable Peroxide Solution

Zero Blunt® TOPO® PCR Cloning Kit Invitrogen
pCR™-BluntII-TOPO® vector
Salt solution, 1.2 M NaCl, 0.06 M $MgCl_2$
dNTP Mix
M13 Forward Primer
M13 Reverse Primer
OneShot TOP10 chemically competent *E.coli* cells

2.8 Agars and media

Media	Supplier
GM17	Oxoid

Medium
- 37.25 g M17
- dH_2O to 1 litre
- Sterilized in a certoclav for 15 min at 115°C
- After sterilization, the medium was cooled to ~60 °C before adding 0.5 % glucose (sterile filtered using 0.2 µm pore size)

Agar
- M17 medium with 1.5 % (w/v) agar
- After sterilization, the medium was cooled to ~60 °C before adding 0.5 % glucose (sterile filtered using 0.2 µm pore size) and appropriate antibiotics; subsequently, the medium was poured into petri dishes

Media	Supplier
BHI (Brain-Heart-Infusion)	Oxoid

Medium
- 37g BHI
- dH_2O to 1 litre
- Sterilized in a certoclav for 15 min at 115°C

Agar
- BHI medium with 1.5 % (w/v) agar
- After sterilization, the medium was cooled to ~60 °C before adding appropriate antibiotics; subsequently, the medium was poured into petri dishes

Media	Supplier
MRS (de Man, Rogosa, Sharpe)	Oxoid

Medium
- 52 g MRS
- dH_2O to 1 litre
- Sterilized in a certoclav for 15 min at 115°C

Agar
- MRS medium with 1.5 % (w/v) agar
- After sterilization, the medium was cooled to ~60 °C before adding appropriate antibiotics; subsequently, the medium was poured into petri dishes

MRSSM medium

5.2 g MRS
17.1 g Sucrose (0.5 M)
2.0 g $MgCl_2$ x $6H_2O$ (0.1 M)
dH_2O to 100 ml
Sterile filtrated using 0.2 µm pore size

RPMI-1640

500 ml 1 x RPMI-1640	PAA The Cell Culture Company
5 ml 100 mM Sodium pyruvate	PAA The Cell Culture Company
5 ml 100 x Non.-ess. amino acids	PAA The Cell Culture Company
50 ml Inactivated fetal calf serum (FCS)	PAA The Cell Culture Company
(250 µl 50 mg/ml Gentamicin)	Sigma

SGM17 medium

GM17 medium
0.5 M sucrose (sterile filtered using 0.2 µm pore size)

S.O.C medium

2 g Bacto™ Tryptone	BD (Becton, Dickinson and Company)
0.5 g Bacto™ yeast extract	BD (Becton, Dickinson and Company)

0.057 g NaCl
0.019 g KCl
0.247 g $MgSO_4$
60 ml dH_2O
Sterilized in certoclav for 15 min at 115 °C
After cooling to room temperature, the following was added:
1ml 2 M glucose (sterile filtered using 0.2 µm pore size)
Sterile dH_2O to 100 ml

2x TY medium

15 g Bacto Tryptone
10 g Bacto yeast extract
5 g NaCl
dH_2O to 1 litre
Sterilized in a certoclav for 15 min at 115 °C

2.9 Buffers and solutions

Buffer/ solution	Content
GTE	50 mM glucose 25 mM Tris-HCl, pH 8 10 mM EDTA, pH 8
PBS (Phosphate Buffered Saline), pH 7.4	8 g/l NaCl 0.2 g/l KCl 1.44 g/l Na_2PO_4 0.24 g/l KH_2PO_4
TAE, 50 x	242 g Tris Base 57.1 ml acetic acid 100 ml 0.5 M EDTA, pH 8 dH_2O to 1 l
TBS (Tris Buffered Saline), pH 7.4	150 mM NaCl 10 mM Tris-HCl, pH 8
TTBS	TBS 0.1 % (v/v) Tween 20

3. METHODS

3.1 Growing of bacteria

Bacteria were cultured in liquid media or grown on agar plates. Antibiotics were added to the media in order to select for bacteria containing plasmids with an antibiotic resistance gene.

E. coli was cultured overnight in BHI either in liquid media at 37 °C with shaking or on agar plates at 37 °C. For *E. coli* harbouring pSIP-derivatives 200 µg/ml erythromycin was added to the media for selection. For *E. coli* harbouring the TOPO-vector, 100 µg/ml and 50 µg/ml kanamycin was added to liquid and solid media, respectively.

L. plantarum was cultured overnight in liquid or solid MRS medium at either 30 °C or 37 °C without shaking. When grown on solid media the incubation time was extended to two days. 10 µg/ml erythromycin was added to the medium for selection.

L. lactis was cultured overnight in liquid or solid GM17 medium at 30 °C without shaking. When grown on solid media the incubation time was extended to two days. 10 µg/ml erythromycin was added to the medium for selection.

3.2 Storage of bacteria

For long term storage of bacteria, the bacteria were kept as glycerol stocks at -80 °C. The glycerol prevents the cells from bursting when frozen.

Materials:

Bacteria

Culture media

85 % (v/v) glycerol

Procedure:

The bacteria were cultured overnight in an appropriate growth medium. 1 ml of the culture was transferred to a 1.5 ml cryovial, 300 µl 85 % (v/v) sterile glycerol was added and after inverting the vial a few times for mixing it was kept in a -80°C freezer.

To cultivate cells from glycerol stocks, a toothpick was used to scrape off some of the frozen culture, which was then transferred to a tube or flask containing appropriate growth medium.

3.3 Plasmid isolation from bacteria

The NucleoSpin® Plasmid miniprep Kit was used for isolation of plasmids from bacteria. When isolating plasmid from *L. lactis*, which is a gram positive bacterium, the bacteria were first pre-treated with lysozyme to break down the cell wall, as described in section 3.3.2.

<u>Materials:</u>

NucleoSpin® Plasmid miniprep Kit, Macherey-Nagel (section 2.7)

<u>Procedure:</u>

The isolation was carried out according to the protocol provided by the supplier of the kit

1. 1-5 ml of the overnight bacterial culture was centrifuged in an eppendorftube at 11000 x g for 30 sec at room temperature and the supernatant was discarded.
2. The resulting cell pellet was resuspended in 250 µl of Buffer 1A.
3. The cells were lysed by adding 250 µl of Buffer A2. The tube was inverted 6-8 times and then incubated for up to 5 min at room temperature or until the lysate appeared clear.
4. 300 µl of Buffer A3 was added to precipitate cell debris and the tube was inverted 6-8 times. The solution was then centrifuged at 11000 x g for 5min at room temperature to pellet the precipitate.
5. A NucleoSpin® Plasmid Column was placed in a collection tube and the supernatant was transferred to the column. The column was centrifuged at 11000 x g for 1 min to flush the supernatant through the column and bind the DNA to the membrane in the column.
6. The flow through was discarded and the column placed back in the collection tube. The column was washed with 500 µl of Buffer AG and recentrifuged. (When isolating plasmid from *L. lactis* or *E. coli* TG1, Buffer AG was preheated to 50 °C before addition.)
7. 600 µl of Buffer A4 was added to the column, which was then recentrifuged. The flowthrough was discarded and the column was put back in the empty collection tube and centrifuged for 2 min to get rid of residual ethanol.

8. The column was placed in a clean eppendorf tube and 50µl of Buffer AE was added followed by incubation for 1 min at room temperature. The column was then centrifuged at 11000 x g for 1 min. (When isolating plasmid from *L. lactis* or *E.coli* TG1, Buffer AE was preheated to 70 °C before addition)
9. The eluted plasmid was stored at -20 °C.

3.3.1 Treatment of plasmid DNA isolated from *E. coli* TG1

Plasmid DNA isolated from *E. coli* TG1 was treated with trypsin after plasmid isolation to degrade nucleases and prevent loss of DNA.

Materials:

Trypsin

Isolated plasmid

Procedure:

1. 2 µl trypsin was added to 50 µl of plasmid immediately after plasmid purification. The solution was incubated in a water bath at 37 °C for 1.5 hour.
2. After the incubation the solution was heated to 90 °C for 10 min to denature the trypsin.
3. The plasmid was stored at -20 °C.

3.3.2 Isolation of plasmids from *L. lactis*

Materials:

GTE buffer

Lysozyme 100 mg/ml

Mutanolysin 5000 U/ml in 0.1 M K-phosphate buffer, pH 6.2. Sterile filtered.

RNase 10 mg/ml, boiled for 10 min and cooled down before use.

Procedure:

1. 5-10 ml of the bacterial culture was centrifuged at 5200 x g for 7 min to collect the cells.
2. The supernatant was discarded and the cells were washed with 500 µl GTE buffer and recentrifuged; the supernatant was discarded.

3. The cell pellet was resuspended in 100 µl GTE buffer, 120 µl lysozyme, 25 µl RNase and 5 µl mutanolysin. The mixture was incubated in a water bath at 37 °C for 1 hour;during the incubation the mixture was vortexed occasionally.
4. The rest of the isolation was done as described in section 3.3 from step 3 (addition of buffer A2).

3.4 Nucleic acid precipitation with Pellet Paint® Co- Precipitant

In some reactions a higher concentration of plasmid was needed, for example before restriction enzyme digestion or ligation. In order to increase the concentration, precipitation of DNA using Pellet Paint® was performed after plasmid purification or after extraction of digested DNA from agarose gels.

Materials

Pellet Paint® Co- Precipitant
3 M sodium acetate, pH 5.2
96 % ethanol
70 % ethanol

Methods

1. 2 µl of room tempered Pellet Paint and 0.1 volume of room tempered 3 M sodium acetate were added to the sample of DNA and the sample was mixed well.
2. 2 volumes of 96 % ethanol were added followed by incubation for 2 min at room temperature.
3. The solution was then centrifuged at 16 000 x g for 5 min at 4 °C to pellet the precipitated DNA and the supernatant was aspired.
4. The pink pellet was washed with 500 µl 70 % ethanol and recentrifuged.
5. The supernatant was aspired and the pellet was dried in a vacuum centrifuge for 5 min to remove residual ethanol.
6. An appropriate amount of sterile dH_2O was used to dissolve the dried pellet
7. The DNA solution was either used directly or stored at -20 °C.

3.5 PCR

The Polymerase Chain Reaction is a method that is used for *in vitro* amplification of a specific DNA fragment. This method relies on the use of thermo-cycling. When heated, the DNA strands in double-stranded DNA will separate and can be used as templates for synthesis of a new strand. When the temperature is lowered, specific oligonucleotides (primers) bind to the single stranded DNA and serve as primers for a thermally stable polymerase which extends the strand by incorporating deoxynucleotides (dNTPs). This cycle of heating, annealing and extension may be repeated in a thermal cycling device, leading to an exponential increase in the amount of synthesized fragments.

3.5.1 Phusion High-Fidelity DNA polymerase

Phusion High-Fidelity DNA polymerase has a very low error rate and was therefore used for all PCR-amplification of DNA fragments that were to be cloned.

Materials:
Phusion High Fidelity DNA polymerase
5 x Phusion HF buffer
dNTP mix
Primers (see materials, section 2.5)

Procedure:

1. The PCR reactions were carried out as suggested by the suppliers of the Phusion High-Fidelity DNA polymerase (NEB). The reactants were mixed in a sterile PCR tube (0.2 ml) as shown in table 3.1 (Reactions were mixed in the order indicated in the table, i.e. water first). The PCR tube was kept on ice during the mixing.

Table 3.1. Phusion PCR reactants

Reactants	Volume (µl)	Final conc.
H_2O	To 50	
5x phusion HF buffer	10	1x
10mM dNTP mix	1	200 µM
Forward primer	1	0.5 µM
Reverse primer	1	0.5 µM
Template	0.5	
Phusion DNA polymerase	0.5	0.02 U/µl

2. The reaction mixtures were put in a thermal cycler and the settings in table 3.2 were applied.

Table 3.2. Phusion cycling program

Program	Temperature (^oC)	Time (sec)	Cycles
Initial denaturing	98	30	1
Denaturing	98	5	25
Annealing	50*	20	25
Elongation	72	30	25
Final elongation	72	5	1

*** The annealing temperature was varied and depended on the primers that were used**

3.5.2 VWR Red Taq DNA Polymerase Master Mix

The VWR Red Taq DNA Polymerase Master Mix was used when doing colony PCR to check for the presence of correct plasmids. The VWR Red Taq DNA Polymerase Master Mix contains an inert red dye and a stabilizer so that it is possible to apply the PCR product directly on to a gel for analysis, without having to add a loading dye.

Materials:

Taq Master Mix RED

Primers (see materials, section 2.5)

Procedure:

1. A colony was picked up from the agar plate using a sterile toothpick and a small clump of cells was transferred directly to a sterile 0.2 ml PCR tube.
2. The tube was kept on ice and the reactants listed in table 3.3 were added and mixed gently by pipetting up and down.

Table 3.3. Red Taq PCR reactants

Reactants	Volume (µl)	Final conc.
Taq Master Mix RED	25	1x
Forward primer	1	0.1-1.0 µM
Reverse primer	1	0.1-1.0 µM
Destilled Water	23	
Template DNA	Variable	

3. The tubes were put in a thermal cycler and the settings in table 3.4 were applied.

Table 3.4. Red Taq cycling program

Program	Temperature (°C)	Time (min)	Cycles
Initial denaturing	94	3	1
Denaturing	94	1	30
Annealing	55*	2	30
Elongation	72	3	30
Final elongation	72	10	1

*** The annealing temperature was varied and depended on the primers that were used**

3.6 Agarose gel electrophoresis

Agarose gel electrophoresis is a method that is used to separate DNA fragments according to size. The fragments are separated by the use of an electric current that moves the fragments through the agarose gel towards the positive pole; small fragments migrate faster than larger ones. To separate fragments >200 bp a 1.2 % agarose gel was used, for fragments smaller than 100 bp a 4 % agarose gel was used.

Materials:

SeaKem® LE Agarose (for 1.2% gels)
NuSieve®GTG®Agarose (for 4% gels)

1 x TAE Buffer (section 2.9)
10 mg/ml ethidium bromide
Loading dye
DNA ladder

Procedure:

1. For preparing a 1.2 % agarose gel, 12 g of SeaKem® LE Agarose was dissolved in 1 l of 1 x TAE buffer. A 4 % agarose gel was prepared by dissolving 2.4 g of NuSieve®GTG®Agarose in 60 ml of 1 x TAE buffer. The solutions were sterilized in a certoclav for 15 min at 115 °C. The stock solution for 1.2 % agarose gels was kept at 60 °C, whereas 4 % gels were prepared one at the time (60 ml).
2. For making of gels, 60 ml of the solution was mixed with 1 µl 10 mg/ml ethidium bromide and then poured into a moulding tray with well combs.
3. The gel was left to harden for approximately 20 min before the combs were removed; the gel was then transferred to an electrophoresis chamber and covered with 1x TAE buffer.
4. 0.1 volume of loading dye was mixed with the DNA samples before they were loaded on the gel. An appropriate DNA ladder was loaded in at least one of the wells. The gel was run at 90 V or 60 V for 15 to 40 min

3.7 Purification of DNA and extraction of DNA from agarose gels

The NucleoSpin® Extract II kit was used both for purification of DNA treated with restriction enzymes or ligases (removal of enzymes and buffers) and for DNA extraction from agarose gels. The purification and extraction steps were carried out according to the protocol provided by the supplier of the kit

Materials

NucleoSpin® Extract II Kit, Macherey-Nagel (section 2.7)

Methods

1. 200 µl of NT1 buffer per 100 mg of agarose gel was added to the agarose piece, in an eppendorf tube. (In the case of DNA purification 2 volumes of NT1 buffer were added to 1 volume of sample)
2. The eppendorf tube was incubated in a water bath at 50 °C for 5-10 min and the tube was vortexed every 2-3 min until the gel slice was dissolved. (This step was skipped when the kit was used for DNA purification).

3. A NucleoSpin® Extract column was placed in a collection tube and the sample was loaded on to the column. The column was centrifuged at 11000 x g for 30 sec and the flow through was discarded.
4. 700 µl of buffer NT3 was added to the column before centrifugation at 11000 x g for 30 sek and discarding the flow through.
5. The column was dried by centrifugation at 11000 x g for 1 min and then placed into a clean eppendorf tube.
6. 15-30 µl of buffer NE was added to the dry column and after incubation at room temperature for 1 min the DNA was eluted from the column by centrifugation at 11000 x g for 1 min.
7. The eluted DNA was used directly or stored at -20 °C

3.8 Restriction enzyme digestion of DNA and ligation of DNA fragments

Restriction enzyme digestion of DNA is a method used for cleaving double stranded DNA at specific restriction sites. Restriction enzymes make cuts in DNA at sites containing a particular sequence. The two DNA strands can be cut at exactly the same position, generating blunt ends, or at slightly different positions, generating overhangs of a few bases, the so-called sticky ends. The reaction conditions needed for the enzymes to work optimally differ between the enzymes. The digesting reaction has to be carried out at a certain temperature and with a specific buffer adjusted to the enzyme used. Some restriction enzymes additionally require bovine serum albumin (BSA) in the mixture in order to digest efficiently. If there is a need to use multiple restriction enzymes having incompatible reaction conditions, the reaction must be carried out in multiple steps. A purification step to remove the previous enzyme and buffer has to be done before addition of the next enzyme and buffer.

3.8.1 Restriction enzyme digestion of DNA

Restriction enzyme digestion was performed according to the following protocol. The buffer used in the reaction depended on the type of restriction enzyme used and was selected based on the supplier's specifications.

Materials:

DNA to be cut	
Restriction enzyme/enzymes	(section 2.3)
10 x restriction buffer	(section 2.3)
10 x BSA	(if needed)

Procedure:

1. DNA was mixed with dH_2O in a 0.2 ml PCR tube (the amount of DNA used depended on the experiment). The reaction volume was usually 50 µl.
2. 1/10 volume of the appropriate restriction buffer was added to the tube. If required 1/10 volume of 10x BSA was added.
3. At most 1/10 volume of restriction enzyme was added to the mixture and the tube was then placed in a water bath holding the appropriate temperature (normally 37 °C). The reaction was left in the water bath for 1-2 hours.
4. When a two step restriction cutting was performed the restriction buffer and enzyme from the first reaction were removed by using the NucleoSpin® Extract II Kit (procedure described in section 3.7)
5. To separate the generated DNA fragments, the reaction mixture was run on an agarose gel. If the digestion was done to prepare a DNA fragment for cloning a small piece of agarose containing the desired fragment was excised from the gel after the completion of the electrophoresis run.
6. The NucleoSpin® Extract II kit was used to extract the fragment from the gel, as described in section 3.7.

3.8.2 Determination of DNA concentration

The concentration of DNA after purification was determined using the Qubit® dsDNA BR Assay Kit.

Materials

Qubit™ Assay tubes
Qubit™ dsDNA BR buffer
Qubit™ dsDNA BR reagent
Qubit™ dsDNA BR standard 1 and 2
Qubit™ fluorometer

Methods

1. A stock solution of 200 µl per sample was made by diluting the Qubit^{TM} reagent 1:200 in Qubit^{TM} buffer.
2. 190 µl of the stock solution was added to 10 µl of each of the Qubit^{TM} standards. The solution was vortexed and incubated for 2 min at room temperature.
3. 199 µl of the stock solution was added to 1 µl of each of the samples. The solution was vortexed and incubated for 2 min at room temperature.
4. The Qubit^{TM} fluorometer was equilibrated using the prepared standards and then the DNA concentrations in each sample were determined.

3.8.3 Ligation of DNA fragments

Ligation of DNA fragments is done by the use of DNA ligase. DNA ligase is an enzyme that joins DNA strands by forming a phosphodiester bond between the 5’ phosphate- and 3’ hydroxyl- ends of adjacent DNA bases. The enzymatic reaction uses ATP and the cofactor Mg^+, which are both supplied in the ligation buffer. Both blunt and sticky end DNA fragments can be joined by T4 DNA ligase and Quick T4 DNA ligase.

3.8.3.1 T4 DNA ligation

Materials:

DNA to be ligated (fragment + vector)
10 x T4 Ligation Buffer
T4 DNA Ligase

Procedure:

1. The ligation was carried out according to the protocol provided by the supplier of the ligase enzyme (NEB), in 1.5 ml eppendorf tubes in a total volume of 20 µl. Usually, approximately 50 ng of vector and 3 times more insert were used.
2. 2 µl of 10 x T4 Ligation Buffer and 1 µl of T4 DNA Ligase were added to the mixture and after adjusting the volume to 20 µl with dH_2O, the mixture was vortexed briefly. The mixture was incubated over night at 16 °C.
3. The reaction mixture was either stored at -20 °C or transformed directly into bacteria.

3.8.3.2 Quick T4 DNA ligation

Materials:

DNA to be ligated (fragment + vector)

2 x Quick Ligation Buffer

Quick T4 DNA Ligase

Procedure:

1. The ligation was carried out according to the protocol from the supplier of the ligase enzyme (NEB) Approximately 50 ng of vector and 3 times more insert was added to a 1.5 ml eppendorf tube.
2. 10 µl of 2 x Quick Ligation Buffer was added to the mixture and after adding 1 µl of Quick T4 DNA Ligase the reaction mixture was incubated at room temperature for 5 min, before it was placed on ice.
3. The reaction mixture was either stored at -20 °C or transformed directly into bacteria.

3.9 Drop dialysis

Drop dialysis is an easy way of removing salt ions from ligation mixtures. The removal of salt ions is necessary when the ligation mixture is to be used in electroporation. High salt concentrations may lead to electrical discharge which again leads to reduced viability of the bacterial cells.

Materials:

MF-Millipore™ Membrane Filters, 0.025 µm pore size

TE-buffer

Ligation mixture

Methods:

1. TE-buffer was poured into petri dishes and membrane filters were gently applied to the surface of the buffer.
2. The ligation mixture was applied as a droplet onto a membrane filter and incubated at room temperature for 30 min.
3. The ligation mixture was then collected by a pipette and was ready to use in electroporation.

3.10 Preparation of competent bacterial cells

Competent bacterial cells are cells that are able to take up plasmid from the extracellular environment. In gene technology, DNA uptake is promoted either by applying an electric pulse or by exposing the bacteria to a heat shock. Bacterial cells can become competent in the exponential growth phase and are therefore harvested when they are in this particular phase.

3.10.1 Preparation of chemically competent *Escherichia coli* TOP10

Materials:

E. coli TOP10
TY medium
0.05M $CaCl_2$
0.05M $CaCl_2$ with 15 % glycerol

Procedure:

1. *E. coli* TOP10 from a glycerol stock was grown over night in 5ml TY medium at 37 ^{o}C with shaking.
2. The culture was transferred to 200 ml of prewarmed TY medium and grown at 37 ^{o}C until the OD_{600} reached ~0.5.
3. The culture was transferred to a centrifuge tube and placed in an ice bath for 10 min. Then the culture was centrifuged at 4000 rpm, at 4 ^{o}C for 10 min.
4. The supernatant was discarded and the cell pellet was resuspended in 10 ml ice cold 0.05M $CaCl_{2,}$. After incubation on ice for 15 min, the tube was centrifuged at 4000 rpm, at 4 ^{o}C for 10 min.
5. The supernatant was discarded and the cell pellet was resuspended in 10 ml ice cold 0.05M $CaCl_2$ with 15 % glycerol, and then kept in an ice bath for 10 min.
6. The cell suspension was aliquoted in eppendorf tubes (200 µl in each tube) and then immediately frozen at -80 ^{o}C.

3.10.2 Preparation of electrocompetent *Lactobacillus plantarum* WCFS1

Electrocompetent *L. plantarum* and *L. lactis* cells were made by growing them in a medium containing glycine. The incorporation of glycine in the cell wall of these bacteria makes the cell wall more permeable for uptake of plasmids. The procedure was done in accordance to the protocol described by Aukrust et al. (1995).

<u>Materials:</u>

L. plantarum
MRS medium
MRS + 1 % glycine
30 % PEG_{1450}
MRS + 0.5 M sucrose + 0.1 M $MgCl_2$

<u>Procedure:</u>

1. *L. plantarum* from glycerol stock was cultured over night in 10 ml MRS at 37 °C.
2. The overnight culture was used to make a serial dilution (from 10^{-1} to 10^{-10}) in MRS + 1 % glycine and these fresh glycine-containing cultures were incubated over night at 37 °C
3. The next day, 1ml of the culture with an OD_{600} of 2.5 ± 0.5 was further diluted in 20 ml MRS + 1 % glycine. The diluted culture was then grown until it reached the logarithmic phase (OD of 0.7 ± 0.07) and then placed on ice for 10 min.
4. The culture was centrifuged at 4500 rpm at 4 °C for 10 min. The supernatant was discarded and the pellet resuspended in 5 ml ice cold 30 % PEG_{1450}. After adding an additional 20 ml ice cold 30 % PEG_{1450} the tube was inverted gently and placed on ice for 10 min.
5. The cells were collected by centrifugation at 4500 rpm at 4 °C for 10 min and the pellet was resuspended in 400 µl ice cold 30 % $PEG_{1450.}$
6. 40 µl portions of the cells were pipetted into sterile eppendorf tubes and immediately frozen. The cells were stored at -80 °C.

3.10.3 Preparation of electrocompetent *Lactococcus lactis* MG1363

The procedure was done in accordance to the protocol by Holo and Nes (1989).

Materials:

GM17 medium

SGM17 medium

Glycine 20 %

0.5 M sucrose

0.5 M sucrose with 10 % glycerol

Procedure:

1. *Lactococcus lactis* was grown over night in 10 ml GM17 medium not containing antibiotics, at 30 °C.
2. The overnight culture was diluted 100 times in 10 ml GM17 and incubated at 30 °C until the OD_{600} was ~0.5.
3. 100 µl portions of this culture were then used to start a series of new 10 ml cultures in SGM17 medium with different concentrations of glycine (see Table 3.5). These cultures were incubated overnight at 30 °C.

Table 3.5. Overview of SGM17 dilution series

Glycin concentration (%)	20 % glycine (µl)	SGM17 (µl)
2.4	1200	8800
2.6	1300	8700
2.8	1400	8600
3.0	1500	8500
3.2	1600	8400
3.4	1700	8300
3.6	1800	8200
3.8	1900	8100
4.0	2000	8000

4. The next day, the OD_{600} was measured in all the cultures the culture with the highest concentration of glycine and an OD_{600} between 0.3 and 0.4 was selected.
5. This culture was chilled on ice for 5 min before the cells was harvested by centrifugation at 5500 x g for 5 min at 4 °C.

6. The supernatant was discarded and the cells were washed with 15 ml ice cold 0.5 M sucrose and collected by centrifugation at 5500 x g for 2 min at 4 °C. The supernatant was discarded and the washing step was repeated 2 times.
7. After the last washing step, the cell pellet was resuspended in 350 µl ice cold 0.5 M sucrose containing 10 % glycerol. The cells were added to eppendorf tubes in 40 µl portions and immediately frozen.
8. The cells were stored at -80 °C.

3.11 Transformation of chemically competent *E. coli* TOP10

Materials:

Competent *E.coli* TOP10

Plasmid

Soc medium

BHI agar plates with 200 µg/ml erythromycin

Procedure:

1. A portion of thawed chemically competent *E.coli* TOP10 cells was transferred to a 14 ml Falcon 2059 Polypropylene Round Bottom tube. 20 µl of cold ligation mixture or 1-5 µl of plasmid solution was added to the cells and the mixture was incubated on ice for 30 min.
2. The tube was placed in a water bath holding 42 °C for 1 min for heat shocking of the cells.
3. The tube was immediately placed on ice for 2 min.
4. 250 µl of room tempered S.O.C. medium was added to the cells followed by incubation for approximately 1 hour at 37 °C with shaking.
5. The cells were then spread on agar plates and the plates were incubated at 37 °C over night.

3.12 Transformation of electrocompetent *Lactobacillus plantarum* and *Lactococcus lactis*

The electroporation settings and the growth conditions used for recovering and growing transformants are summarized in table 3.6.

Materials:

Agar plates with appropriate antibiotic
Bio-Rad GenePulser® II
Bio-Rad Pulse controller plus
Electrocompetente cells (*L. plantarum* or *L. lactis*)
Electroporation cuvette
Ligation mixture
MRSSM / SGM17 media

Procedure:

1. 20 µl of ice cold desalted (see section 3.9) ligation mixture or 1-5 µl of plasmid solution were added to 40 µl fresh or freshly thawed competent cells and mixed carefully.
2. The cell mixture was immediately transferred to a cold electroporation cuvette, which was then inserted into the electroporation apparatus and given the electric pulse.
3. Preheated media was directly transferred to the cuvette and the mixture was then transferred to a sterile eppendorf tube for incubation.
4. The cell suspension was spread on agar plates, normally100 µl on each plate.

Table 3.6. Overview of the electroporation settings and growth conditions during and after electroporation

Bacterial strain	Electroporation settings	Media and incubation after electroporation	Agar plates and incubation
L. plantarum	Capacitance: 25 µF Voltage: 1.5 kV Resistance: 400 ohm	950 µl MRSSM 2-4 hours at 37 °C	MRS plates with 10 µg/ml erythromycin 2-3 days at 37 °C
L. lactis	Capacitance: 25 µF Voltage: 2.0 kV Resistance: 200 ohm	700 µl SGM17 2-4 hours at 30 °C	GM17 plates with 10 µg/ml erythromycin 2-3days at 30 °C

3.13 TOPO-cloning and transformation

TOPO-cloning is used for the insertion of a blunt end PCR-product into a plasmid-vector. Topoisomerase I is an enzyme whose natural role is to cleave and rejoin DNA during replication. The topoisomerase in the Zero Blunt® TOPO® PCR Cloning Kit is a topoisomerase from *Vaccinia* virus which recognizes the sequence 5'-(C/T)CCTT-3'. The topoisomerase cleaves one DNA strand in the 3' end and forms a covalent bond with the phosphate group on the 3' end. Attack of this bond by a 5' hydroxyl group leads to reversing of the reaction and ligation of the two DNA strands. The TOPO® vectors are linearized and contain bound topoisomerase and are thus ready to join PCR product and vector. Fig. 3.1 illustrates the procedure.

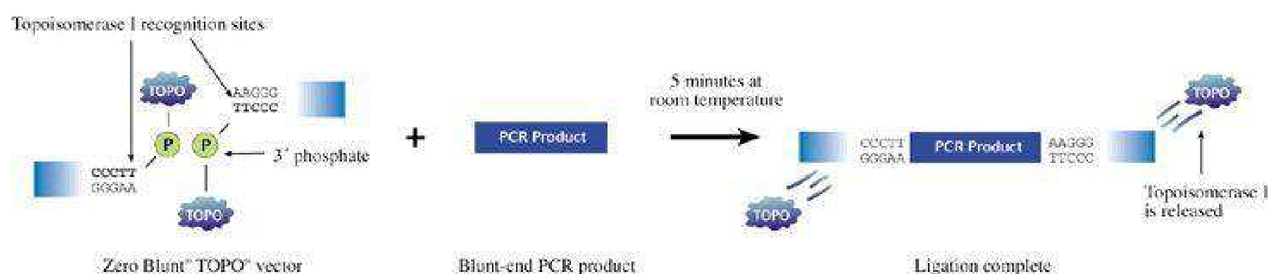


Figure 3.1. Illustration of the TOPO cloning reaction. The figure is taken from the homepage of Invitrogen™, the supplier of the Zero Blunt® TOPO® PCR Cloning Kit. http://www.invitrogen.com/site/us/en/home/brands/Product-Brand/topo/The-Technology-Behind-TOPO-Cloning.html

Materials

Zero Blunt® TOPO® PCR Cloning Kit

pCR™-BluntII-TOPO® vector

Salt solution, 1.2 M NaCl, 0.06 M $MgCl_2$

OneShot TOP10 chemically Competent *E. coli* cells

S.O.C. medium

BHI agar plates with 50 µg/ml kanamycin

Methods

Table 3.7. Reagents in the TOPO®-cloning reaction

Reagent	Volume
PCR-product	2 µl
Salt solution	1 µl
dH_2O	2 µl
pCR™-BluntII-TOPO® vector	1 µl

1. The reagents listed in table 3.7 were mixed and incubated at room temperature for 5 min.
2. Portions of chemically competent *E. coli* TOP10 cells were thawed and transferred to cold Falcon tubes; 2 µl of the TOPO®-cloning reaction was added to each tube and the cells were then incubated on ice for 30 min.
3. The tube was heat shocked at 42 °C for 1 min before immediately cooling on ice for 2 min.
4. 250 µl of S.O.C medium was added to the cells, followed by incubation for approximately 1 hour at 37 °C with shaking.
5. The cell suspension was spread on BHI agar plates containing 50 µg/ml kanamycin; approximately 100 µl of cell suspension were spread on each plate. The plates were incubated over night at 37 °C.

3.14 Analysis of gene products in *Lactobacillus plantarum*

Gene expression in *L. plantarum* was induced by the addition of an inducer peptide and the cells were harvested in order to analyse the production of the protein of interest.

3.14.1 Cultivation and harvesting of *L. plantarum*

Materials:

MRS medium
Erythromycin 10 mg/ml
Inducing peptide SppIP
PBS buffer
50 mM PMSF (phenylmethylsulphonyl fluoride) dissolved in isopropanol

Procedure:

1. *L. plantarum* strains were grown at 37°C over night in MRS containing 10 µg/ml erythromycin.
2. The overnight cultures were diluted in 50 ml prewarmed MRS to an OD_{600} of 0.13-0.15.
3. The cultures were further incubated at 37°C to an OD_{600} of 0.3 when they were induced with 25 ng/ml (final concentration) of the inducing peptide SppIP.
4. After 2 hours incubation the cultures were chilled on ice for 10 min to stop the growth.

5. Harvesting of cells was done by centrifugation at 5000 x g for 10 min at 4 °C.
6. After addition of 1 mM PMSF the supernatant was transferred to a new 50 ml tube and frozen at -20 °C for further analysis.
7. The resulting cell pellet was washed twice with ice cold 1 x PBS and recentrifuged. The pellet was either frozen at -20 °C or directly disrupted with glass beads.

3.14.2 Cell disruption by glass beads

Harvested cells of *L. plantarum* were disrupted by glass beads to prepare a cell free protein extract for analysis of intracellular and surface proteins.

Materials:

PBS buffer

50 mM PMSF (phenylmethylsulphonyl fluoride) dissolved in isopropanol

FastPrep tubes

Glass beads (106 microns and finer)

Procedure:

1. The cell pellet was resuspended in 1 ml of ice cold 1 x PBS and 1mM PMSF and transferred to a cold FastPrep tube containing 1.5 g of glass beads.
2. The FastPrep tube was placed in a FastPrep® FP120 Cell Disrupter and shaken at a speed of 6.5 m/s for 45 sec.
3. The tube was then centrifuged at 16100 x g for 5 min at 4 °C and the supernatant was transferred to a clean eppendorf tube.
4. The eppendorf tube was recentrifuged to remove the remaining glass beads and the supernatant was transferred to a new eppendorf tube.
5. The cell free protein extracts were frozen at -20 °C or analysed directly.

3.14.3 TCA precipitation of proteins in culture supernatants

In order to analyse the proteins in the culture supernatant of *L. plantarum* the proteins were precipitated using trichloroacetic acid (TCA).

Materials:
6 M NaOH
Sodium Deoxycholate
100 % (w/v) trichloracetic acid (TCA)
Acetone

Procedure:

1. The pH in the supernatant was adjusted to pH 7 with 6 M NaOH, before the addition of 0.2 mg/ml of Sodium Deoycholate. The samples were kept on ice for 30 min.
2. 1 volume of 100 % TCA was added to 4 volumes of sample and the mixture was vortexed. The mixture was placed on ice for 20 min.
3. The precipitated protein was collected by centrifugation at 16 100 x g for 5 min at 4 °C; the supernatant was discarded.
4. 200 µl of ice cold acetone was added and the tube and after mixing by vortexing the pellet was collected by centrifugation.
5. The pellet was dried in a vacuum centrifuge for 5 min to remove residual acetone.
6. The dried pellet was dissolved in 20 µl of 50 mM NaOH before analysis by SDS-PAGE.

3.15 Gel electrophoresis of proteins

Polyacrylamide gel electrophoresis (PAGE) is a method used to separate proteins according to their size. In this study the NuPAGE® Novex Bis- Tris Electrophoresis System from Invitrogen™ was used. Protein samples are mixed with LDS Sample Buffer and a Reducing Agent containing dithiothreitol (DTT). The sample buffer, which contains lithium dodecyl sulphate, an analogue of SDS, disrupts non covalent interactions in the protein while the reducing agent breaks the sulphide bonds, leaving the protein denatured. LDS also gives the protein a fixed negative charge per residue. The samples with denatured and negatively charged proteins are applied to the gel and separated by an electric current which moves the proteins towards the positive pole. Small proteins move faster in the gel than larger proteins,

which leads to separation. The size of the proteins can be estimated by comparing with a suitable protein standard. Visualization of proteins can be done by either staining the gel or doing a western blot.

Materials:

NuPAGE® Novex Bis- Tris Gels

NuPAGE® LDS Sample Buffer (4x)

NuPAGE® Reducing Agent (10x)

Procedure:

1. 7 µl of Sample Buffer and 3 µl of Reducing agent were added to 20 µl of protein sample.
2. The samples were heated at 90 °C for 10 min in order to denature proteins.
3. The electrophoresis chamber was assembled and the gel was placed in the inner chamber. The two chambers were filled with 1 x MOPS running buffer.
4. The heated samples were applied to the gel and the gel was run for 50 min at 200V.
5. After completing the run, the chambers were disassembled and the gel was transferred to a plastic tray with dH_2O before analysis by western blotting.

3.16 Western blotting

Western blotting is a method for detection of proteins in a sample by the use of antibody hybridisation. First a mixture of denatured proteins is run on a gel that separates the different proteins according to size (see section 3.15). The proteins are then transferred from the gel to a membrane by the use of an electric current ("blotting"). To prevent unspecific binding of antibodies to the membrane, the membrane is incubated in a blocking solution containing BSA which binds to potential sites of nonspecific interactions. The membrane is then incubated with a primary antibody that binds to an epitope on the protein of interest. The membrane is rinsed to remove unbound primary antibody and a secondary antibody with specificity for the primary antibody is added. This secondary antibody contains a reporter enzyme that will give a detectable fluorescent signal when an appropriate substrate is added. In the present study, two different blotting systems were used.

3.16.1 Blotting with thank transfer system

Materials:

Mini Trans-Blot® Electrophoretic Transfer Cell (section 2.7)

- 2 gel holder cassettes
- 4 fiber pads
- Modular electrode assembly
- Blue cooling unit
- Lower buffer chamber
- Lid with cables

Methanol

1 x TBS

Towbin buffer

Procedure:

1. The gel was washed in dH_2O for 15 min.
2. A PVDF-membrane and filter paper were cut in approximately the same size as the gel and the membrane was soaked in methanol.
3. The gel and the membrane were equilibrated in Towbin buffer for 15 min.
4. The filter papers and fiber pads were soaked in Towbin buffer before assembly of the gel sandwich. The assembly order is showed in figure 3.2. A blot roller was used to get rid of air bubbles trapped between the gel and the membrane.

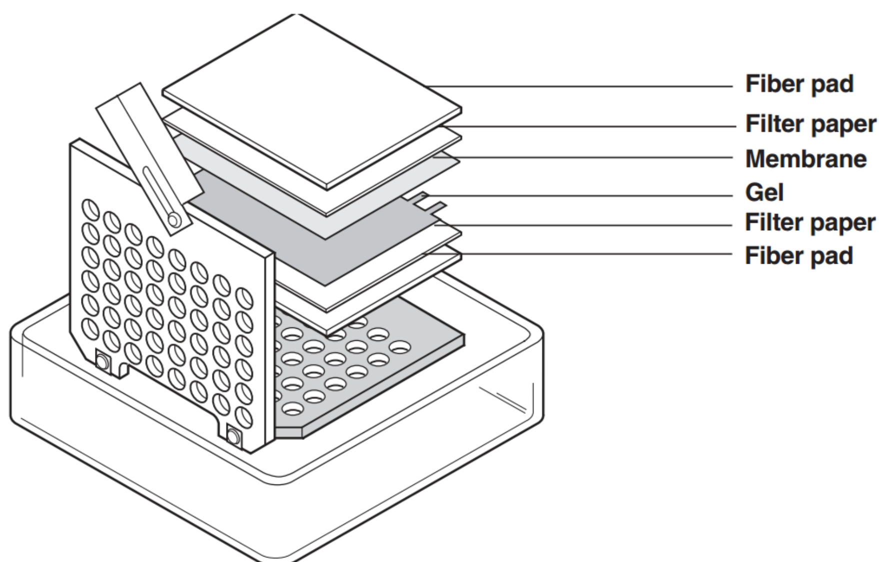


Figure 3.2. Assembly of the gel sandwich. The figure shows the assembly order of the "gel sandwitch" in the cassette. The figure is taken from the BioRad Mini Trans-Blot® Electrophoretic Transfer Cell Instruction Manual.

5. The assembled cassette was placed in the electrode module and the electrode module and a cooling unit were placed in the buffer thank.
6. The buffer thank was filled with Towbin buffer and a magnet was added for stirring.
7. Protein transfer was then accomplished by applying power (100 V) for 60 min.
8. After blotting the membrane was washed twice with 1 x TBS before immunoblotting.

3.16.2 Blotting with iBlot™ Dry Blotting system

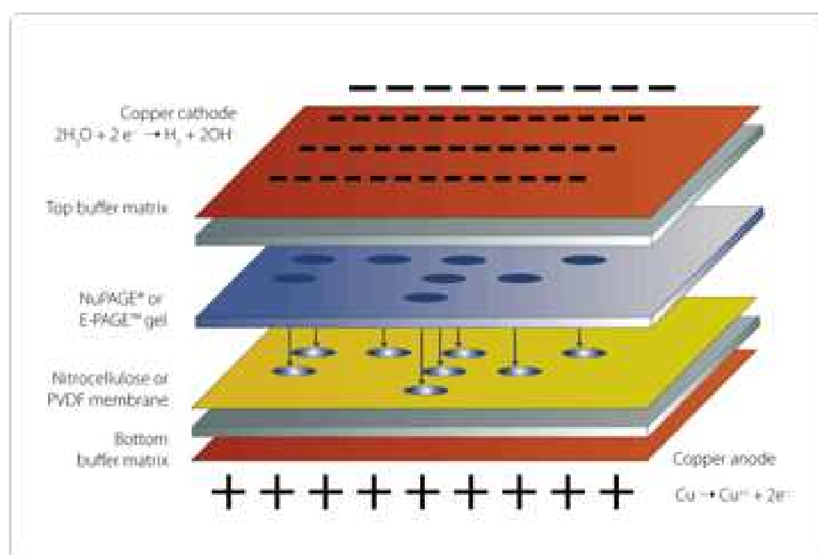


Figure 3.3. Assembly of the iBlot™ Transfer stack. The transfer stack consists of: 1. The anode stack with a copper anode, the anode buffer incorporated into a gel matrix and the nitrocellulose membrane. 2. The gel which is placed on top of the membrane and covered with a wetted filter paper. 3. The cathode stack with a copper anode and the cathode buffer incorporated into a gel matrix. The figure is taken from the iBlot Dry Blotting system user manual (Invitrogen™)

<u>Materials:</u>

iBlot™ Dry Blotting system (section 2.7.)

- Blotting roller
- iBlot™ Gel Transfer Stack, Regular and Mini
  - iBlot™ Cathode Stack, top
  - iBlot™ Anode Stack, bottom
  - iBlot™ Disposable sponge
  - iBlot™ Filter paper
- iBlot™ Gel Transfer Device

Procedure:

1. The gel was washed for 5 min in dH_2O
2. The anode stack (Fig. 3.3) (Containing the nitrocellulose membrane) was placed in the gel transfer device and the gel was placed on top of the membrane. Air bubbles trapped between the gel and the membrane were removed by using a blotting roller.
3. A filter paper wetted in dH_2O was placed on top of the gel. The cathode stack (Fig 3.3) was removed from its plastic tray and placed on top of the filter paper with the copper-electrode side facing up.
4. A disposable sponge was placed in the lid of the transfer device and the lid closed.
5. The blotting was carried out at 23 V (program 2) for 7 min.

3.16.3 SNAP i.d.® immunodetection

The SNAP i.d.® Protein detection system is a system for hybridization of antibodies to proteins on the membrane. Unlike traditional immunodetection where the membrane has to be incubated with the various reagents over a longer time period, the SNAP i.d.® Protein detection system utilizes vacuum to pull the reagents through the membrane and is thus less time consuming.

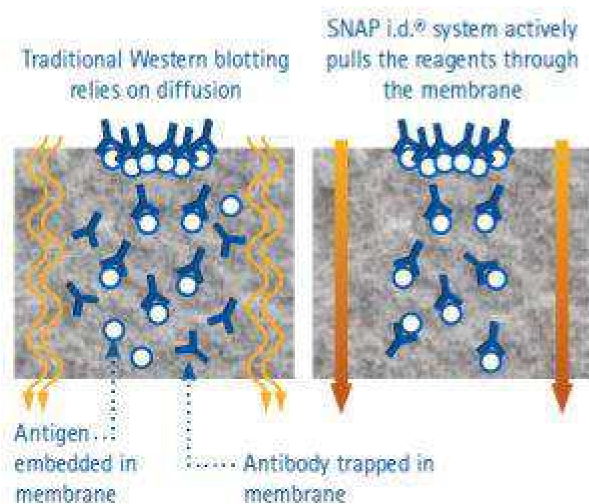


Figure 3.4. Illustration of the SNAP i.d. immunoblotting principle. The reagents applied on the surface of the membrane are actively pulled through the membrane by vacuum and does not rely on diffusion as traditional immunodetection. The figure is taken from the homepage of Millipore, the supplier of the SNAP i.d.® Protein detection system:
http://www.millipore.com/life_sciences/flx4/western_blotting&tab1=5&tab2=2#:tab1=5:tab2=2

Materials:

SNAP i.d.® Protein detection system (section 2.7)

TTBS

TTBS/ 0.5 % BSA

Blocking buffer (TTBS/ 1 % BSA)

Primary antibody ESAT6 Mouse mcAb (ab26246)

Secondary antibody HRP-Rabbit Anti-Mouse IgG

Procedure:

1. The blot holder was wetted with dH_2O and the blotted membrane was placed in the middle of the blot holder with the protein side facing down. A wetted filter paper was placed on top of the membrane and any air bubbles were removed by using a blotting roller.
2. The blot holder was firmly closed and placed in the SNAP i.d. Protein detection system device.
3. 30 ml of blocking buffer was added and the vacuum was immediately turned on. When the well had emptied the vacuum was turned off.
4. 3 ml of TTBS/ 0.5 % BSA with 2 µg/ml primary antibody was added to the well and incubated for 10 min.
5. The blot was washed 3 times with TTBS with the vacuum running continuously. After the last wash the vacuum was turned off.
6. 3 ml of TTBS/ 0.5 % BSA with 0.04 µg/ml secondary antibody was added and incubated for 10 min.
7. The washing in step 5 was repeated.
8. The membrane was removed from the blot holder for incubation with an appropriate detection agent.

3.16.4 Chemiluminescent detection of proteins

Chemiluminescent detection is used to visualize the proteins of interest on the membrane. The secondary antibody which was added during the immunoblotting is linked to a horseradish peroxidase (HRP). When a chemiluminescent substrate is added the HRP will oxidize the substrate which results in the emission of light. The emitted light can be captured on an x-ray film.

Materials:

SuperSignal® West Pico Chemiluminescent Substrate

  Luminol/Enhancer Solution

  Stable Peroxide Buffer

CL-Xposure™ Film

Developer solution

Fixing solution

Procedure:

1. The substrate working solution was made by mixing 12.5 ml of Luminol/Enhancer Solution with 12.5 ml Stable Peroxide Buffer.
2. The substrate working solution was added to the membrane in a plastic tray and the membrane was incubated with shaking for 5 min.
3. After the incubation the membrane was covered in plastic foil and placed in a lightproof film cassette with the protein side facing up.
4. The following steps were done in a darkroom.
5. CL-Xposure™ Film was cut in an appropriate size and placed on the top of the membrane; the cassette was closed and incubated for 1-15 min depending on the strength of the chemiluminescent signal.
6. The film was placed in a tray containing developer solution and incubated until the protein bands became visible.
7. The film was then transferred to a tray containing fixing solution and incubated for 2 min.
8. After the fixing the film was washed in a tray with water and air dried.

3.17 Preparation of cells for freeze drying

Materials:

1 x PBS

Freeze drying medium

Skim milk 11% (w/v)

Fructose 12.5 g/l

Procedure:

1. Bacterial cells were harvested according to step 1-5 in section 3.14.1. Approximately10^9 cells were harvested; this amount was calculated according to Fig. A1 in the appendix.
2. The cells were washed with 10 ml 1 x PBS and collected by centrifugation at 5000 x g for 10 min at 4 °C; the supernatant was discarded.
3. The cell pellet was resuspended in 1 ml cold freeze drying medium and then frozen at -20 °C before the freeze drying.
4. The cells were freeze dried and stored at room temperature in the dark.
5. Rehydration of the cells was done by adding 1 ml of sterile dH_2O.

3.18 Detection of surface antigens using a FITC-labelled secondary antibody

The FITC-labelled secondary antibody used in this study is an anti-mouse IgG antibody labelled with the fluorochrome fluorescein isothiocyanate (FITC) that binds to the primary mouse antibody that is specific for the protein of interest. In this study, detection of cells labelled with FITC was done by either flow cytometry or confocal laser scanning microscopy.

Flow cytometry detects cells when they pass through a laser beam one at a time. When a cell pass through the light beam the light is scattered in all directions and can be detected by detectors. Forward scatter gives information on the size of the cell, while sideways scatter gives information of the granularity and complexity inside the cell. When the laser beam hits the fluorochrome on a fluorochrome labelled cell it excites the fluorochrome and causes it to emit light at a specific wave length. All these data are collected and give information about subsets in the analysed cell population. Confocal laser scanning microscopy utilizes a principle similar to flow cytometry to create an image of the cells.

3.18.1 Staining cells for flow cytometry

Materials:

PBS

PBS/ 2 % BSA

Primary antibody ESAT6 Mouse mcAb (ab26246)

Secondary antibody Anti-Mouse IgG FITC

Procedure:

1. *L. plantarum* strains harbouring different plasmids were cultured and induced according to step 1-3 in section 3.14.1.
2. 2 hours after induction the cells were harvested. The amount of bacterial culture harvested was adjusted according to their OD_{600} at the time of harvest. 1 ml of bacterial culture was used for cultures with an OD_{600} of 0.5. In some of the experiments 10^9 cells of each culture were harvested; this amount was calculated according to Fig. A1 in the appendix.
3. The bacteria were harvested by centrifugation at 5000 x g for 3 min at 4 °C and the supernatant was discarded.
4. The cell pellet was washed twice with 1ml of ice cold PBS and cells were collected by centrifugation at 5000 x g for 2 min at 4 °C. All of the supernatant was aspired after the final wash.
5. Cells were resuspended in 50 µl of PBS/ 2 % BSA and 0.4 µl of primary antibody. The samples were incubated on ice for 30 min.
6. After the incubation the samples were washed 3 times with 1 ml PBS/ 2 % BSA and centrifuged between each wash at 5000 x g for 2 min at 4 °C. All of the supernatant was aspired after the final wash.
7. The samples were resuspended in 50 µl PBS/ 2 % BSA and 0.8 µl of FITC secondary antibody and incubated for 30 min on ice in the dark.
8. The samples were washed as in step 6.
9. The stained cell pellets were suspended in 100 µl of PBS and the samples were immediately analysed by MacsQuant® Analyser and the MacsQuantify™ software.

This procedure was also used for staining of cells for confocal laser scanning microscopy.

3.18.2 Staining of bacterial cells on glass slides for confocal laser scanning microscopy

Confocal laser scanning microscopy was used to visualize the bacteria and detect FITC labelled cells. The bacterial cells were stained on glass slides to achieve a higher degree of stained cells and prevent motion of the bacteria during analysis.

Materials:

PBS

PBS/ 2 % BSA

Primary antibody ESAT6 Mouse mcAb (ab26246)

Secondary antibody Anti-Mouse IgG FITC

Glass slides

Cover slips

Procedure:

1. *L. plantarum* strains harbouring different plasmids were harvested and washed as described in step 1-4 in section 3.18.1. Approximately 10^9 bacterial cells of each sample were harvested; this amount was calculated according to Fig. A1 in the appendix.
2. The cell pellet was resuspended in 2 ml of PBS and 100 µl of the suspension was put on a glass slide. A square was drawn on the glass slide using a PAP pen (the pen creates a hydrophobic barrier), before application of the sample.
3. The bacterial suspension on the glass slide was left to dry in a heating cabinet at 37 °C for 1 hour.
4. 100 µl of PBS/ 2 % BSA and 0.8 µl of primary antibody were applied on top of the dried bacterial suspension. The glass slides were placed in a plastic box together with some moist tissue to prevent the antibody solution from drying and incubated at 4 °C over night.
5. The glass slides were washed 3 times with 200 µl PBS/ 2 % BSA.
6. 100 µl of PBS/ 2 % BSA and 1.6 µl of FITC secondary antibody were applied to the samples and they were incubated at 4 °C for 2 hours in the dark.
7. After the incubation the slides were washed as in step 5. A cover slip was put on the glass slides directly after the last wash.
8. The microscopy analysis was performed on a Zeiss LSM 700 Confocal Microscope by using Zen software.

3.18.3 Treatment with lysozyme

Lysozyme degrades the peptidoglycan in the cell wall of bacteria and this was utilized in an attempt to enhance the fluorescent signal detected by flow cytometry analysis and confocal microscopy analysis.

Materials:

Lysozyme 100 mg/ml

PBS

Procedure:

1. *L. plantarum* strains harbouring different plasmids were harvested and washed as in step 1-4 section 3.18.1. Approximately 10^9 pelleted bacterial cells of each sample were used; this amount was calculated according to Fig. A1 in the appendix.
2. The cell pellet was dissolved in 300 µl of PBS followed by addition of 200 µl of lysozyme, the sample was mixed. The control sample was dissolved in 500 µl of PBS. All the samples were incubated in water bath at 37 °C for 30 min.
3. After the treatment the cells were washed 3 times with PBS and centrifuged between each wash at 5000 x g for 2 min at 4 °C.
4. The treated cells were either stained for flow cytometry (from step 5 in section 3.18.1) or stained for confocal microscopy (from step 2 in section 3.18.2).

3.19 Effects on dendritic cells

Induced *L. plantarum* strains were incubated with dendritic cells (DC) followed by evaluation of two types of responses, (1) internalization of bacteria and (2) DC maturation.

3.19.1 Isolation of CD14+ cells from human peripheral blood leukocytes (PBL)

CD14+ cells are monocytes that express the CD14 molecule on their surface. These cells were isolated from peripheral blood leukocytes (PBL) using CD14 magnetic microbeads that bind to CD14+ cells. Labelled and unlabelled cells are separated by the use of a column which is placed in a magnetic field. Cells binding magnetic beads will be retained in the column due to the magnetism, while unlabelled cells are washed out. The labelled cells are eluted when the column is removed from the magnet. CD14+ cells have the ability to develop into DCs when incubated with (commercially available) interleukin 4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Materials:

Macs CD14 MicroBeads (human)
Macs Column
Macs Separator
PBS/ 0.5 % BSA, 2 mM EDTA
RPMI-1640/ 10 % fetal calf serum (FCS) and gentamicin 25 µg/ml
IL-4
GM-CSF

Methods:

1. Tubes containing human peripheral blood leukocytes were thawed and the cells were transferred to a 50 ml cellstar tube; RPMI supplemented with 10 % FCS and gentamicin was added to a total volume of 10 ml.
2. The cell suspension was centrifuged at 1300 rpm for 10 min in a Megafuge and the supernatant was aspired.
3. 10 ml of RPMI/ 10 % FCS and gentamicin was added and cells ranging in size from 3 µm - 9 µm were counted using Colter Counter® Z1
4. The cell suspension was recentrifuged and the supernatant was aspired.

5. The cells were resuspended in 80 µl/10^7 cells PBS/0.5 % BSA, 2mM EDTA and 20 µl/10^7 cells of the CD14 Microbead suspension was added. The suspension was mixed and incubated on ice for 15 min.
6. After collecting the cells by centrifugation, the cells were washed with 5 ml PBS/ 0.5 % BSA, 2mM EDTA and recentrifuged. The supernatant was aspired and the cells were resuspended in 500 µl PBS/ 0.5 % BSA, 2 mM EDTA
7. The column was placed in a magnet and equilibrated with 500 µl PBS/ 0.5 % BSA, 2 mM EDTA
8. The cell suspension was added to the column and the unmarked cells were collected in a suitable tube.
9. The column was washed 3 times with 500 µl PBS/ 0.5 % BSA, 2 mM EDTA
10. The column was removed from the magnet and placed in a 15 ml cellstar tube. 1 ml PBS/ 0.5 % BSA, 2 mM EDTA was added and the labelled cells were released from the column using a plunger to press out the cell suspension.
11. RPMI-1640/ 10 % fetal calf serum and gentamicin 25 µg/ml was added to the cells to a total volume of 10 ml and the cells were counted (see step 3)
12. The cell suspension was centrifuged and the supernatant was aspired.
13. The cells were suspended in RPMI-1640/ 10 % FCS and gentamicin 25 µg/ml. The differentiation factors IL-4 and GM-CSF were added to final concentrations of 25 ng/ml and 50 ng/ml, respectively.
14. The cell suspension was dispersed on a microtiter plate (48 wells); 1 ml of cell suspension, containing approximately 2 million cells, was added per well.
15. The microtiter plate was incubated in a Steri-Cycle incubator at 37 °C and under 5% CO_2
16. At day 4 (start day = day 0) the media was aspired and 1 ml fresh RPMI-1640/ 10 % FCS and gentamicin 25 µg/ml with 25 ng/ml IL-4 and 50 ng/ml GM-CSF was added to each well.
17. At day 6 the cells were checked for differentiation using a Leica microscope and were ready to be used in further experiments (see section 3.19.2 and 3.19.3)

3.19.2 Gentamicin assay of dendritic cells

The internalization of bacteria by DC was analysed by doing a gentamicin survival assay, mainly following the procedure developed by Innocentin, Silvia et al. (2009). Gentamicin will kill the bacteria outside the DC, but not harm the DC or the bacteria present inside it. After the bacteria and DCs have been in contact, gentamicin kills the non-internalized bacteria. Subsequently, the DCs are lysed by triton to release the bacteria inside and the solution is plated on agar plates to determine the number of internalized bacteria.

Materials:
RPMI-1640/ 10 % FCS
Gentamicin 10 mg/ml
PBS / 0.1 % triton
MRS plates with 10 mg/ml erythromycin

Procedure:

1. *L. plantarum* strains were grown and induced as in step 1-5 section 3.14.1. 10^7-10^9 bacterial cells were harvested; this amount was calculated according to Fig. A1 in the appendix.
2. The medium from wells containing differentiated DCs (section 3.19.1) was aspired. The bacterial pellet was dissolved in 1.1 ml of RPMI-1640/ 10 % FCS and 1 ml of the bacterial suspension was added to each well with DCs. The remaining 100 µl of the bacterial suspension was diluted in Ringers solution and dispersed on MRS plates containing 10 µg/ml erythromycin (to determine the total number of bacteria).
3. The microtiterplate containing DCs and bacteria was incubated for 2-4 hours at 37 °C under 5 % CO_2.
4. After the incubation, the cells were washed 4 times with 1 ml RPMI-1640/ 10 % FCS. Then, 1 ml RPMI-1640/ 10 % FCS with 400 µg/ml gentamicin was added to each well followed by incubation for 1.5 hour, at 37 °C, under 5 % CO_2.
5. The gentamicin solution was aspired and plated on MRS plates containing 10 µg/ml erythromycin to check for surviving bacteria.
6. 100 µl PBS / 0.1 % triton was added to each well to lyse the DCs.
7. The triton solution was aspired from each well and diluted in Ringers solution before plating on MRS plates containing 10 µg/ml erythromycin.
8. The MRS plates were incubated at 37 °C for 2 days before counting of bacteria.

3.19.3 Flow cytometry of maturation markers expressed on dendritic cells

DCs that have internalized bacteria will go into a maturing phase where they lose their ability to take up bacteria and start expressing proteins involved in priming of T-cells. Cells expressing these proteins can be detected by flow cytometry using directly conjugated fluorescent antibodies specific for these proteins.

Materials:

RPMI-1640/ 10 % FCS
Trypsin EDTA 1 x
PBS
Rabbit serum
Antibodies
- PE-cy conjugated CD83
- FITC conjugated HLA DR
- FITC conjugated CD80
- Alexa fluor conjugated CD86

Positive control (i.e. compounds known to induce maturation)
- LPS 100 ng/ml (final concentration)
- TNFα 15 ng/ml (final concentration)
- PGE2 5 µM (final concentration)

Procedure:

1. DCs and bacteria were co-incubated as in step 1-3 section 3.19.2. A positive control cocktail consisting of 100 ng/ml LPS, 15 ng/ml TNFα and 5 µM PGE2 in 1 ml of RPMI-1640/ 10 % FCS was added to one of the wells with DCs. One well with DCs was not incubated with anything and was used as a negative control. The incubation time was 2 hours.
2. After incubation, the DCs were washed 4 times with RPMI-1640/ 10 % FCS; after the last wash, 1 ml of RPMI-1640/ 10 % FCS with 25 µg/ml gentamicin was added to each well.
3. The DCs were incubated over night at 37 °C with 5 % CO_2.
4. The cells were washed with PBS and 200 µl trypsin was added to each well. The microtiter plate was then incubated for 10 min at 37 °C, or until the cells had detached from the bottom of the wells.

5. 200 µl RPMI-1640/ 10 % FCS was added to each well to stop the trypsinization.
6. 200 µl of cell suspension were dispersed into a 96 well plate with conical bottoms.
7. The cells was washed 2 times with PBS and centrifuged at 1300 rpm for 2 min between each wash.
8. 100 µl PBS / 5 % rabbit serum was added to each well for blocking of Fc receptors. The blocking was carried out at ice for 30 min.
9. The samples were centrifuged at 1300 rpm for 2 min and the blocking solution was discarded.
10. 20 µl of CD83 antibody and 20 µl of HLA DR antibody were added to each sample in one of the parallels.
11. 20 µl of CD80 antibody and 5 µl of CD86 antibody were added to each sample in the other parallel. 40 µl PBS was added to the remaining negative control well.
12. The samples were incubated in the dark for 1 hour at room temperature.
13. After the incubation, the cells were washed 3 times with PBS and centrifuged at 1300 rpm for 2 min between each wash.
14. The cells were then suspended in 100µl PBS and analysed by a MacsQuant® Analyser and the MacsQuantify™ software.

4 RESULTS

The antigen used in this study was the fusion protein AG85B-ESAT6 which has been shown to induce protective immune responses in several animal models (Hall et al. 2009; Langermans et al. 2005; Olsen et al. 2004). Vectors for expression and anchoring of this antigen that are based on the pSIP-system (Sørvig et al. 2003) had previously been constructed by (Tjåland 2011). Strains carrying some of these vectors had been tested in a limited mice experiment, and the results indicated that they did not elicit an immune response as monitored by measuring production of IFN-gamma. In the present study, some of these vectors were developed further by coupling a sequence coding for a dendritic cell binding peptide to the AG85B-ESAT6 encoding sequence.

4.1 Fusion of the DC-pep to secreted Ag85B-ESAT6

By screening a phage display library, Curiel et al. (2004) identified a 12-mer peptide that binds to both human and mouse DCs. In a study by Mohamadzadeh et al. (2009), it was shown that fusing this peptide to a *B. anthracis* protective antigen strongly improved the efficacy of this antigen in protecting mice against *B. anthracis* infection. Based on these studies, the 12-mer peptide FYPSYHSTPQRP (from now on called DC-pep) was selected for fusion with AG85B-ESAT6. In all cases described below, this peptide was fused directly to Ag85B-ESAT6, without the introduction of any additional amino acids.

The DC binding sequence (DC-seq) was fused to the Ag85B-ESAT6 gene by using overlap-extension PCR with three different forward primers to stepwise build up the DC-sequence (Fig. 4.1). The plasmid pLp_3050-Ag85B-E6 (Tjåland, 2011) was used as a template in the first PCR-reaction. The three different forward primers, P1-DCF, P2-DCF and P3-DCF have different and partially overlapping 5’ tails that elongate the template until the whole DC-sequence is added to the antigen gene. The PCR-product from the first reaction, obtained with P1-DCF, was used as template in the next reaction, with P2-DCF, and so on. The primer P3-DCF (forward primer) contains a SalI restriction site in the 5’ end, which can be used to insert the DC-Ag85B-ESAT6 fragment into the vector (Fig. 4.1). The reverse primer, AgE6PurR, binds downstream of the Ag85B-ESAT6 sequence and introduces the restriction site HindIII

for cloning (Fig.4.1). To minimize the risk of introducing errors in the sequence during the PCR reactions, the PCR amplification for the first two reactions was stopped after just 7 cycles. The final PCR fragment was subcloned into TOPO-vector before it was excised by the restriction enzymes SalI and HindIII and ligated into the 5.7 kb fragment of pLp_3050-Ag85B-E6 that was digested with the same restriction enzymes, yielding pLp_3050-DC-Ag85B-E6. This plasmid should yield secreted AG85B-ESAT6 N-terminally labeled with the DC-pep; secretion is driven by the leader peptide of Lp_3050 from *L. plantarum* WCFS1. The final cloning step was conducted in *E. coli* TOP10 cells and the obtained transformant was used to multiply the constructed plasmid prior to electroporation into *L. plantarum.* All PCR amplified sequences were confirmed by DNA sequencing before transformation to *L. plantarum*. This procedure was the same for all constructs described below.

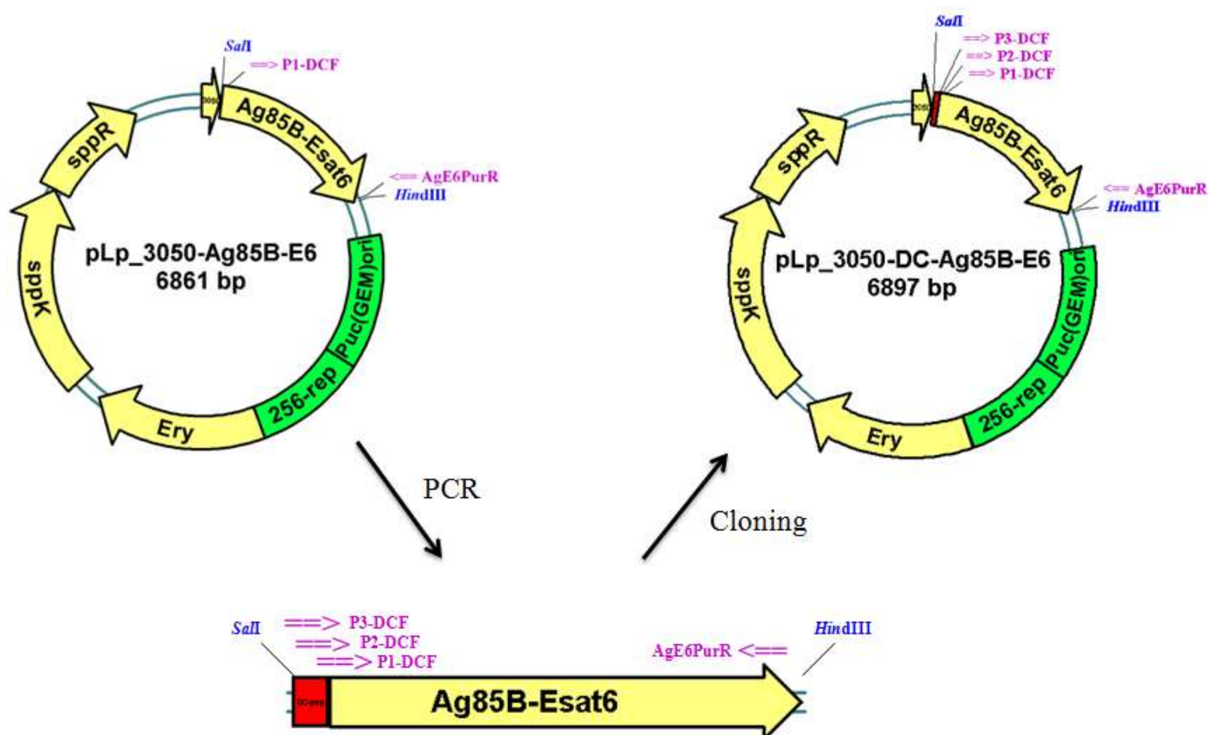


Figure 4.1. Cloning strategy for the construction of pLp_3050-DC-Ag85B-E6 for secretion of DC-Ag85B-ESAT6. Colour coding: yellow, erythromycin resistance gene (*Ery*), histidine proteinase gene (*sppK*), response regulator gene (*sppR*) and the target gene (*Ag85B-ESAT6*); red, sequence encoding the DC-binding peptide (DC-seq); green, *E. coli* replicon (pUC(GEM)ori), *Lactobacillus* replicon (256-rep). The names of the primers used are shown in purple and relevant restriction enzyme sites are shown in blue. This colour scheme is used in all the figures describing plasmids.

4.2 Construction of a plasmid for cell wall anchoring of DC-Ag85B-ESAT6

The DC-seq was also fused to the Ag85B-ESAT6 gene in a construct designed for cell wall anchoring of antigen, pLp_3050-Ag85B-E6-cwa2 (Tjåland 2011). The cwa2 anchor is a LPxTG- type anchor (see introduction for more details), meaning that the preceding protein will be anchored through its C-terminal end. The DC-seq was therefore fused to the N-terminal end of the antigen, which presumably would lead to maximal exposure and, thus optimal interaction with DCs. The target gene with the DC-peptide was amplified by PCR using the primers P3-DCF and AgMluR using pLp_3050-DC-Ag85B-E6 (section 4.1) as template (Fig.4.2). The AgMluR primer contains a MluI restriction site at its 5'-end and the amplified fragment will therefore also contain this restriction site. The P3-DCF primer contains a SalI site. The 1191bp PCR fragment was subcloned into a TOPO-vector before it was excised by the restriction enzymes SalI and MluI and ligated into the 6381bp fragment of pLp_3050-Ag85B-E6-cwa2 that had been treated with the same restriction enzymes, resulting in pLp_3050-DC-Ag85-E6-cwa2.

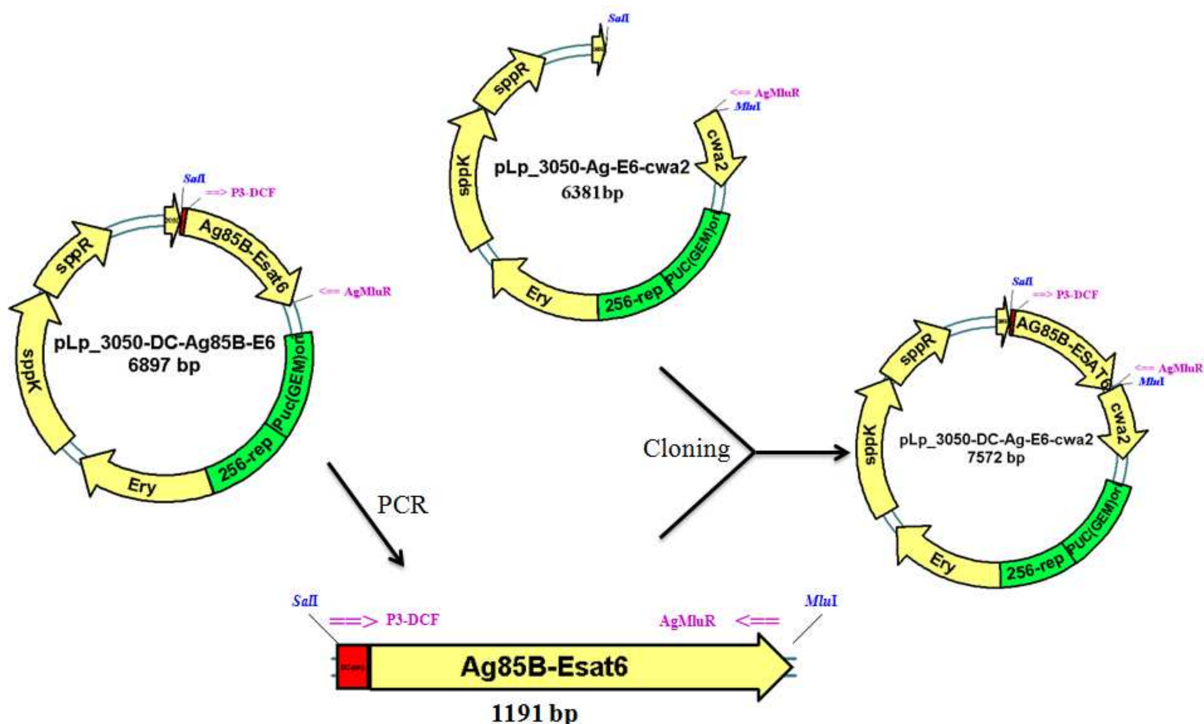


Figure 4.2. Construction of a plasmid for cell wall anchoring of DC-Ag85B-ESAT6. DC-seq fused with the antigen-encoding sequence was inserted into pLp_3050-Ag85B-E6-cwa2 by the use of PCR and cloning (see text for more details).

4.3 Construction of membrane-anchored Ag85B-E6-DC

The plasmid pLp_1261-Ag85B-E6 contains a lipobox for anchoring the antigen N-terminally to the cell membrane. In order for the DC-pep to protrude from the cells, thus potentially maximizing interactions with DCs, it needs to be inserted at the C-terminal end of the antigen. Three PCR-reactions were performed in order to insert the DC-pep encoding sequence into pLp_1261-Ag85B-E6 (Fig. 4.3 and 4.4). In the first cloning phase (Fig. 4.3), the plasmid pLp_3050-DC-Ag85B-E6 was used as a template for the amplification of the DC-pep. The amplification was done by PCR with E6-DCF as a forward primer and Hind-DCR as a reverse primer (Fig. 4.3B). E6-DCF has a 5'-tail that corresponds to 18 nuclotides encoding the very C-terminus of AG85B-ESAT6. Likewise Hind-DCR has a 5'-tail that corresponds to the 12 first nucleotides downstream of the Ag85B-ESAT6 sequence. The plasmid pLp_1261-Ag85B-E6 was used as a template to amplify the sequence between primer pSekF and DC-E6R (Fig. 4.3A). A 5'-tail in primer DC-E6R corresponds to the 20 first nucleotides of the DC-pep. Thus, it is possible to fuse the two fragments generated as shown in Fig. 4.3 by overlap-extension PCR.

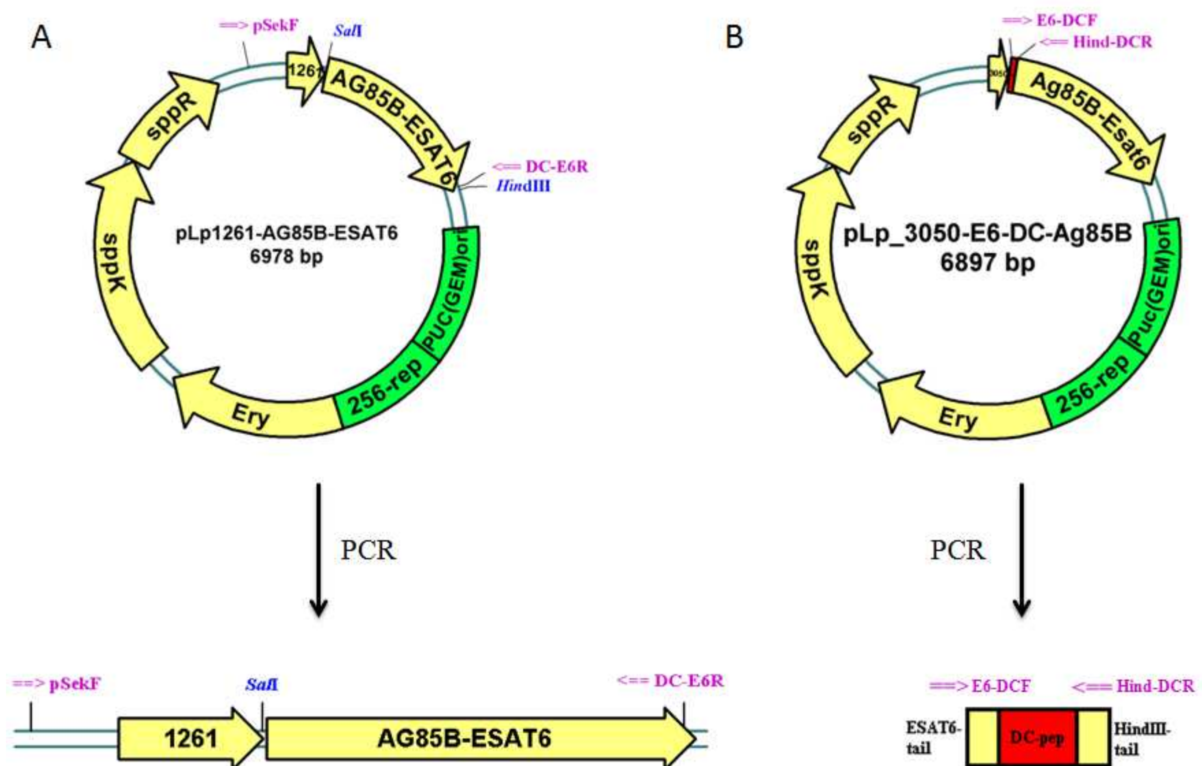


Figure 4.3. Construction of plasmid for membrane anchoring of antigen; step 1 and 2. The figure shows the procedure for amplification of the Ag85B-ESAT6 sequence with a lipobox N-terminal domain (A) and the DC-seq (B) by PCR. See text for details.

In the final phase of the cloning process a third PCR-reaction was carried out using the two fragments from the previous PCR-reactions as overlapping templates and pSekF and DC-E6R as primers (Fig. 4.4). The resulting 1194 bp PCR-fragment was cut by the restriction enzymes SalI and HindIII and ligated into the 5820 bp fragment of pLp_1261-Ag85B-E6 digested with the same restriction enzymes, resulting in pLp_1261-Ag85B-E6-DC.

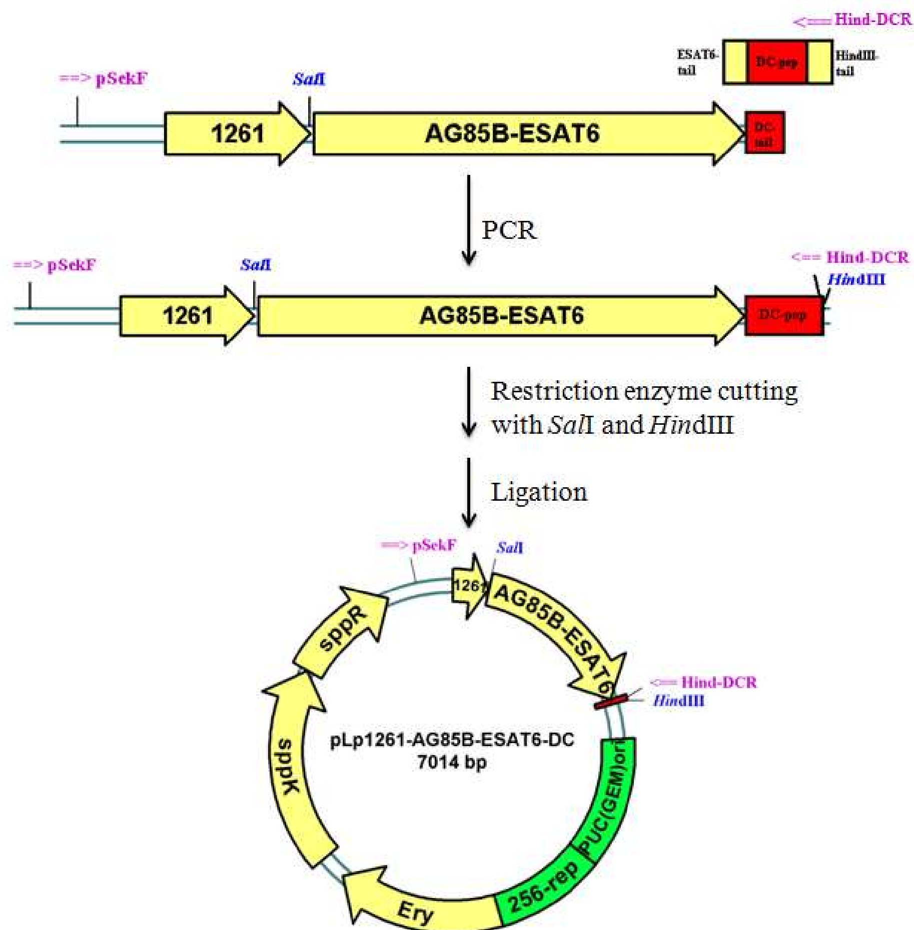


Figure 4.4. Construction of a plasmid for membrane anchoring of antigen, step 3. The figure shows the fusion of the DC-seq and the antigen-endocing sequence by overlapping PCR and ligation of the fused fragment into the “vector part” of pLp_1261-Ag85B-E6, resulting in pLp_1261-Ag85B-E6-DC.

4.4 Construction of plasmids containing the SH71-replicon

Maudal (2012) showed that changing the host-specific replicon 256 with the broad spectrum and higher copy number replicon SH71 may increase the expression of the protein of interest in *L. plantarum*. Changing the 256 replicon to the broad spectrum SH71 relicon allows for utilization of the constructed plasmids in other *Lactobacillus* spp. The use of other *Lactobacillus* species than *L. plantarum*, to obtain other adjuvant effects could be a method for improving the effect of an oral vaccine.

L. lactis harbouring the plasmid pLp_0373-NucA-sh71 constructed by Maudal (2012) was first used as a template to obtain the SH71 replicon. The isolation and restriction digestion of this plasmid from *L. lactis* turned out to be difficult because it was not possible to obtain plasmid material of sufficient quality (illustrated by “smears” observed during agorse gel electrophoresis). Therefore the SH71 replicon was retrieved from *E. coli* TG1 harbouring the plasmid pSIP411, which also contains the SH71 replicon. The plasmid was isolated by using the mini Prep kit (described in section 3.3) and trypsin was added directly after the isolation to stop the nucleases from degrading the plasmid. The trypsin was inactivated by boiling for 10 minutes to prevent breakdown of restriction enzymes. Plasmid pSIP411 was then digested by the restriction enzymes BsaI and HindIII and the fragment containing the SH71 replicon was ligated into fragments of BsaI and HindIII digested pLp_3050-DC-Ag85B-E6, pLp_3050-DC-Ag85B-E6-cwa2 or pLp_1261Ag85B-E6-DC (Fig.4.5). The ligation mixtures were transformed directly into *L. lactis.* This strategy only succeeded for pLp_1261-Ag85B-E6-DC. Several attempts were made to change the replicon in the two remaining plasmids, but none of the obtained colonies contained both the new replicon and the antigen Ag85B-ESAT6. Transformation of ligation mixtures directly into *L. plantarum* was also tried, but no colonies were obtained.

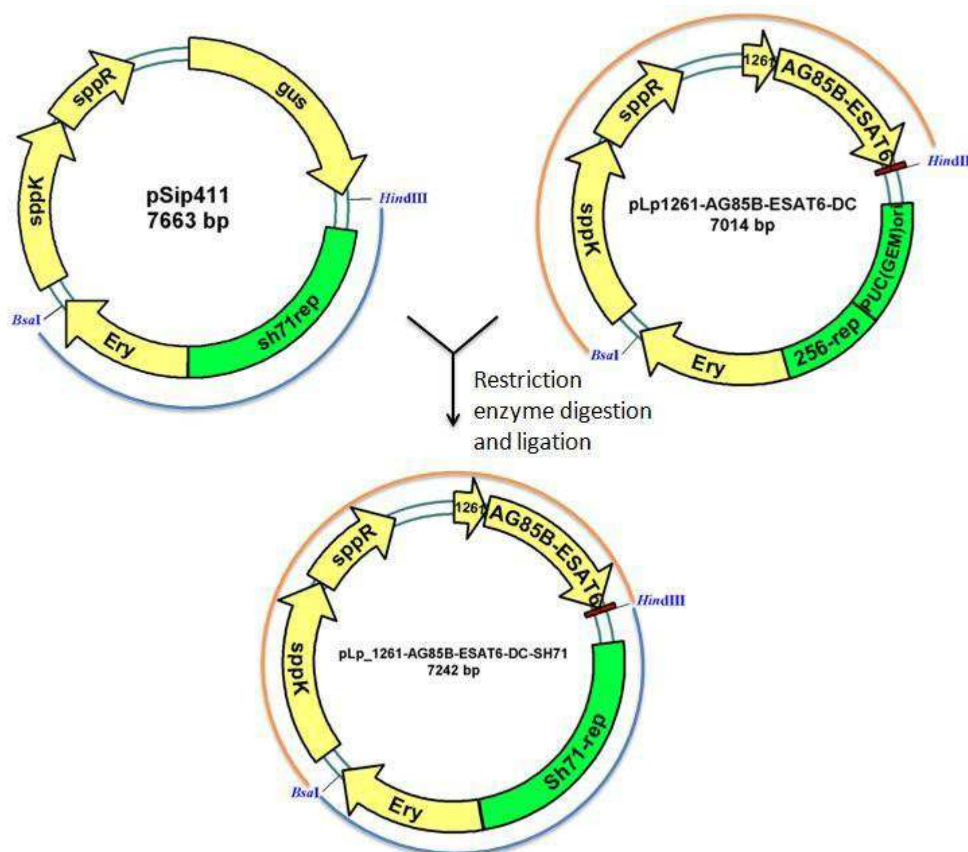


Figure 4.5. Construction of a plasmid for production of lipo-anchored Ag85B-ESAT6-DC with the SH71 replicon. The figure describes the procedure of exchanging the 256 replicon with the sh71 replicon. pSIP411 and pLp_1261-Ag85B-E6-DC were double digested by BsaI and HindIII. The 3093 bp fragment (indicated by blue line) from pSIP411 containing the sh71 replicon and the 4149 bp fragment (indicated by orange line) from pLp_1261-Ag85B-E6-DC containing the antigen were ligated together by the use of T4-ligase.

4.5 Summary of expression vectors used in functional analysis

Table 4.1 shows an overview of the expression vectors used in this study. The abbreviations shown in this table will be used in the text from this point.

Table 4.1. Overview of the constructed plasmids and their function

Name	Abbreviation	Function
pLp_1261-Ag85B-ESAT6	pLp_lipo	Production of membrane anchored Ag85B-ESAT6
pLp_1261-Ag85B-E6-DC	pLp_DC-lipo	Production of membrane anchored Ag85B-ESAT6 with a C-terminally fused DC-pep
pLp_1261-AG85B-E6-DC-sh71	pLp_DC-lipo-sh71	Production of membrane anchored Ag85B-ESAT6 with a C-terminally fused DC-pep; containing the SH71 replicon
pLp_3050-Ag85B-ESAT6	pLp_sec	Production of secreted Ag85B-ESAT6
pLp_3050-Ag85B-ESAT6cwa2	pLp_cwa2	Production of cell wall anchored Ag85B-ESAT6
pLp_3050-DC-Ag85B-E6	pLp_DC-sec	Production of secreted Ag85B-ESAT6 with a N-terminally fused DC-pep
pLp_3050-DC-Ag85B-E6-cwa2	pLp_DC-cwa2	Production of cell wall anchored Ag85B-ESAT6 with a N-terminally fused DC-pep

4.6 Growth curves for *L. plantarum* harbouring different plasmids

Growth curves for *L. plantarum* strains harbouring the different plasmids were recorded to investigate if addition of DC-pep had an effect on the growth rate and also to compare the growth rate between the different constructs. For previous studies it was known that expression of Ag85B-ESAT6 led to a considerable reduction in growth rate, depending on the type of construct (Tjåland, 2011).

Overnight cultures of *L. plantarum* harbouring the different constructs were diluted in MRS to OD_{600} ~ 0.15. The cultures were incubated at 37°C until the OD_{600} reached ~ 0.3 and were then induced with 25ng/ml of the inducing peptide, SppIP. The cultures were incubated further for 6 hours and OD_{600} values were measured every hour (for more details, see section 3.14.1 step 1-3). A strain harbouring the plasmid pEV, which lacks any target gene, was used as a control. Fig. 4.6 shows the growth curves for the different recombinant *L. plantarum* strains. All of the *L. plantarum* strains harbouring plasmids for expression of AG85B-ESAT6 grew slower than the "empty" control strain (pEV). The growth rates differed a lot between strains harbouring different plasmids. The fused DC-peptide did not seem to have any influence on the growth rates of the strain producing cell wall or lipo-anchored AG85B-ESAT6, but addition of the DC-pep clearly reduced the growth rate of the strain producing secreted antigen (Fig. 4.6). From two hours after induction and onwards, the OD_{600} for the bacteria containing plasmid without DC-seq was still increasing, while the OD_{600} for bacteria containing plasmid with DC-seq maintained stable. Interestingly, the *L. plantarum* strain harbouring a plasmid where the 256 rep was exchanged with the sh71 rep (pLp_DC-lipo-sh71) was not growing as fast as the corresponding strain with the 256-rep (pLp_DC-lipo).

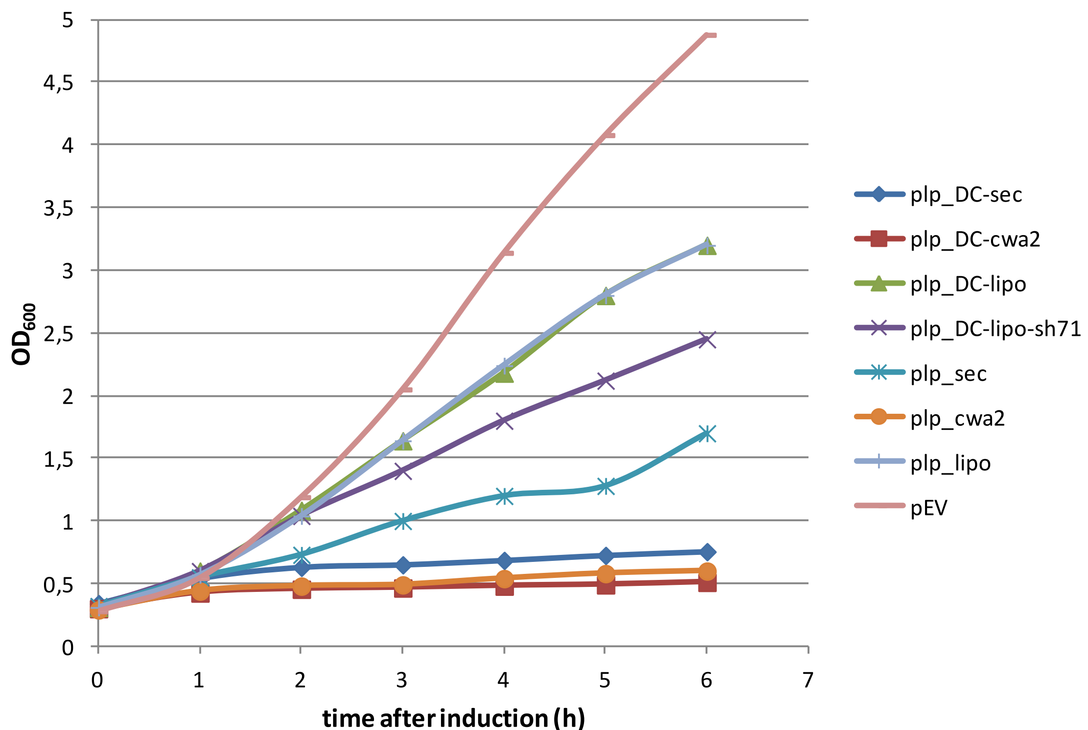


Figure 4.6. Growth curves for *L. plantarum* harbouring different plasmids for production of AG85B-ESAT6. *L. plantarum* strains were induced with 25 ng/ml SppIP at time 0 and incubated at 37 °C. OD_{600} values were measured every hour for a total of 6 hours.

4.7 Analysis of Ag85B-ESAT6 production using Western blotting

Western blotting of cell free protein extracts was done to determine the total amount of Ag85B-ESAT6 produced in induced recombinant *L. plantarum* strains. The induced cultures were harvested 2 hours post induction as described in section 3.14.1. The protein extract was obtained by disrupting the cells with glass beads using a FastPrep®FP120 Cell Disrupter. In order to better compare the different samples, the volume of sample applied to the gel was adjusted according to the OD_{600} at the time of harvest in order for the samples to represent approximately the same amount of cells. All the blotting analyses were run several times and showed similar trends as those illustrated by the pictures in this thesis.

Fig. 4.7 shows a Western blot of the cell-free protein extracts of *L. plantarum* strains harbouring the different plasmids for production of Ag85B-ESAT6. The intensities of the bands obtained with the different plasmids indicate that approximately the same amount of protein is produced in the different strains, although some variation does occur. The results do not reveal a systematic effect of the presence of DC-pep on intracellular Ag85B-ESAT6 levels. *L. plantarum* harbouring pLp_DC-sec (Fig. 4.7, lane 7) seemingly shows a slightly

lower production of the antigen than the strain harbouring pLp_sec (lane 8 in Fig.4.7). The strain harbouring pLp_DC-lipo (Fig. 4.7, lane 5) shows approximately the same amount of antigen as the strain harbouring pLp_lipo (Fig. 4.7, lane 6), but, surprisingly, the former strain shows two bands around 50 kDa. Western blotting of these two constructs was repeated several times with samples from different harvestings and the additional band was observed in all the blots. Additional protein bands with masses below the expected values are most likely degradation products of Ag85B-ESAT6 and were observed for all constructs in all blots, except for the extracts from the strain carrying pEV (Fig. 4.7, lane 2). Interestingly, while the DC-pep is only 1.3 kDa, thus generating a size difference that is not expected to be easily visible on the western blot, the size difference does seem to be visible in lane 7 vs. 8, and perhaps also in lanes 5 versus 6.

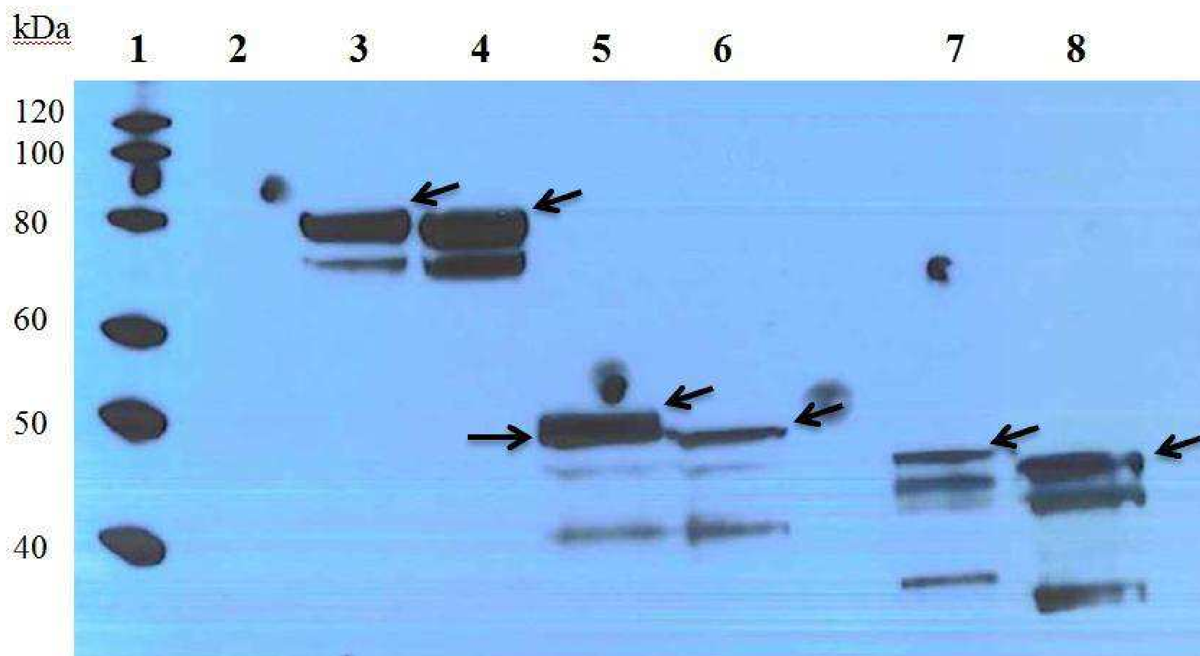


Figure 4.7. Analysis of Ag85B-ESAT6 in cell free protein extracts. The blot shows cell-free protein extracts from induced *L. plantarum* strains harbouring the different plasmids for production of Ag85B-ESAT6. **(1)** MagicMark protein ladder; **(2)** pEV **(3)** pLp_DC-cwa2 (73 kDa); **(4)** pLp_cwa2 (72 kDa); **(5)** pLp_DC-lipo (52 kDa): **(6)** pLp_lipo (51 kDa); **(7)** pLp_DC-sec (48 kDa); **(8)** pLp_sec (47 kDa). The theoretical molecular masses of the proteins are given in parentheses and include the mass of the signal peptide. Correct N-terminal processing would reduce these masses by 4 kDa and 2 kDa for 3050 and 1261, respectively. Bands that presumably represent AG85B-ESAT6 are indicated by arrows; see text for more details.

A Western blot analysis was also done on the supernatant fractions of induced *L. plantarum* strains harbouring different Ag85B-ESAT6 plasmids (Fig. 4.8). As for the cell free protein extract the supernatant was obtained from induced *L. plantarum* cultures that had been incubated for two hours post induction. 1.4 ml of the supernatant was transferred to a 2 ml eppendorf tube and frozen at -20°C before the proteins were precipitated using TCA (described in section 3.14.3). The sample sizes applied to the gel were not adjusted to according to $OD_{600.}$ The blot in Fig. 4.8 shows that the antigen is present in the supernatants of all the tested strains, albeit in quite different amounts. No antigen was detected in the negative control (pEV; results not shown). As expected, the supernatant from *L. plantarum* harbouring plasmids for secretion of the antigen, with and without the DC-seq (lane 1 and 2 in Fig. 4.8), gave clearly stronger signals than the other supernatants, which are from strains where the antigen is supposed to be retained on the cell surface.

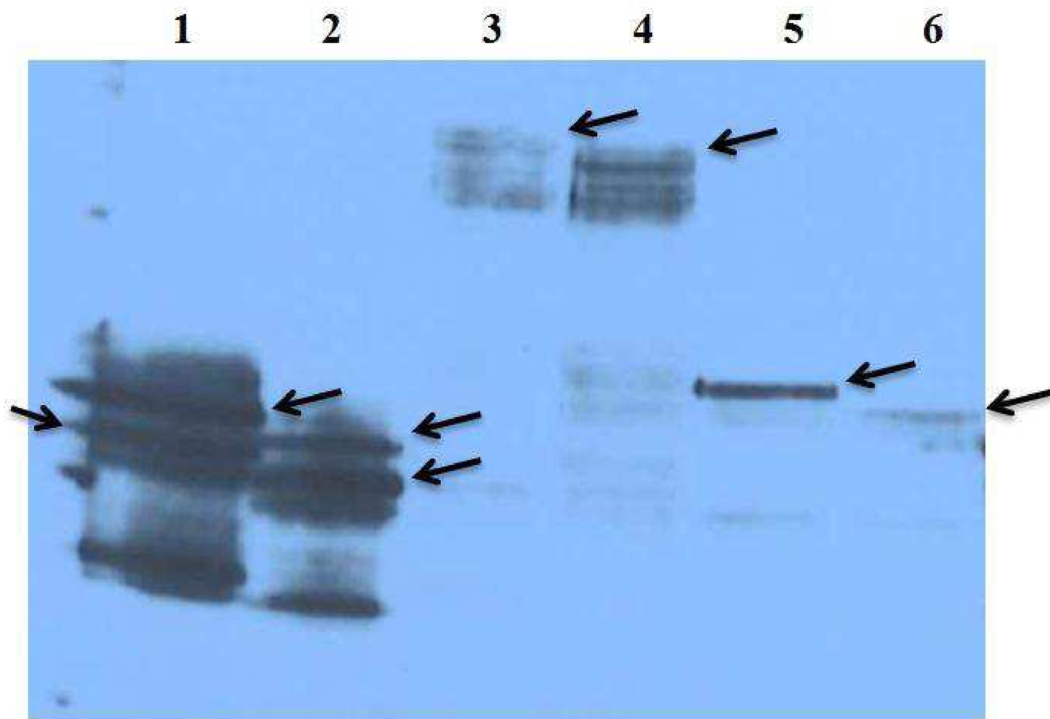


Figure 4.8. Analysis of Ag85B-ESAT6 in culture supernatants. The blot shows TCA-precipitated protein from the supernatants of induced *L. plantarum* strains harbouring different plasmids for production of AG85B-ESAT6. **(1)** pLp_DC-sec (44 kDa); **(2)** pLp_sec (43 kDa); **(3)** pLp_DC-cwa2 (69 kDa); **(4)** pLp_cwa2 (68 kDa); **(5)** pLp_DC-lipo (50 kDa) ; **(6)** pLp_lipo (49 kDa). The theoretical molecular masses are denoted in the parenthesis. Bands that presumably represent AG85B-ESAT6 are indicated by arrows; see text for more details.

To obtain better insight into the presence of the DC-labelled Ag85B-ESAT6 proteins in the cellular and supernatant fractions a comparative analysis of these fractions were performed. (Fig. 4.9) The cellular and supernatant fractions for each construct are from the same cell culture. *L. plantarum* harbouring pLp_DC-sec (Fig. 4.9, lane 2) has the highest amount of antigen in the supernatant while the lowest amount of antigen is found in *L. plantarum* harbouring pLp_DC-lipo.

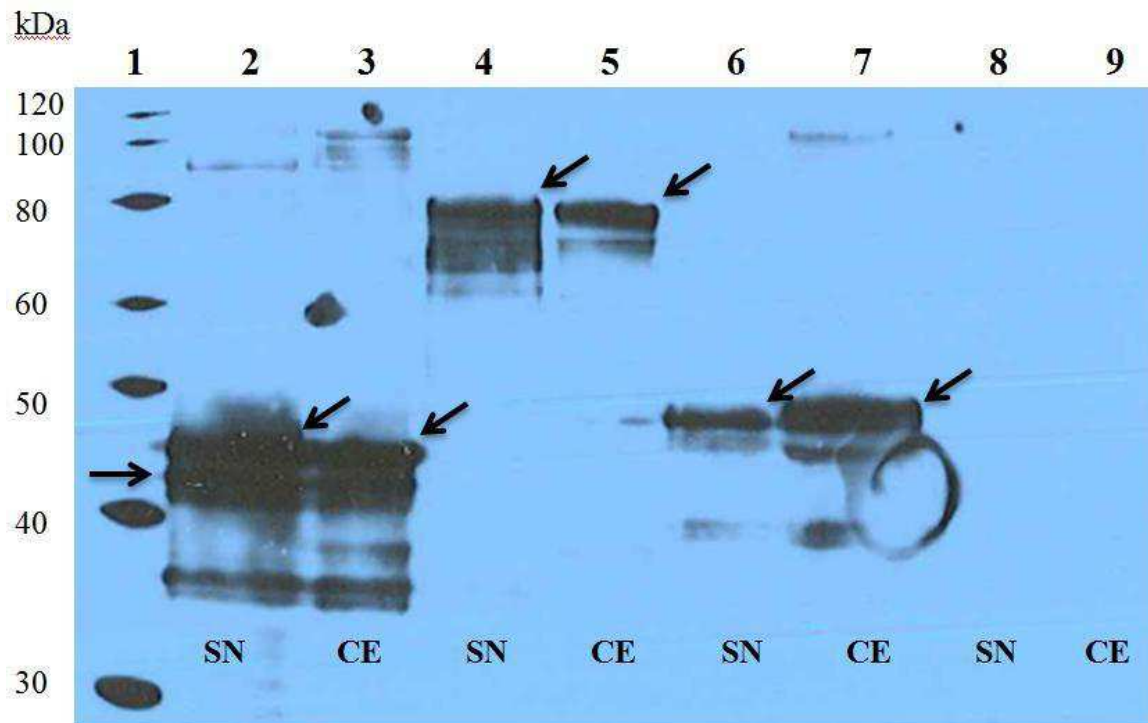


Figure 4.9. Comparison analysis of Ag85B-ESAT6 in the supernatants (SN) and cell free protein extracts (CE). The blot shows TCA precipitated protein from supernatants (SN) and cell free protein extracts from induced *L. plantarum* strains harbouring different plasmids for expression of Ag85B-ESAT6. **(1)** MagicMark protein ladder; **(2)** pLp_DC-sec (SN, 44 kDa); **(3)** pLp_DC-sec (CE, 48 kDa); **(4)** pLp_DC-cwa2 (SN, 69 kDa); **(5)** pLp_DC-cwa2 (CE, 73 kDa); **(6)** pLp_DC-lipo (SN, 50 kDa); **(7)** pLp_DC-lipo (CE, 52 kDa); **(8)** pEV (SN); **(9)** pEV (CE). The theoretical molecular masses are denoted in the parenthesis. Bands that presumably represent AG85B-ESAT6 are indicated by arrows; see text for more details. To enable semi-quantitative comparison, the amount of sample loaded onto the gel was adjusted to the OD600 of the original culture so that the samples represent approximately the same amount of cells. The amount of supernatant loaded onto the gel corresponds to 7 times more cell culture than the loaded amount of cell free protein extract.

A comparative Western blot analysis for studying the effect of the replicon exchange in the plasmid constructed for lipo-anchored DC-labelled Ag85B-ESAT6 is shown in Fig.4.10. The results suggest that use of the Sh71 leads to slightly higher intracellular protein levels (Fig. 4.10, lane 3 vs. lane 1). Interestingly, the blots also indicate that the protein produced with the Sh71- containing vector is less degraded (Fig.4.10 lane 3 vs. lane 1, and lane 6 vs. lane 4).

The blots also visualize a minor size difference between the antigen with and the antigen without the DC-pep.

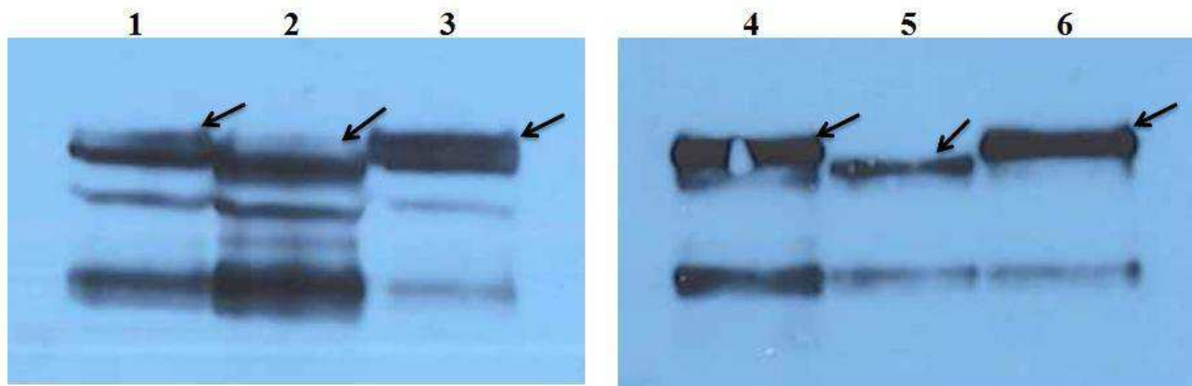


Figure 4.10. Western blot of cell free protein extracts and supernatants from *L. plantarum* strains harbouring plasmids for membrane anchoring of antigen. Lanes 1-3 show cell free protein extracts and lanes 4-6 show supernatants. **(1)** pLp_DC-lipo (52 kDa); **(2)** pLp_lipo (51 kDa); **(3)** pLp_DC-lipo-sh71 (51 kDa); **(4)** pLp_DC-lipo (50 kDa); **(5)** pLp_lipo (49 kDa); **(6)** pLp_DC-lipo-sh71 (50 kDa). The theoretical molecular masses are denoted in the parenthesis, bands that presumably represent AG85B-ESAT6 are indicated by arrows; see text for more details.

4.8 Detection of Ag85B-ESAT6 on the surface of *L. plantarum* using flow cytometry

Flow cytometry was used to analyse surface display of antigen in *L. plantarum.* *L. plantarum* was harvested two hours after induction and stained with primary antibody against ESAT6 (Mouse mcAb ab26246), and subsequently with a FITC conjugated secondary antibody, which will hybridize to the antigen bound primary antibody. The detailed procedure for preparation of samples and flow cytometry is described in section 3.18.1.

Fig. 4.11 shows the results for the flow cytometry analysis of *L. plantarum* cells harbouring plasmids with DC-pep antigen. No fluorescent signal was detected for pEV (Fig 4.11A), whereas all three constructs harbouring antigen showed fluorescent signals, indicated by a shift of the peaks in Fig. 4.11 to the right compared to the strain carrying pEV (negative control). The cell wall anchored antigen (pLp_DC-cwa2) gave the strongest shift, while the membrane anchored antigen (pLp_DC-lipo) gave the weakest shift (see Fig. 4.11, lower right diagram). Notably, a shift to right is also observed for the cells that are expected to secrete antigen (Fig. 4.11B). The histograms for pLp_DC-lipo (Fig. 4.11D) and pLp_lipo (Fig. 4.11E) show the same shifts. The results shown in Fig. 4.11 are consistent with the results

found by Tjåland (2011) for the corresponding plasmids without DC-seq, which indicates that the DC-peptide does not have any impact on the surface display of antigen.

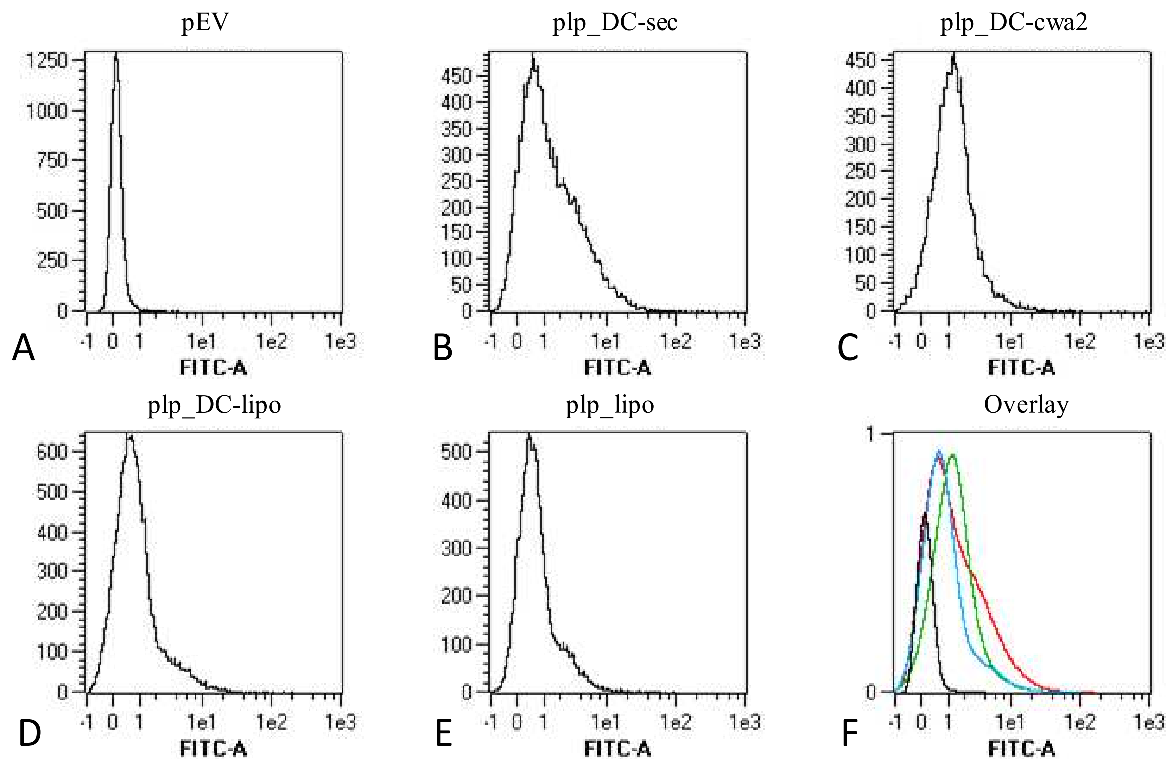


Figure 4.11. Flow cytometry analysis of FITC stained *L. plantarum* cells. The results are shown as histograms with the relative fluorescence (x-axis) plotted against the number of fluorescent cells (y-axis). The name of the construct analysed is denoted above the histograms. The last histogram shows an overlay of all the results for the DC-peptide containing constructs: Black, pEV; blue, pLp_DC-lipo; red, pLp_DC-sec; green, pLp_DC-cwa2.

4.9 Visualization of surface displayed antigen by flow cytometry and confocal laser scanning microscopy (CLSM) before and after treatment with lysozyme

Attempts were made to enhance the fluorescent signal by treating the cells with lysozyme before the immunostaining. Lysozyme breaks down the cell wall and this could reveal hidden antigen from under the cell wall and make it available for antibody to bind. Cells treated with lysozyme and cells not treated with lysozyme were analysed by both CLSM and flow cytometry.

Fig. 4.12 shows the results of the flow cytometry analysis. A tiny shift to the right is observed for the lysozyme treated pEV, but the shift is so small that it will probably not affect the other results. A more prominent shift to the right is observed for the secreted antigen (pLp_DC-sec) (Fig. 4.12B) and for the cell wall anchored antigen (pLp_DC-cwa2) (Fig.4.12C). The flow cytometry results for the two strains producing membrane anchored antigens (pLp_DC-lipo and pLp_DC-lipo-sh71) (Fig. 4.12D and E) show a large increase in fluorescent intensity for the lysozyme treated cells. The degree of fluorescence for these cells is similar or even higher than for the secreted and cell wall anchored antigens. The strain producing membrane anchored antigen from the plasmid containing the SH71-replicon shows a stronger fluorescent signal than the strain carrying the corresponding plasmid with the 256-replicon, both before and after the treatment with lysozyme. This may indicate higher production and/or better secretion of the antigen when using the SH71-replicon.

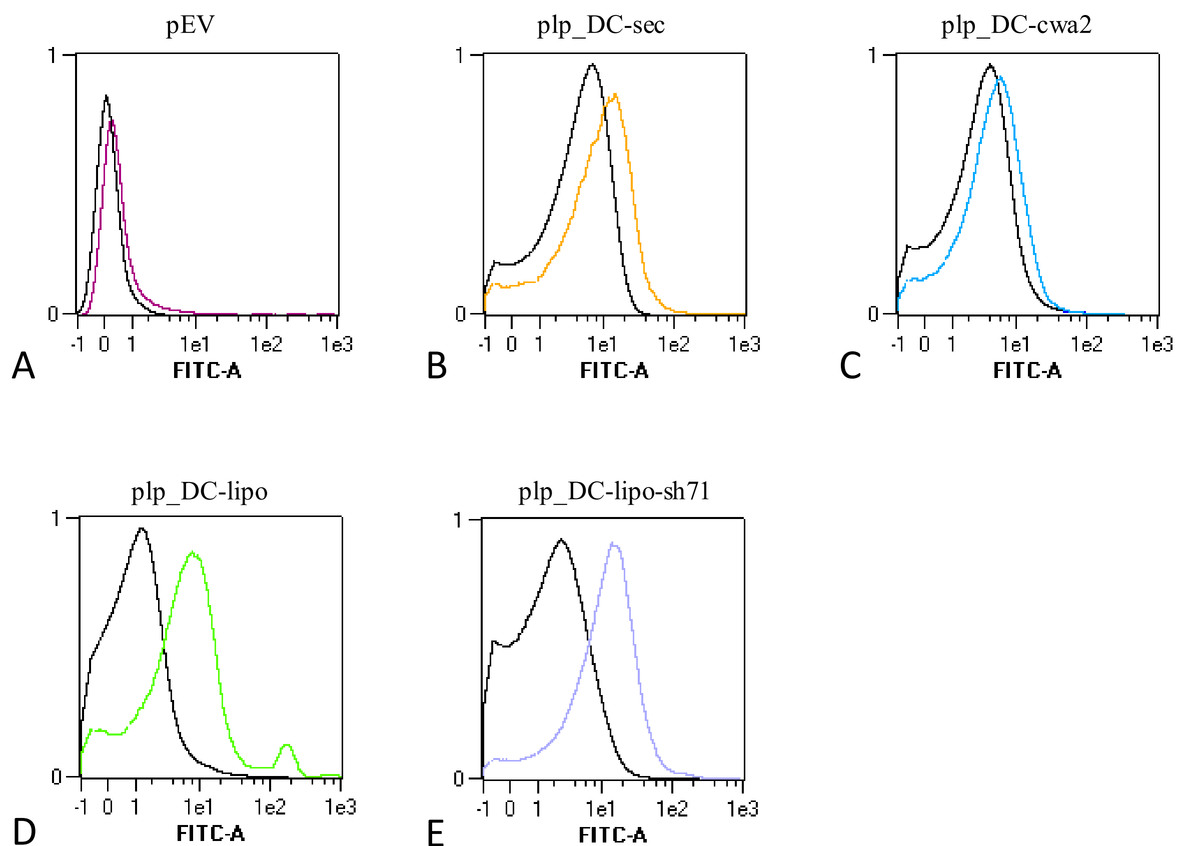
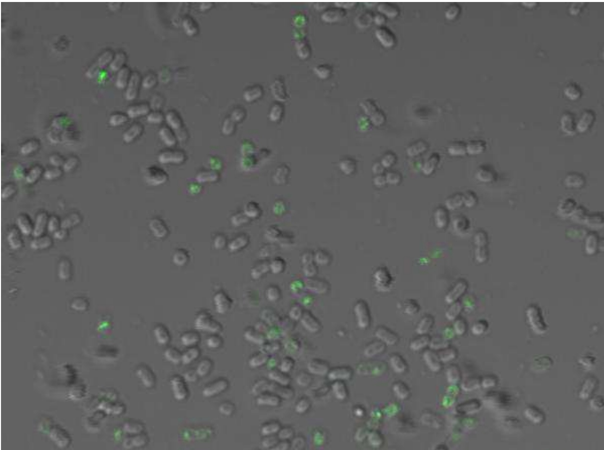
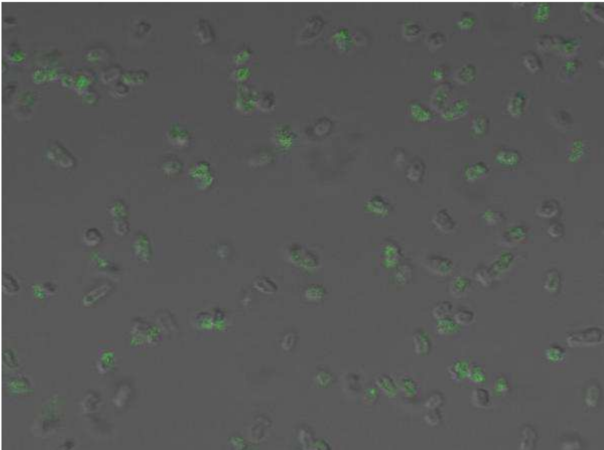


Figure 4.12. Flow cytometry analysis of *L. plantarum* cells treated and not treated with lysozyme. The results are shown as histograms with the relative fluorescence (x-axis) plottet against the number of fluorescent cells (y-axis). The name of the construct analysed is denoted above the histograms. The histograms shown in black are non treated cells whereas the coloured histograms show lysozyme treated cells.

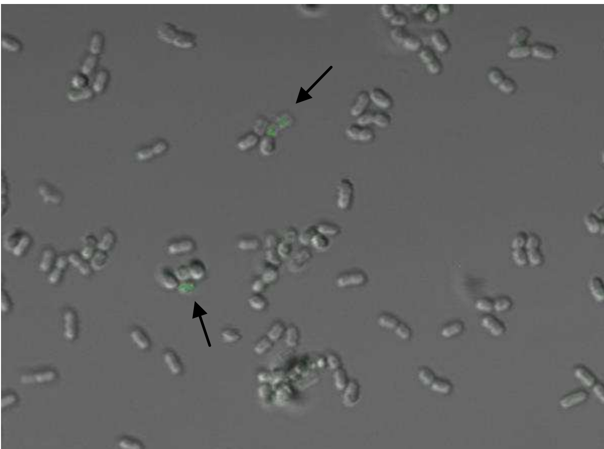
A confocal laser scanning microscope was also used to visualize fluorescent staining of bacterial cells. Induced and harvested *L. plantarum* cells were stained on microscope slides with antigen specific primary antibody and FITC-conjugated secondary antibody as described in section 3.18.2. The results from the CLSM analyses (Fig. 4.13) are roughly consistent with the results from the flow cytometry analysis (Figs. 4.11 and 4.12). Before treatment with lysozyme, the highest amounts of fluorescent cells are observed for *L. plantarum* strains harbouring pLp_DC-sec or pLp_DC-lipo-sh71. A surprisingly low amount of fluorescent cells are visible for the strain carrying pLp_DC-cwa2, considering the strong fluorescent signal detected for this strain with flow cytometry (Fig. 4.11C and 4.12C). Very few fluorescent cells are visible for the strain carrying pLp_DC-lipo, which are consistent with the results from the flow cytometry analyses (Fig. 4.11D and 4.12D). Several fluorescent cells can be observed after treatment with lysozyme compared to without the treatment. The secreted antigen and the membrane anchored antigen containing the SH71-replicon shows several clearly stained cells also before the treatment. The most evident effect of the lysozyme treatment is the results for cells carrying pLp_DC-lipo, which changes from very few visible fluorescent cells to have almost all cells stained after treatment. None fluorescent cells were observed for the pEV neither before nor after treatment with lysozyme.



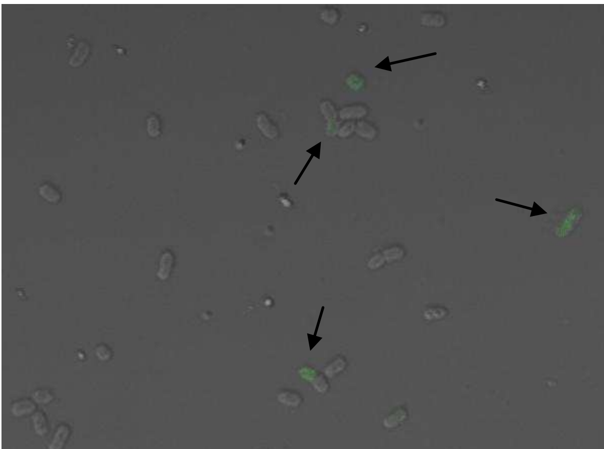
pLp_DC-sec



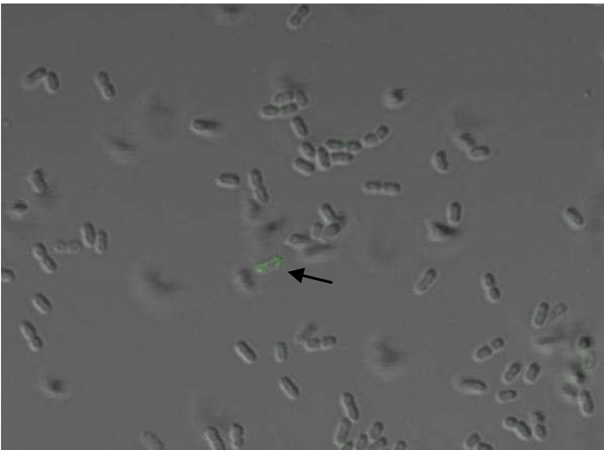
pLp_DC-sec - lysozyme treated



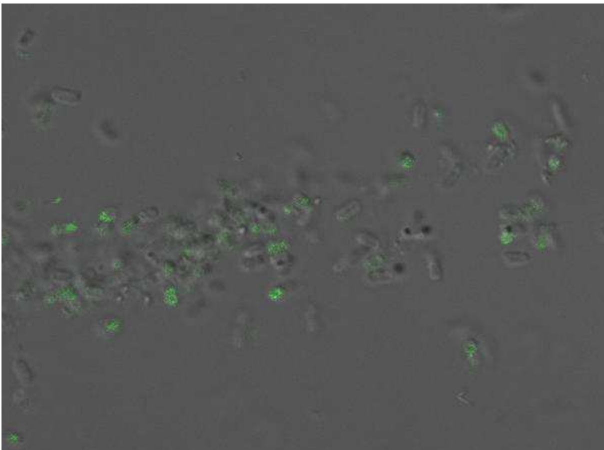
pLp_DC-cwa2



pLp_DC-cwa2 - lysozyme treated



pLp_DC-lipo



pLp_DC-lipo – lysozyme treated

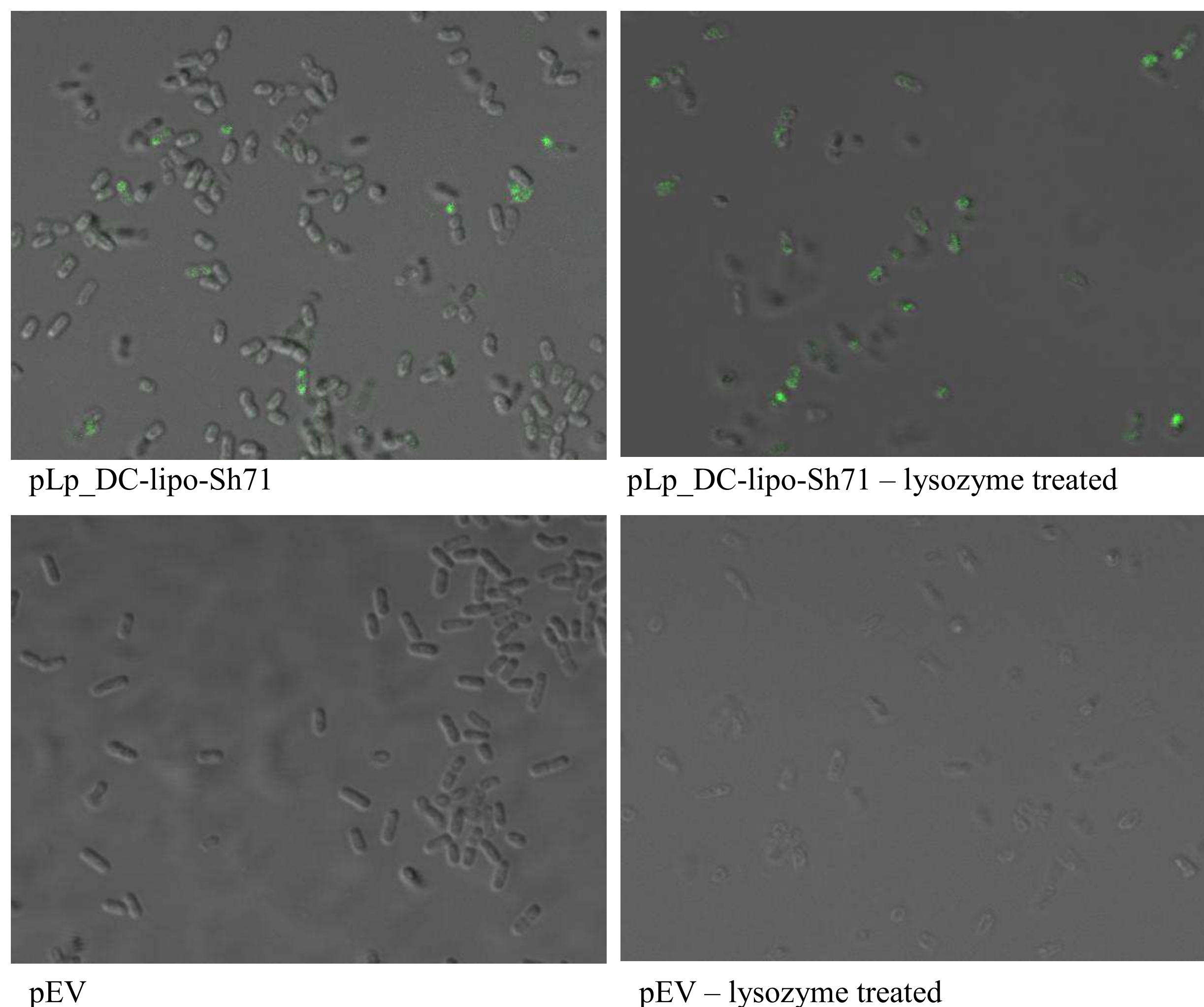


Figure 4.13. CLSM of FITC stained *L. plantarum* cells harbouring different plasmids for expression of Ag85B-ESAT6. The pictures to the right are of lysozyme treated cells, the names of the constructs are denoted under each picture. The pictures to the left are of cells that were not treated with lysozyme. The arrows in some of the pictures indicate fluorescent cells.

4.10 Storage of induced *L. plantarum*

Considering the fact that the bacteria were to be used as an oral vaccine, easy storage of induced harvested bacteria is important. Freezing or freeze drying the bacteria is an easy way of making it possible to store the bacteria. Freezing of bacteria was first tested in order to check if it affected the surface display of the antigen.

L. plantarum cells were induced and harvested as described in section 3.14 step 1-5 before they were frozen in PBS with 20 % glycerol at -80 °C. After thawing the cells, flow cytometry was used to check if the antigen was still displayed at the surface. If this treatment didn't affect the results, it would be possible to harvest the cells several days before the functional studies and also use the same batch of cells in different analyses. Fig. 4.14 shows

flow cytometry analysis of cells after freezing at -80°C for 4 days. Clearly, the treatment did not affect the display of antigen on the surface of the cells.

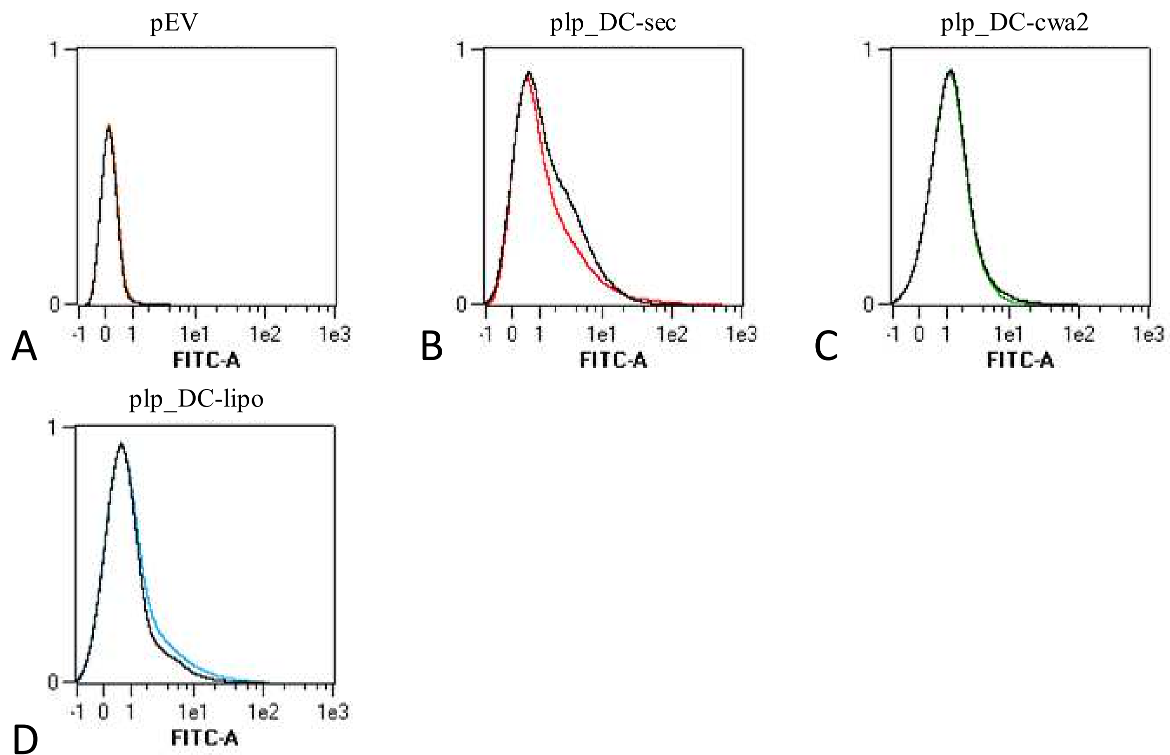


Figure 4.14. Flow cytometry of cells frozen in PBS with 20 % glycerol. The results are shown as histograms with the relative fluorescence on the x-axis. The names of the constructs analysed are denoted above the histograms. The histograms shown in black are the fresh harvested cells whereas the coloured histograms are from cells that had been frozen in PBS with 20 % glycerol.

A method which allows storage of bacteria at room temperature is freeze drying. The freeze drying medium that the bacteria were suspended in before freeze drying was selected based on an article by Carvalho et al. (2002), showing that skim milk containing different sugars or sugar alcohols protected *L. plantarum* during freeze drying and prevented bacterial cell death during storage. The freeze drying medium tested here contained: Skim milk 11 % (w/v) solids and 12.5 g/l of fructose. Induced and harvested bacteria were prepared and freeze dried as described in section 3.17. Fig. 4.15 shows the viability of the *L. plantarum* cells suspended in freeze drying medium just before freeze drying and two months after freeze drying. For *L. plantarum* strains harbouring antigen-encoding constructs the percentage of surviving bacteria varied between 5 and 25 %. For the strain harbouring pEV the survival percentage was as high as 68 %. These results show a higher percentage of surviving bacteria than the results from Carvalho et al. (2002), which showed that 1 % of the bacteria survived after two months.

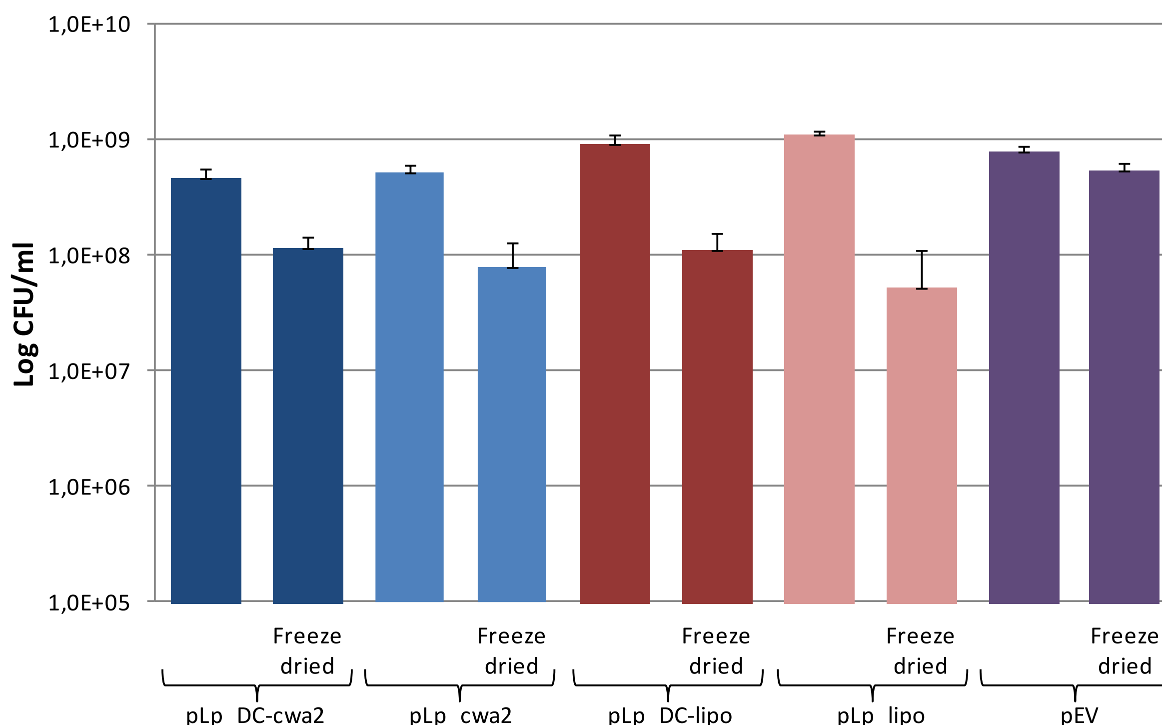


Figure 4.15. Survival of freeze dried *L. plantarum*. The figure shows the amount of viable bacteria before freeze drying and after freeze drying followed by two months storage. The names of the constructs harboured by the *L. plantarum* strains are denoted under the columns.

Freeze dried samples of bacteria were dissolved in PBS and analysed by flow cytometry to analyse if the antigen still was present on the surface of bacteria. The results from this analysis are shown in Fig. 4.16. No difference in fluorescent signal was observed in the freeze dried samples compared to the non freeze dried samples. It should be noted though that the analysis of the freeze-dried samples was done only one day after the freeze drying and that an analysis needs to be done on samples stored for several months. Carrying out this experiment was unfortunately not possible in the present study due to time limitations. It is therefore not possible to conclude if freeze drying could be a good method for preservation of induced bacteria over longer periods.

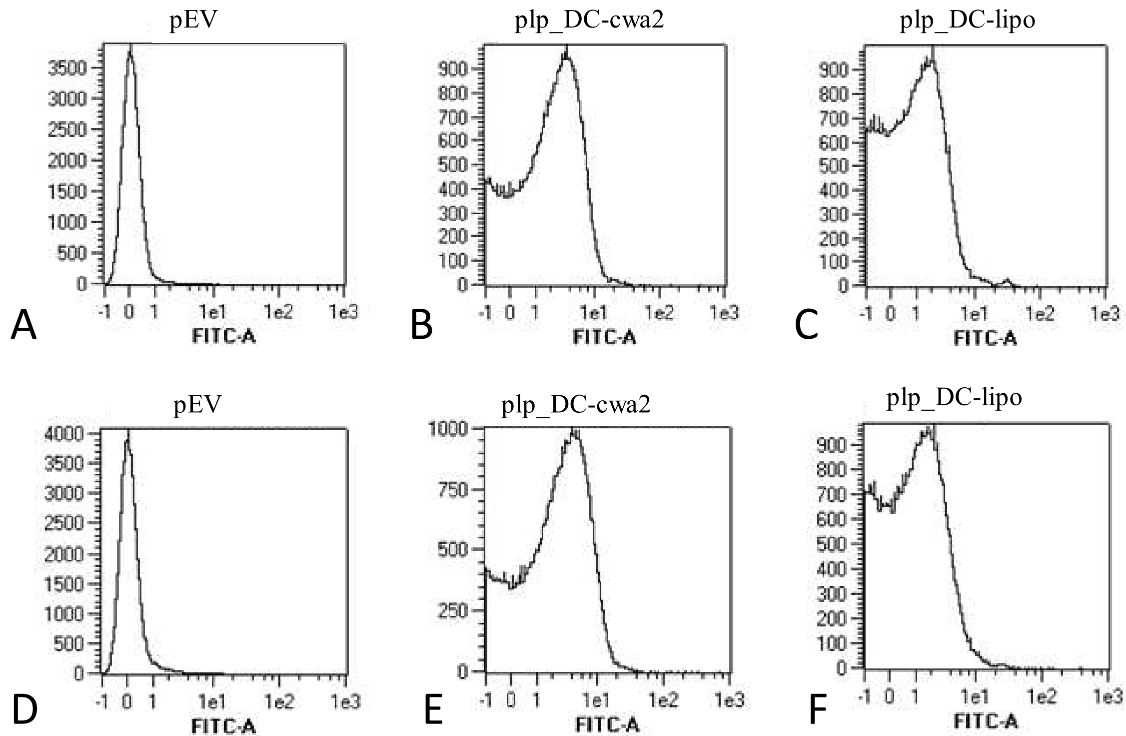


Figure 4.16. Flow cytometry analyses of *L. plantarum* cells frozen in skim milk and freeze dried. The results are shown as histograms with the relative fluorescence (x-axis) plotted against the number of fluorescent cells (y-axis). **A-C)** Results for cells suspended in freeze drying medium before flow cytometry analysis. **D-F)** Results for cells that were freeze dried before flow cytometry analysis. The analysis was done one day after the freeze drying.

4.11 Internalization of *L. plantarum* strains by dendritic cells

Dendritic cells play a central role in the regulation of an immune response. The reason for fusing the DC-pep with Ag85B-ESAT6 was to hopefully enhance the uptake of antigen presenting bacteria by DCs. To check for increased uptake, *L. plantarum* strains producing DC-labelled Ag85B-ESAT6 were compared to strains producing the antigen without DC-pep. The uptake of bacteria was analysed by doing a gentamicin survival assay (Innocentin, S. et al. 2009) (see section 3.19.2). First, DCs were prepared by maturation of $CD14^+$ cells. In short, $CD14^+$ cells were isolated from human peripheral blood leukocytes and incubated for 6 days in RPMI-1640 with IL-4 and GM-CSF for development into dendritic cells (section 3.19.1). Some preliminary experiments were then carried out in order to optimize the conditions for uptake of bacteria. Different ratios between DCs and bacteria were tested; the amount of DCs was kept constant while the amount of bacteria was varied. Various incubation times were also tested. Initially, only the two constructs containing cell wall anchored antigen (pLp_DC-cwa2 and pLp_cwa2) were used in the optimization work. Fig.

4.17 shows the results from an experiment with different ratios of DCs and bacteria. As the figure shows, the uptake of bacteria increased proportionally with the amount of bacteria added. In addition, the number of internalized bacteria was generally higher for the strain harbouring pLp_DC-cwa2 at all tested bacterial dosages. Based on these results, it was decided to apply10^9 bacterial cells per 2 million DCs in the following experiments.

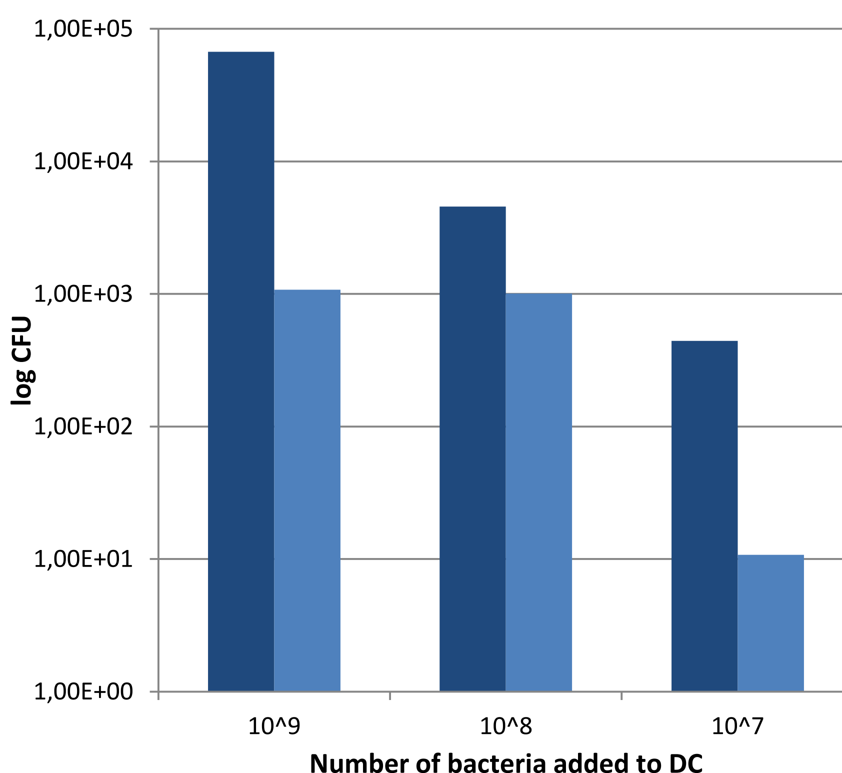


Figure 4.17. Uptake of *L. plantarum* by dendritic cells at different ratios of bacteria and DCs. The amount of bacteria added to 2 million DCs are indicated below the bars and correspond to ratios between dendritic cells and bacteria of 1:500, 1:50 and 1:5, from left to right, respectively. The incubation time of bacteria and dendritic cells was 2 hours (see section 3.19.2 for more details). The bars represent the number of internalized bacterial cells for strains harbouring pLp_DC-cwa2 (dark blue) or pLp_cwa2 (light blue).

Next, different incubation times were tested, whereas, the ratio between DCs and bacteria was kept at 1:500. The largest difference in uptake for the two constructs was after 1 hour; over time the difference was reduced (Fig.4.18). Since the purpose of these experiments was to detect the effect of the DC-pep on the uptake of bacteria by DCs, an incubation time of 1 hour was used in the following experiments.

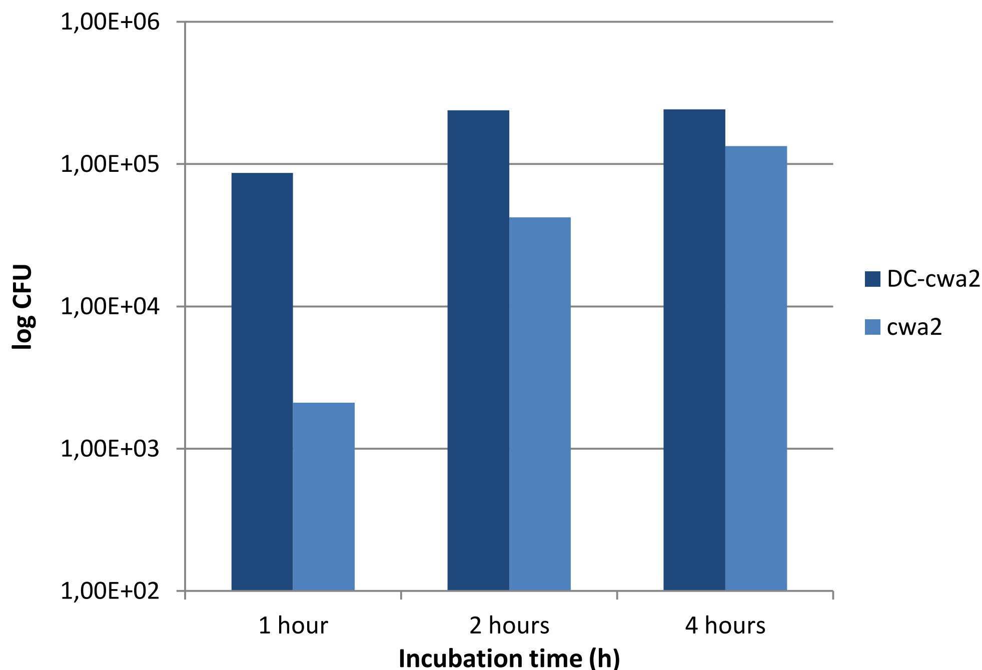


Figure 4.18. Uptake of *L. plantarum* strains by dendritic cells, different incubation times. The ratio between DCs and bacteria was 1:500 and the incubation times were 1, 2 and 4 hours. The bars represent the number of internalized bacterial cells for strains harbouring pLp_DC-cwa2 (dark blue) or pLp_cwa2 (light blue).

Fig. 4.19 shows the results for uptake of various *L. plantarum* strains by DCs after 1 hour incubation with a DC:bacteria ratio of 1:500. The numbers shown are the averages from several experiments. The highest uptake was obtained for the membrane anchored DC-antigen (approximately 7 bacteria per 100 DCs) followed by the cell wall anchored DC-antigen (approximately 2 bacteria per 100 DCs). The data also indicate that the DC-pep has a bigger impact on the uptake of bacteria with the cell wall-anchored antigen compared to bacteria carrying the membrane-anchored antigen. The results varied a lot between each individual experiment; however each individual experiment showed a clear trend towards a positive effect of the DC-pep on internalization. A paired students T-test was performed on the values shown in Fig. 4.19, but it didn't show significant results ($p \leq 0.05$)

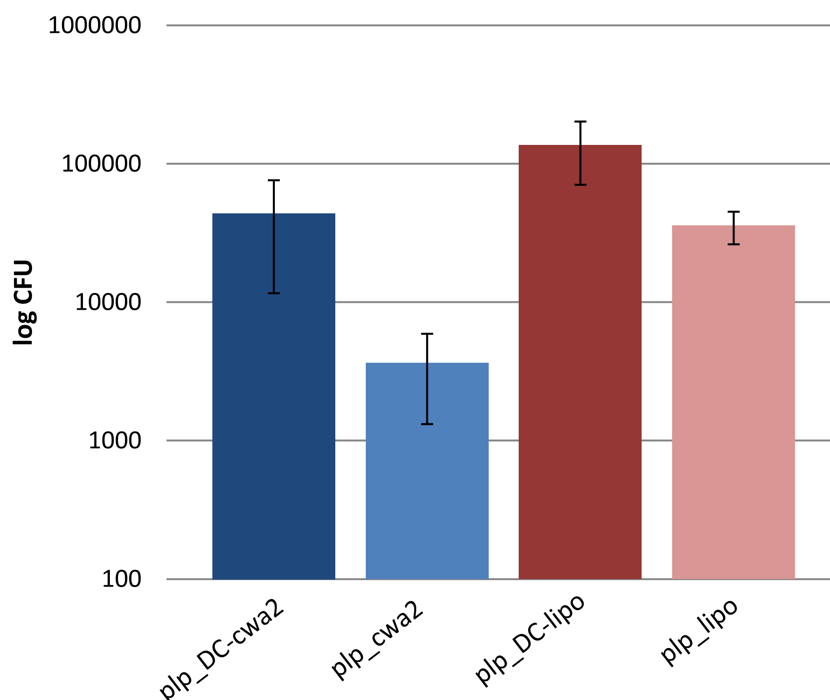


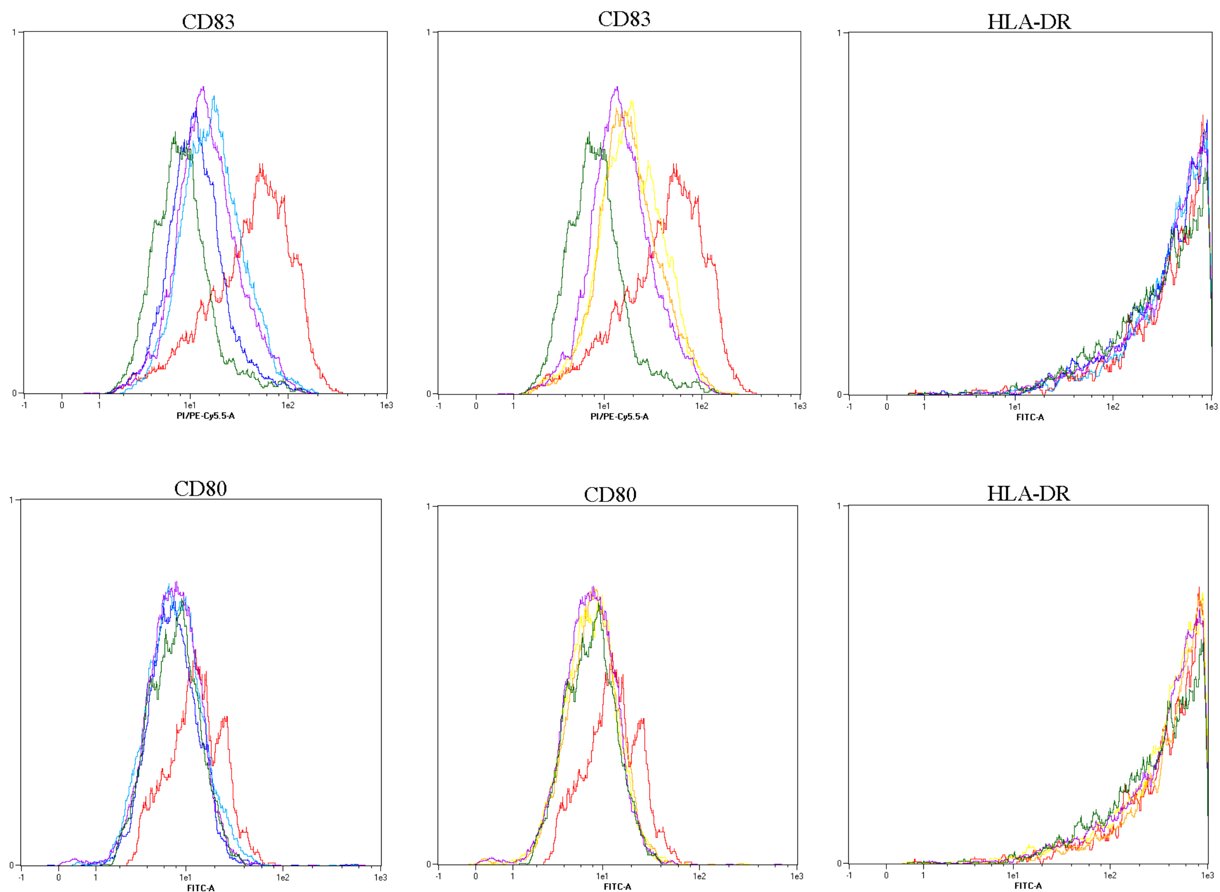
Figure 4.19. Uptake of *L. plantarum* strains by dendritic cells. The ratio between DCs and bacteria was 1:500 and the incubation time was 1 hour. The bars represent the number of internalized bacterial cells. The names of the constructs tested are denoted beneath the bars. The error bars represent the standard error; the results for the cell wall anchored constructs are derived from 3 individual experiments with 1, 1, and 3 parallels. The results for the membrane anchored constructs are derived from 2 individual experiments with 2 parallels each.

4.12 Maturation of dendritic cells

Immature DCs capture and internalize antigens and whole bacteria which lead to the DCs evolving from immature DCs to mature DCs. The mature DCs are antigen presenting cells which are capable of priming naive T-cells. A mature DC expresses specific molecules necessary for the priming of T-cells; some marker proteins for this development are MHC-class II, CD80, CD86 and CD83. Maturation of DCs after being exposed to bacteria was tested by staining the DCs with directly conjugated fluorescent antibodies specific for these marker proteins and the results were analysed by flow cytometry. The negative control consisted of DCs with no added bacteria and a positive control was generated by inducing DC maturation with a mixture of LPS, TNF-α and PGE2 (section 3.19.3).

Figure 4.20 shows the results of the flow cytometry analyses. The analysis of the CD83 marker shows fluorescent signal for both the negative (green line) and positive control (red line), but with a higher shift to the right for the positive control. The histograms for DCs incubated with bacteria all show a slight shift to the right relative to the negative control, but

there is not a clear difference between cells containing the expression constructs and cells containing the empty vector (purple line). The results for the CD80 analysis show that the shift for the positive control is only slightly higher than for the negative control. In this case, the histograms for DCs incubated with bacteria have the same intensity as the negative control, indicating that expression of CD80 is at the same level as in the negative control. The analysis of both HLA-DR (an MHC-class II molecule) and CD86 showed that the positive control and the DCs incubated with bacteria all had the same degree of fluorescent intensity as the negative control.



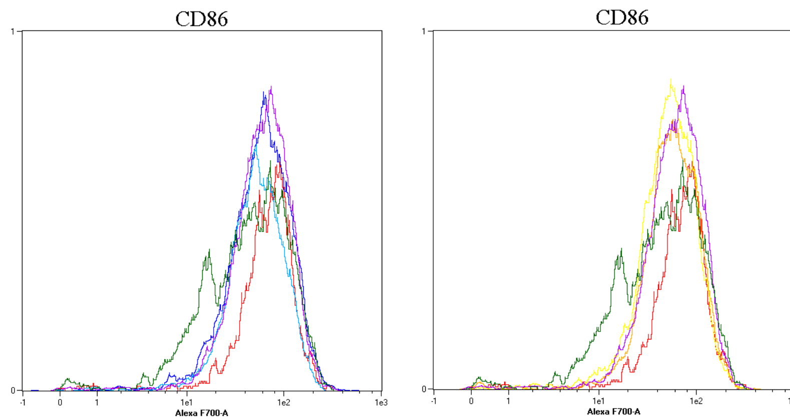


Figure 4.20. Flow cytometry analyses of maturation markers on DCs. The results are shown as histograms with the relative fluorescence on the x-axis; the heights of the histograms are adjusted after the negative control. The names of the markers tested are denoted above each picture. The colour coding is as follows: Negative control (green), positive control (red), pEV (purple), pLp_DC-cwa2 (blue), pLp_cwa2 (light blue), pLp_DC-lipo (orange), pLp_lipo (yellow). Note that there are two panels for each marker, one showing data for the cell wall anchored constructs and one showing data for the membrane anchored constructs.

Taken together these experiments shows that all the *L. plantarum* strains, including the strain containing pEV, were able to induce higher expression of CD83 in DCs than the negative control. Higher expression of the other tested markers was not observed in any of the samples.

4.13 Mice experiments

Mice experiments were performed in order to evaluate the ability of *L. plantarum* strains producing Ag85B-ESAT6 to elicit an immune response against *M. tuberculosis* antigens. Mice experiments were performed by Rajna Dimitrijevic at the Institute of Virology, Vaccines and Sera, Torlak, Belgrade. All experiments were approved by the Ethics Committee for the welfare of experimental animals at the Institute of Virology, Vaccines and Sera in Torlak, Serbia, and conformed to Serbian laws and European regulations on animal welfare (Approval No. 011-00-00510/2011-05/4).

Immune responses were monitored by measuring the levels of relevant cytokines produced by peripheral blood mononuclear cells (PMBCs). The PBMCs were isolated from blood of mice immunized with *L. plantarum strains* harbouring different plasmids for production of Ag85B-ESAT6. The mice were divided into 8 groups with 5 mice in each group; the setup of the experiment is shown in table 4.2. At day 1, 12 and 33 of the experiment, the mice in five of the eight groups were intranasally given 10^{11} frozen bacteria. One of the groups was given 10^{11} dried BCG bacterial cells intradermally to mimic the regular BCG vaccine, while another

group was given 10^{11} dried BCG bacterial cells intranasally as a positive control. Mice in the negative control group were not given any bacteria. At day 60 all the mice were challenged by giving them $5*10^4$ dried BCG bacterial cells intranasally. At day 72, two of the mice were sacrificed and peripheral blood mononuclear cells (PBMC) were isolated. The PBMC were then stimulated with 3 µg/ml purified Ag85B-ESAT6, 10 µg/ml dried BCG, or not stimulated and kept in a humidified 5% CO_2 atmosphere at 37 °C for 48 hours. Supernatants were collected and cytokine profiles of the supernatants were analysed using ELISA. The results are shown in Fig. 4.21-4.24.

Table 4.2. Mice experiment setup. The table shows at which days the mice were treated with bacteria or BCG. Each group contained five mice. At day 72, two of the mice were sacrificed. At day 80, the rest of the mice were sacrificed.

Group	Day 1	Day 12	Day 33	Day 60	Day 72	Day 80*
neg-control	-	-	-	BCG i.n.	½ X	½ X
pEV	pEV i.n.	pEV i.n.	pEV i.n.	BCG i.n.	½ X	½ X
pLp_lipo	pLp_lipo i.n.	pLp_lipo i.n.	pLp_lipo i.n.	BCG i.n.	½ X	½ X
pLp_cwa2	pLp_cwa2 i.n.	pLp_cwa2 i.n.	pLp_cwa2 i.n.	BCG i.n.	½ X	½ X
pLp_DC-lipo	pLp_DC-lipo i.n.	pLp_DC-lipo i.n.	pLp_DC-lipo i.n.	BCG i.n.	½ X	½ X
pLp_DC-cwa2	pLp_DC-cwa2 i.n.	pLp_DC-cwa2 i.n.	pLp_DC-cwa2 i.n.	BCG i.n.	½ X	½ X
BCG i.d.	BCG i.d.	BCG i.d.	-	BCG i.n.	½ X	½ X
BCG i.n.	BCG i.n.	BCG i.n.	-	BCG i.n.	½ X	½ X

Abbreviations: i.n. = intranasal; i.d. = intradermal
* Samples from day 80 had not been analysed at the time of writing of this thesis.

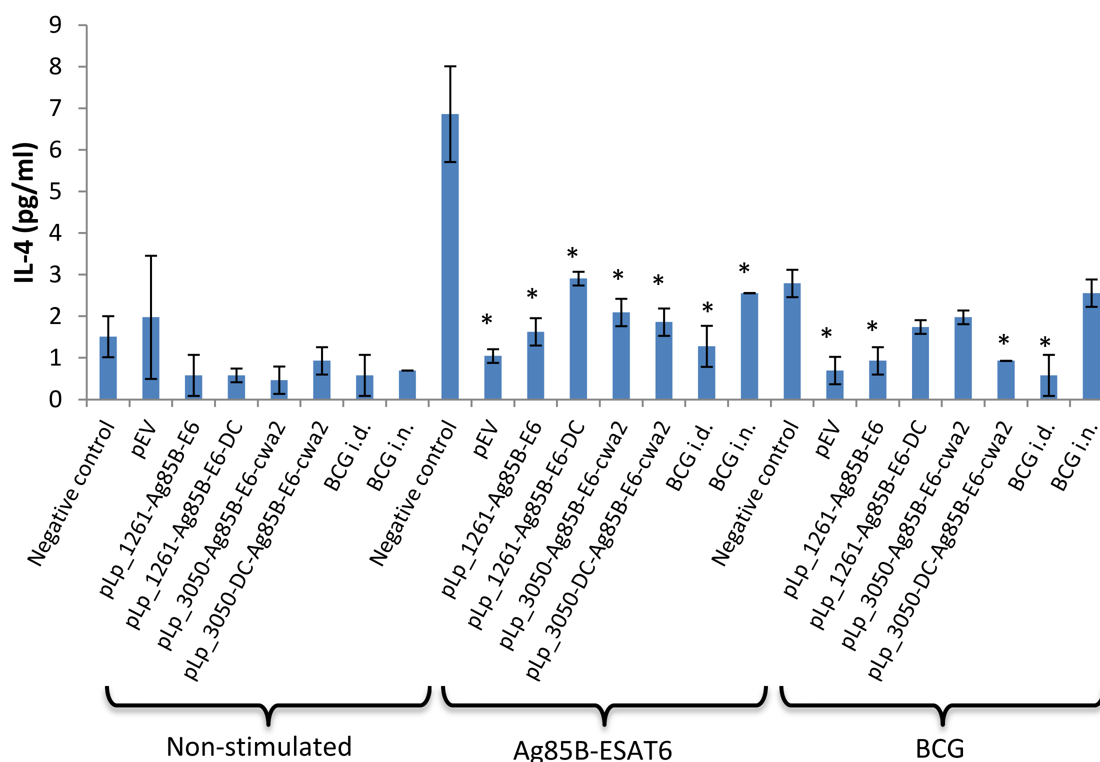


Figure 4.21. Analysis of IL-4 production by PBMCs. The columns show the amount of IL-4 secreted by PBMCs isolated from immunized and control mice. Each well contained approximately $6*10^5$ PMBCs in a total volume of 1ml. The PBMCs were stimulated with BCG, Ag85B-ESAT6 or not stimulated, as indicated by the brackets. The standard deviation is derived from two parallel analyses of mixed samples from two mice and is indicated as a vertical line at the top of each bar.
* indicate statistical significance ($p \leq 0.05$) compared to the negative control, using Student T-test.

IL-4 was analysed because increased IL4 levels upon stimulation gives an indication of the type of immune response elicited. A Th2 response is characterized by an up-regulation of IL-4, whereas a Th1 response most likely would leave IL-4 levels unchanged. A Th2 response is most effective against extracellular pathogens and parasites, however the immune response needed to control an infection with *M. tuberculosis* is a Th1 response. Fig. 4.21 shows that IL-4 levels generally were low for all the samples. All the cells stimulated with Ag85B-ESAT6 showed a significant reduction of IL-4 compared to the negative control. None of the PBMCs from immunized mice showed an increase in IL-4 production.

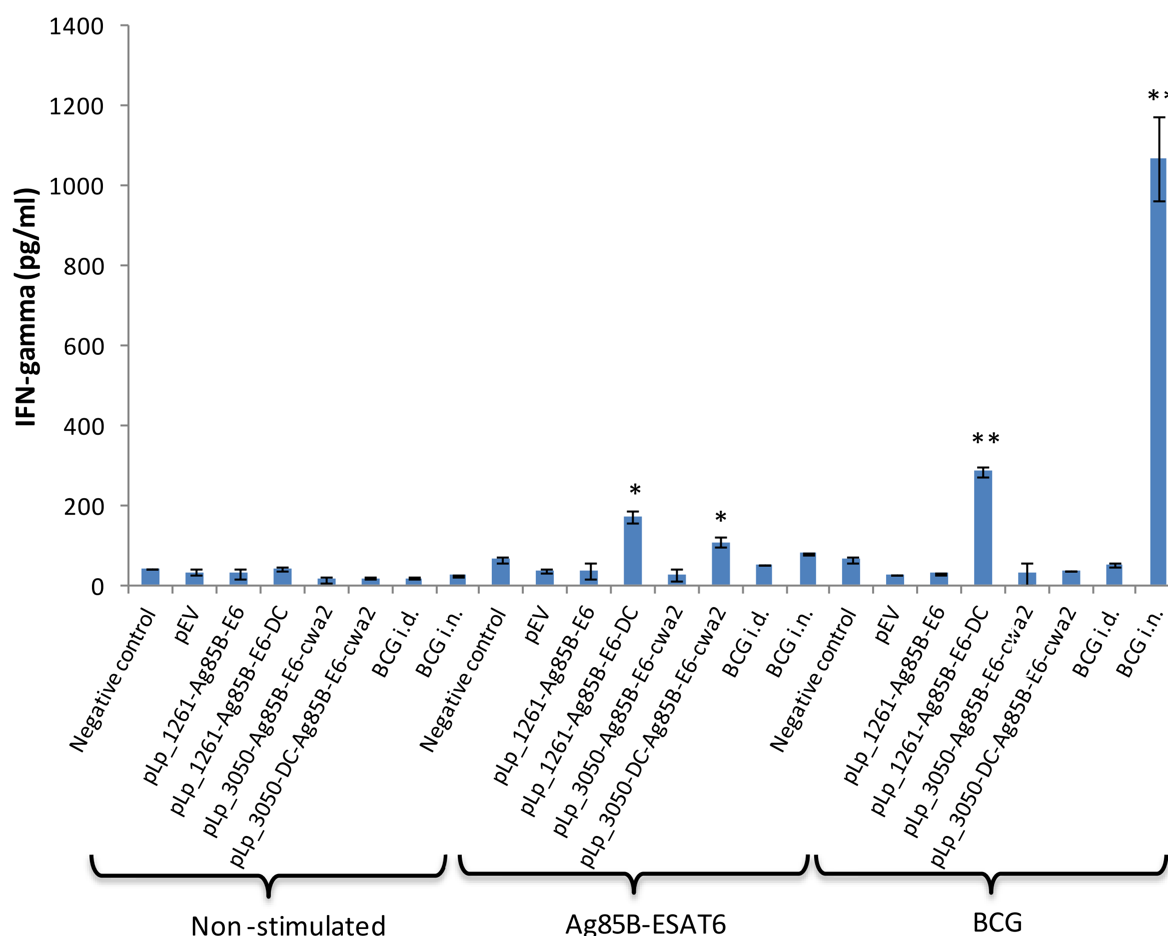


Figure 4.22. Analysis of INF-gamma production by PMBCs. The columns show the amount of IFN-gamma secreted by PBMCs isolated from immunized and control mice. Each well contained approximately $6*10^5$ PMBCs in a total volume of 1ml. The PBMCs were stimulated with BCG, Ag85B-ESAT6 or not stimulated, as indicated by the brackets. The standard deviation is derived from two parallel analyses of mixed samples from two mice and is indicated as a vertical line at the top of each bar.
* and ** indicate statistical significance ($p \leq 0.05$ and $p \leq 0.01$, respectively) compared to the negative control, using Student T-test.

Elevated production of IFN-γ indicates initiation of a Th1 response. The production of IFN-γ by PMBCs is shown in Fig. 4.22. The results show that PMBCs from mice that were intranasally immunized with BCG produced high amounts of IFN-γ, upon stimulation with BCG only. PBMCs from mice immunized with *L. plantarum* harbouring pLp_lipo-DC clearly stand out from the rest, showing a very significant ($p \leq 0.01$) and a slightly less significant ($p \leq 0.05$) production of IFN-γ, upon stimulation with BCG and Ag85B-ESAT6, respectively. PBMCs from pLp_DC-cwa2 immunized mice also show a significant production of IFN-γ ($p \leq 0.05$). Notably, both recombinant LABs giving effects express DC-pep-labeled antigen; the equivalent strains expressing the antigen without the DC-pep did not show these effects.

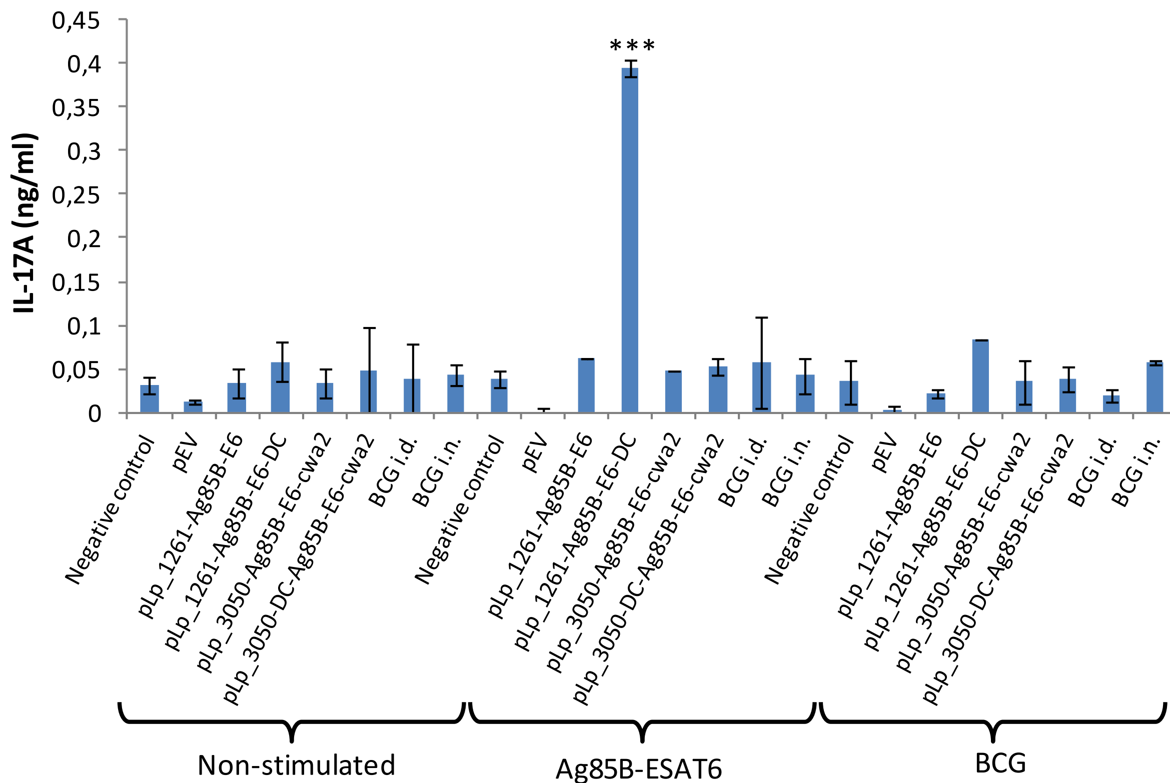


Figure 4.23. Analysis of IL-17A production by PBMCs. The columns show the amount of IL-17A secreted by PBMCs isolated from immunized and control mice. Each well contained approximately $6*10^5$ PMBCs in a total volume of 1ml. The PBMCs were stimulated with BCG, Ag85B-ESAT6 or not stimulated, as indicated by the brackets. The standard deviation is derived from two parallel analyses of mixed samples from two mice and is indicated as a vertical line at the top of each bar.
*** indicates statistical significance ($p \leq 0.001$) compared to the negative control, using Student T-test.

Fig. 4.23 shows IL-17A production by the various groups of PBMCs. IL-17A is a pro-inflammatory cytokine which mainly responds to invasion of extracellular pathogens (Iwakura et al. 2008). Again PMBCs isolated from mice immunized with pLp_lipo-DC stand out. Upon stimulation with antigen, they show a very significantly elevated IL-17A level. In all other samples, IL-17A levels were much lower, without significant differences between the samples.

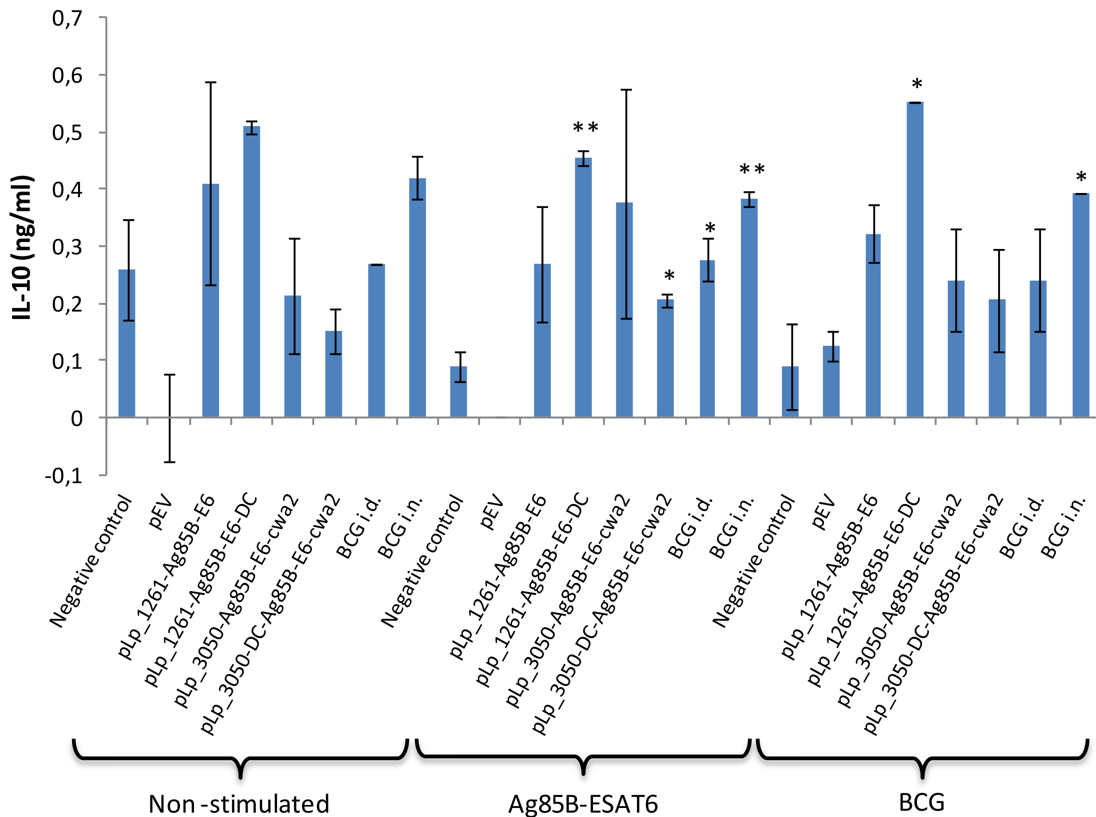


Figure 4.24. Analysis of IL-10 production by PMBCs. The columns show the amount of IL-10 secreted by PBMCs isolated from immunized and control mice. Each well contained approximately $6*10^5$ PMBCs in a total volume of 1ml. The PBMCs were stimulated with BCG, Ag85B-ESAT6 or not stimulated, as indicated by the brackets. The standard deviation is derived from two parallel analyses of mixed samples from two mice and is indicated as a vertical line at the top of each bar.
* and ** indicate statistical significance ($p \leq 0.05$ and $p \leq 0.01$, respectively) compared to the negative control, using Student T-test.

IL-10 is an anti-inflammatory cytokine which down-regulates the Th1 response and up-regulate the Th2 response. Fig. 4.24 shows the results for the analysis of IL-10. All samples from mice immunized with bacteria harbouring antigen or immunized with BCG show elevated values of IL-10 after stimulation with antigen or BCG. pLp_DC-lipo stand out by inducing significant levels of IL-10 both when stimulated with antigen and when stimulated with BCG. When stimulated with antigen, pLp_DC-cwa2 also showed to induce significant production of IL-10. Only PBMC from mice immunized with bacteria harbouring pEV seems to produce a lower amount of IL-10 than the negative control.

Taken together, the results depicted in Figures 4.21 – 4.24 show that pLp_DC-lipo and pLp_DC-caw2 are able to induce pro-inflammatory immune responses, by inducing the production of IFN-γ and IL-17A. However, significantly elevated levels of the anti-inflammatory cytokine IL-10 were also induced by these constructs.

5. DISCUSSION

5.1 Construction of plasmids

A 12 aa peptide (DC-pep) has been shown to bind to dendritic cells (DC) (Curiel et al. 2004) and also to enhance the efficacy of a protective antigen from *Bacillus anthracis* displayed on a lactic acid bacterium (Mohamadzadeh et al. 2009). This DC-seq was fused with the Ag85B-ESAT6 hybrid antigen and expressed in *Lactobacillus plantarum* to hopefully achieve the same effect. Various anchoring methods were tested and constructs were designed to lead to maximum exposure of the DC-peptide.

The DC-seq was successfully fused to the Ag85B-ESAT6 hybrid antigen in the three plasmids pLp_3050-Ag85B-E6 (pLp_sec), pLp_3050-Ag85B-E6-cwa2 (pLp_cwa2) and pLp_1261-Ag85B-E6 (pLp_lipo), constructed for secretion, cell wall anchoring and membrane anchoring of the antigen, respectively. To allow for interaction of the DC-pep with DCs, the DC-seq was inserted in the N-terminal end of cell wall anchored antigen and in the C-terminal end of membrane anchored antigen. Exchanging the 256-replicon with the broad spectrum replicon SH71 was only successfully achieved for the plasmid containing membrane anchored antigen. The colonies obtained during the procedure of changing the replicon in pLp_DC-sec and pLp_DC-cwa2 contained either religated vectors or vectors containing the new replicon but without antigen. Because no correct colonies were obtained after several trials with pLp_DC-sec and pLp_DC-cwa2, the decision was made to only proceed with one SH71-base construct, namely pLp_DC-lipo-sh71. It is known from other studies that plasmids containing the Sh71 replicon are difficult to construct and difficult to handle (Maudal 2012).

5.2 Growth of *L. plantarum* harbouring the different plasmids

Previous studies have shown that heterologous expression and secretion of vaccines in lactobacilli may lead to considerable stress, which is manifested in reduced growth rates and, as discussed further below, protein degradation ((Bolhuis et al. 1999; Fredriksen et al. 2010; Lulko et al. 2007; Mathiesen et al. 2008). Tjåland (2011) had shown that expression and secretion of Ag85B-ESAT6 is no exception. The present results show that addition of the DC-binding peptide did not affect the growth rate (relative to the non-DC-pep containing

counterpart), with the exception of the strains designed for antigen secretion, where addition of DC-pep led to a clear reduction in growth rate. Since the DC-pep occurs immediately after the signal peptide, it is conceivable that its presence affects secretion in a way that causes stress. However, a similar effect was not observed for the strain carrying the cell wall anchored antigen, where the signal peptide is the same and the DC-pep is inserted at the same position. The growth of the two strains for antigen secretion did not diverge until two hours after induction and the growth rate difference will therefore probably not affect the other analyses when the bacteria are harvested two hours after induction.

Interestingly, the growth of *L. plantarum* carrying pLp_DC-lipo-Sh71 was reduced compared to the strain carrying the similar plasmid with the 256-replicon. The SH71 replicon induces a higher copy number of plasmids in the cells (Maudal 2012) which may increase the protein production and this increase might stress the bacteria. A slightly higher production of antigen was also shown in the western blot analysis (Fig. 4.10) for the strain harbouring the SH71 replicon. Concerning the effects of introducing the SH71 replicon, it is important to note that only one such exchange was tested and that the predicted effect on plasmid copy number was not experimentally verified. Furthermore, quantitative effects on protein production were only assessed with Western blots and effects were small. Thus, further work is needed to fully assess the effect of introducing the Sh71 replicon.

5.3 Analysis of Ag85B-ESAT6 production in *Lactobacillus plantarum* by western blotting

Western blot analyses of cell free protein extracts showed that production of Ag85B-ESAT6 was successfully achieved by all the strains and that the DC-pep did not affect the amount of antigen produced, with one exception being the construct for secretion of antigen. The slightly lower amount of antigen produced by the strain harbouring pLp_DC-sec (plasmid for secretion of DC-Ag85B-ESAT6) than the strain harbouring the corresponding plasmid without DC-seq (Fig. 4.7) could be the result of a higher secretion efficiency. The amount of antigen in the supernatant for these strains (Fig 4.8) is in fact slightly higher for the strain harbouring pLp_DC-sec. The difference in growth rate for these constructs, as mentioned earlier, might also be the reason for the slightly lower intracellular production of antigen by the pLp_DC-sec carrying strain.

Degradation products of the antigen were observed in all blots and are likely to be due to the presence of proteases in *L. plantarum.* 19 genes encoding intracellular proteases have been found in *L. plantarum* (Kleerebezem et al. 2003). The translocation capacity of the secretion apparatus is crucial for effective secretion of protein. Too slow translocation of a protein can result in the protein being degraded by intracellular proteases. Increased protein production therefore has to be accompanied by increased efficiency of the secretion apparatus.

The SH71 replicon seems to cause a slightly higher production of protein (Fig. 4.10, lane 3), and the amount of degraded protein seems to be smaller. The lesser amount of degraded protein could be due to a more effective translocation of the protein, but it is difficult to envisage why a change of replicon would cause this. More generally, it is difficult to understand how a higher protein production, most likely causing more stress (as seen by a reduced growth rate) would lead to more efficient translocation and less proteolytic degradation. The results from the flow cytometry analysis (Fig 4.12) support the results from the western blot analysis which indicate higher amounts of extracellular protein. The intensity of a Th1 response in mice, after oral administration of a recombinant antigen producing *L. lactis* strain, has previously been shown to positively correlate with the amount of antigen produced (Bermudez-Humaran et al. 2011). The results for the SH71 construct are therefore promising, considering the possibilities of making a more effective vaccine.

As mentioned in the results, a minor size difference is observed between antigen with and without DC-pep, which appears to be an extra band for the DC-pep antigen. This additional band is also found in the supernatant fraction (Fig 4.8 and 4.10) and it is found for the strain carrying plasmid with SH71 replicon as well. The most plausible reason for the size difference observed in the western blots of the strains with and without DC-pep is the difference constituted by the DC-pep itself which is 1.3 kDa. However, a difference due to the DC-pep is not expected to appear as two bands. Another reason could therefore be that the difference represents processed and unprocessed antigen, which differ by 2 kDa representing the signal peptide.

Ag85B-ESAT6 was found in the supernatant (SN) fraction of all the strains (Fig 4.8).This was not unexpected since the cell wall anchored and membrane anchored antigen were also observed in the supernatant by Tjåland (2011). It has also been observed previously that cell wall and membrane anchored proteins end up in the supernatant (Antelmann et al. 2001;

Fredriksen et al. 2010) and it has been indicated that this could be the result of inefficient retention to the membrane/cell wall or of natural shedding from the membrane and cell wall.

The comparison of antigens in the cell free protein extract (CE) and the supernatant (Fig 4.9) shows that the amount of antigen in the supernatant is considerably lower than in the cell free protein extract, also for the strain harbouring pLp_DC-sec (7 times more SN than CE is loaded onto the gel). The antigen in the CE includes both antigen found inside the cell and antigen anchored to the cell wall or membrane. It is therefore expected that smaller amounts of antigen will be found in the CE for the strain harbouring pLp_DC-sec than for the strains harbouring pLp_DC-cwa2 or pLp_DC-lipo, assuming they produce the same amount of antigen. However, a high amount of antigen is observed in the CE for the strain containing pLp_DC-sec, indicating that the secretion of antigen is not very efficient. The results from the flow cytometry analysis of this strain (Fig. 4.11) indicate that some of the secreted antigen may be retained in the cell wall (see section 5.4), thus contributing to a higher amount in the CE. In theory the signal peptide should not be present in the supernatant as it is supposed to be cleaved off during translocation (Driessen & Nouwen 2008; Kleerebezem et al. 2010; Tjalsma et al. 2004). This means that there should be a slight size difference between antigen found in the CE and the SN as observed for the membrane anchored antigen (Fig. 4.9, lane 6 and 7). This size difference was not observed for the cell wall anchored antigen or the secreted antigen, indicating that the signal peptide has not been cleaved off. However, several bands are observed for all the samples and it is possible that the largest band represent unprocessed antigen whereas the second largest band represent processed antigen.

5.4 Detection of surface anchored Ag85B-ESAT6

Flow cytometry and confocal laser scanning microscopy (CLSM) of induced and FITC-stained *L. plantarum* cells were used to detect Ag85B-ESAT6 presented on the surface of the bacteria. Both flow cytometry and CLSM revealed some degree of stained antigens for all antigen producing constructs tested (Fig. 4.11, 4.12 and 4.13). No fluorescence was detected for *L. plantarum* cells harbouring an empty vector, pEV, which proves that neither the primary nor the FITC-labelled secondary antibody bind non-specifically to other proteins on the surface of *L. plantarum.* The flow cytometry analysis of *L. plantarum* strains harbouring different constructs (Fig. 4.11) showed a similar degree of staining for the strain producing secreted antigen as for the strain producing cell wall anchored antigen. The same result was

obtained by Tjåland (2011) for strains producing the analogous proteins without the DC-pep. This could be due to secreted antigen being trapped or retained in the cell wall of the bacteria. The CLSM images of bacteria harbouring this construct show that several of the cells are stained (Fig.4.13). In comparison, only a few of the cells harbouring cell wall anchored antigen were stained. Flow cytometry is a more sensitive method for detection of fluorescence than CLSM and would probably be able to detect cells with a weaker fluorescent signal which are not visible using CLSM. This could explain why the apparent differences detected with CLSM were not seen with flow cytometry. It could also be possible that the epitope for binding of antibody is more accessible in secreted antigen retained in the cell wall than in the antigen which is anchored to the cell wall.

The flow cytometry (Fig. 4.11 and 4.12) and CLSM analysis (Fig. 4.13) of *L. plantarum* cells producing membrane anchored antigen (pLp_DC-lipo) showed only weak signals. These results are also in accordance with those obtained by Tjåland (2011) for *L. plantarum* producing membrane anchored antigen without the DC-pep. Flow cytometry analysis of *L. plantarum* cells carrying pLp_DC-lipo-sh71 (Fig. 4.12) showed stronger fluorescent signal than cells with pLp_DC-lipo, but still not as strong as the two other strains. The CLSM images of this strain show a stronger fluorescence signal, indicating a higher expression of antigen. These results underpin the findings from the western blot (Fig. 4.10), namely that the strain harbouring the Sh71-construct produces a higher amounts of antigen.

The possibility of the membrane anchored antigen being partially covered or embedded in the cell wall, and consequently hiding the epitope for binding of antibody, was tested by treating the cells with lysozyme. Lysozyme degrades the cell wall and could possibly lead to increased exposure of the antigen hidden in the cell wall. This method had been tested previously by Nygaard (2011) who showed that the weakness of the fluorescent signal from membrane anchored protein could be due to the protein being buried in the cell wall (i.e. treatment with lysozyme improved the signal). All the different *L. plantarum* strains were subjected to treatment with lysozyme and subsequently analysed by flow cytometry and CLSM. The treatment with lysozyme resulted in greatly increased fluorescent signals for both the strains harbouring plasmids for membrane anchoring (Fig.4.13). The signals for the two other strains were also amplified, although not as greatly. It is somewhat remarkable that the cell wall anchored protein also was detected more clearly after lysozyme treatment, since the cell wall is supposed to be disrupted. However, even after treatment with lysozyme, the cell wall might

not necessarily be completely digested and cell wall anchored proteins could remain present, resulting in fluorescent signal. If the Ag85B-ESAT6-cwa2 is hidden in the cell wall and not protruding outwards, lysozyme treatment of the cells could result in easier access to the epitope for the antibody.

Because cells producing secreted antigen gave clear fluorescent signals, the two detection methods used here cannot be used to confirm the anchoring of an antigen. A western blot of the cell wall fraction of strains harbouring cell wall anchored antigen could be used to confirm the anchoring of DC-Ag85B-ESAT6. This was not done in this study; however, Tjåland (2011) performed a western blot of the cell wall fraction of *L. plantarum* strains carrying different plasmids for anchoring of Ag85B-ESAT6, where the presence of Ag85B-ESAT6-cwa2 and Ag85B-ESAT6-cwa3 (Ag85B-ESAT6 containing a shorter anchor than Ag85B-ESAT6-cwa2) were proven. It is therefore likely that the antigen fused with DC-pep is also anchored to the cell wall.

5.5 Storage of induced *L. plantarum*

Freeze drying of induced *L. plantarum* cells was performed in order to investigate the possibility of long term storage of induced bacteria. With the aim of using the bacteria as a vaccine, easy storage is required, especially with regard to distribution in developing countries with poor infrastructure. Compared to the results in the article by Carvalho et al. (2002), which show a 1 % survival rate for freeze dried *L. plantarum* after 2 months of storage, the survival rate for *L. plantarum* strains carrying plasmids for production of antigen was high (5-25 % for the strains carrying plasmids for production of antigen and 68 % for the strain carrying pEV). Interestingly, the pEV-containing strain, i.e. the strain that had not been stressed by the production of antigen before freeze drying, showed the by far highest survival rate, indicating that it is more robust. There are several factors that can affect the survival rate, such as freeze drying medium, drying procedure and the rehydration of the bacteria (Morgan et al. 2006). 100 % survival rate of freeze dried bacteria has been reported when using a special freeze drying technique (Morgan et al. 2004).

Flow cytometry showed that the antigen was still present and accessible at the surface of the bacteria after the freeze drying procedure (Fig. 4.16). These are promising results which show that the antigen is still present on the surface and most likely intact. However, because of time

limitations, the flow cytometry was done on cells stored for only one day. Clearly, further work is needed to assess the effect of long term storage.

5.6 Effect on dendritic cells

The ingestion of antigens and bacteria by DCs is an important step in eliciting an immune response. The internalization level was tested by using a gentamicin assay as previously done with Caco-2 cells by Innocentin, Silvia et al. (2009). The internalization of *Lactobacillus acidophilus* has previously been shown not to be impaired by the expression of DC-pep, meaning DCs are able to internalize bacterial cells containing this peptide (Mohamadzadeh et al. 2009).

The incubation of DCs with *L. plantarum* strains harbouring the two versions of cell wall anchored antigen (DC-AG85B-ESAT6-cwa2 and Ag85B-ESAT6-cwa2) was first tested in order to optimize the conditions for uptake of bacteria. Different amounts of bacteria were added to DCs in order to investigate if the uptake of bacteria could be more effective at a specific ratio or if a high amount of bacteria could saturate the uptake and possibly mask differences in the uptake of the different strains. The different ratios of DCs and bacteria tested were 1:500, 1:50 and 1:5 respectively. The results showed that the highest number of added bacteria gives the highest uptake and that the uptake increases proportionally with the number of added bacteria. This proportional increase indicates that the maximum potential of DCs to ingest bacteria was not exceeded when adding 10^9 bacteria in the assay. The results also showed that strains producing the DC-containing antigen were taken up almost 10 fold more efficiently. This indicates that the DC-pep enhances the uptake of bacteria.

For the strain harbouring pLp_DC-cwa2 the maximum uptake by DCs was reached after 2 hours, and incubation for another 2 hours did not increase the amount of internalized cells. In contrast, the uptake of the strain carrying pLp_cwa2 (without DC-pep) was still increasing after 2 hours, reaching almost the same level as the strain carrying pLp_DC-cwa2 after 4 hours. A possible reason for the early maximum observed for the strain harbouring pLp_DC_cwa2 could be that the DCs have started to degrade the bacteria which were internalized first. This could mask further uptake of bacteria by DCs. In an article by Kolb-Mäurer et al. (2000) the intracellular viability of *Listeria monocytogenes* in DCs was tested. The bacteria were incubated for 1 hour with the DCs before washing and adding gentamicin;

after incubation, a rapid decline in the intracellular viability of the bacteria was shown. Another article showed that after 18 hours of incubation of DCs and *Lactobacillus rhamnosus,* almost all DCs had internalized bacteria and the bacteria showed various stages of degradation (Foligne et al. 2007). To avoid the possibility of masking differences in uptake between the different strains due to degradation of bacteria inside DCs, an incubation time of 1 hour was chosen as standard for the experiments described in this study.

The *L. plantarum* strains expressing cell membrane anchored and cell wall anchored antigens were selected for further testing of internalization in DCs. The strains carrying plasmids for secretion were not included in the internalization studies since the secreted antigen is unlikely to contribute to the uptake of the bacteria. The strain containing pLp_DC-lipo-SH71 was also excluded due to lack of a comparable strain without DC-pep. All the internalization experiments showed higher uptake for the strains expressing DC-pep-labelled Ag85B-ESAT6 than the corresponding strains expressing AG85B-ESAT6 lacking DC-pep. These results were not statistically significant because of variations between individual experiments, but the fact that uptake of strains expressing DC-pep was consistently higher indicates a positive contribution by the DC-pep.

The highest uptake was observed for strains harbouring pLp_DC-lipo, whereas the difference in uptake between the corresponding strains was highest for the strains expressing cell wall anchored antigen. This indicates a greater effect of the DC-pep when attached to a cell wall anchored antigen. As the results from the flow cytometry and CLSM analysis indicated, the membrane anchored antigen could be embedded in the cell wall, thereby hiding the DC-pep and obstructing interaction with DCs. The differences in uptake between the strains expressing cell wall anchored antigens and strains expressing membrane anchored antigens could be due to their difference in growth rate. Strains expressing membrane anchored antigens grew much better than strains expressing cell wall anchored antigens (Fig. 4.6). It is possible that the bacteria could multiply during incubation with dendritic cells, resulting in a higher number of bacteria for the faster growing strain than for the slower growing strain. Based on the numbers in Fig. 4.19, the amount of ingested bacteria is approximately 7 bacteria taken up per 100 DCs for the strain expressing membrane anchored antigen. This is slightly lower compared to a similar study on internalization of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 11129 by DCs, which showed an internalization of 15.8 and 11.5 bacteria per 100 DCs after 30 min of incubation, for *S. aureus* and *E. coli*,

respectively (Nagl et al. 2002). As far as I'm aware, similar experiments have not previously been performed with LAB, thus making it difficult to compare the present results with the results of other studies.

After uptake of bacteria or antigen the DCs will start to mature (Drutman & Trombetta 2010), which is crucial for the initiation of an immune response. During maturation of DCs, several specific molecules involved in the priming of T-cells will be strongly up-regulated. The maturation of DCs incubated with *L. plantarum* strains harbouring different plasmids for production of AG85B-ESAT6 was analysed. DCs were incubated with fluorescent-labelled antibodies specific for the maturation markers CD80, CD83, CD86 and MHC-class II before analysis by flow cytometry. Only the analysis of CD83 showed maturation for some of the strains tested, whereas the analysis of the other markers showed little to no difference between the negative and positive control. The results for CD83 show that all *L. plantarum* strains harbouring plasmids were able to induce expression of this marker, including the strain harbouring pEV. Studies have shown that lactic acid bacteria are able to stimulate expression of the maturation markers CD40, CD86 and MHC-class II. Zoumpopoulou et al. (2009) showed that the three strains *Lactobacillus acidophilus* NCFM*, Lactococcus lactis* MG1363 and *Streptococcus macedonicus* ACA-DC 198 were able to induce a strong up-regulation of CD40 and CD86 on the surface of DCs, whereas the three strains *Lactobacillus rhamnosus* Lr32*, Lactobacillus salivarius* Ls33 and *Lactobacillus fermentum* ACA-DC 179 only induced very low expression of the same markers. One study showed that incubation of DCs with *L. casei* induced a strong up-regulation of MHC-class II and CD86 markers, while incubation with *L. reuteri* only led to a moderate up-regulation. *L. plantarum* 299v and Lb1 were found to induce a high expression of CD86 and a moderate to high expression of MHC-class II (Christensen et al. 2002).

Due to the fact that the antigen producing strains were no better than the pEV in inducing maturation, it appears to be the bacteria itself that are responsible for the induction of maturation. This demonstrates the ability of *L. plantarum* WCFS1to induce an immune response, which may be a beneficial feature for a vaccine delivery vector. The reason for the lacking expression of the maturation markers CD80, CD86 and MHC-class II on DCs in this experiment might be caused by lack of development of DCs from CD14+ cells. It was observed that some of the CD14+ cells isolated for this experiment not had developed into

DCs which resulted in lower number of DCs than expected (pers. comm. Charlotte Kleiveland, IKBM).

5.7 Cytokine response in mice

The kind of immune response which is stimulated can be measured by analysing cytokines released by immune cells. Cytokines govern the immune response by controlling which types of immune cells are activated. The immune response wanted during an infection is the Th1 response which is characterized by the release of the cytokines IFN-γ, IL-2, TNF-α and TNF-β which further activates macrophages that can kill the pathogen (Szabo et al. 2003).

The ability of *L. plantarum* strains harbouring different plasmids for production of Ag85B-ESAT6 to elicit an immune response was tested in mice experiments done by Rajna Dimitrijevic at lnstitute of Virology, Vaccines and Sera, Torlak, Belgrade. The mice were immunized 3 times with the different *L. plantarum* strains, with BCG as a positive control and non-immunized mice as a negative control. To assess the immune response, PBMC from the immunized mice were isolated and subsequently stimulated with Ag85B-ESAT6 or BCG. In mice showing a desirable immune response, one would expect to see elevated levels of pro-inflammatory cytokines. The cytokine response was analysed by ELISA.

All of the PMBC from mice treated with either *L. plantarum* or BCG produce the same or less amount of IL-4 than the negative control (Fig. 4.21). The cytokine IL-4 stimulates the Th2 response, therefore an unchanged production of this cytokine indicates that the Th2 response is not up-regulated. The IFN-gamma production by PBMCs (Fig 4.22) shows that *L. plantarum* strains expressing DC-AG85B-ESAT6 were the only strains which were able to induce a significant ($p \leq 0.01$ and $p \leq 0.05$ for pLp_DC-lipo and pLp_DC-cwa2, respectively) increase of IFN-γ compared to the negative control. The positive control (BCG) was able to induce a significant production of IFN-γ when the PBMCs were stimulated with BCG, but not when stimulated with Ag85B-ESAT6. These are promising results since IFN-γ is mainly produced by Th1 cells, natural killer (NK) cells and cytotoxic T-cells and its production is stimulated by the cytokines TNF-α and IL-12 (Boehm et al. 1997). An increase in IFN-γ concentration thus indicates an activation of the Th1 response.

A significant increase in IL-17A production was only achieved in PBMCs from mice immunized with the strain harbouring pLp_DC-lipo which showed to induce a clearly higher

production than the other strains. IL-17A is a cytokine which is produced by a recently discovered subgroup of CD4+ cells, the Th17 cells. These cells produce the cytokines IL-17A, IL-17F, IL-21 and IL-22 and it is suggested that they are involved in allergic and autoimmune responses and are also important in the defence against infections (Iwakura et al. 2008). The protection against infections require a balance between the Th subsets Th1 and Th17, it is suggested that cytokines produced by these Th subsets are important factors in memory responses to mycobacteria (Hawkridge et al. 2008; Scriba et al. 2008). It has also been suggested that production of IL-17 may be a good indicator of effective vaccination against tuberculosis (Romano et al. 2006; Vordermeier et al. 2009) and that IL-17 is important for the recruitment of Th1 cells producing IFN-γ (Khader et al. 2007). These observations indicate a potentially successful vaccination by the use of the pLp_DC-lipo harbouring strain.

All the samples except PBMCs from mice immunized with bacteria carrying pEV showed an up-regulation of IL-10 (Fig. 4.24). This cytokine has anti-inflammatory abilities and acts by down-regulating the Th1 response and enhancing the Th2 response. In addition to being produced by Th2 cells, IL-10 is also produced in small amounts by Th1 cells, Th17 cells, DCs and macrophages, among others, and it works as a negative feedback on the Th1 response (Saraiva & O'Garra 2010). The results from this experiment are slightly contradictory to the results for IFN-γ and IL-17A; however, it was not observed an increase in IL-4 levels which would have been expected if a Th2 response was initiated.

Based on these experiments, the *L. plantarum* strain expressing membrane anchored DC-Ag85B-ESAT6 is the most promising strain to consider as a vaccine candidate. This was the only strain which led to a significant increase in both IFN-γ, which indicates an up-regulated Th1 response, and IL-17A, which may indicate successful vaccination. Both these cytokines promote inflammation which involves killing of microbes and cells infected with microbes. The strain carrying pLp_DC-cwa2 also led to a significant up-regulation of IFN-γ, but not of IL-17A, in stimulated PMBCs. The observation that only the strains harbouring DC-Ag85B-ESAT6 were able to elicit significant up-regulation of IFN-γ and IL-17A implies that the DC-pep is of great significance for activation of an immune response.

5.8 Conclusion and future perspectives

This thesis describes successful insertion of the DC-seq in plasmids for secretion and display of the fusion protein Ag85B-ESAT6 in *L. plantarum*. Putatively surface anchored DC-Ag85B-ESAT6 was detected using flow cytometry and Confocal Laser Scanning Microscopy. Functional tests of strains carrying various antigen variants showed that the DC-pep enhances the uptake of bacteria by DCs. Another experiment that could be done is to test the effect of the DC-pep on internalization of bacteria is a so-called competition experiment. This experiment involves the addition of single DC-pep to DCs before addition of bacteria. In theory, the DC-pep will bind to and occupy the corresponding receptors on the DCs. This is supposed to even out the differences observed between constructs with and without DC-pep and thus confirm the effect of the DC-pep.

The positive effect of the DC-pep was clearly evident in the results from the mice trial, where only the strains harbouring DC-Ag85B-ESAT6 were able to elicit a significant production of IFN-γ and IL-17A. The construct for membrane anchoring of DC-Ag85B-ESAT6 stands out in both the internalization assay and the mice trials. The strain harbouring this construct showed the highest internalization number and elicited the highest concentrations of IFN-γ and IL-17A, respectively. These results make the continued development of this particular construct promising. The construct pLp_DC-lipo-sh71 was not included in the internalization assay or the mice trials, but it would be interesting to compare the two constructs, pLp_DC-lipo and pLp_DC-lipo-sh71, in order to analyse the possible effects of the replicon change.

Although *L. plantarum* is able to persist for several days in the human GI-tract (Vesa et al. 2000), which is a positive characteristic for a vaccine delivery vector, other host lactic acid bacteria should also be tested, especially since different LAB have been observed to induce various degrees of maturation in DCs (Christensen et al. 2002; Zoumpopoulou et al. 2009). Unfortunately, the experiment regarding maturation of DCs didn't yield significant results for any of the constructs and should be repeated. However, the results from the mice trial indicate that the strains harbouring DC-Ag85B-ESAT6 are able to elicit a correct immune response.

Based on the results that pLp_DC-lipo elicits the strongest immune response, it would be interesting to construct plasmids for production of antigen anchored with other types of membrane anchors, like for example transmembrane anchors.

All in all, the results from this study are very promising regarding the prospects of developing a mucosal vaccine against *M. tuberculosis*. The results certainly warrant further work on this system. Regarding the use of these vectors as a vaccine in humans, it is obvious that more modifications are needed. This includes removal of the antibiotic resistance gene and, possibly, insertion of the gene for heterologous protein production into the genome of the bacterium in order to avoid the use of an inducer peptide.

A future challenge in this project is to not only make a vaccine that is comparable to the BCG vaccine, but to make it better.

6. REFERENCES

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APPENDIX

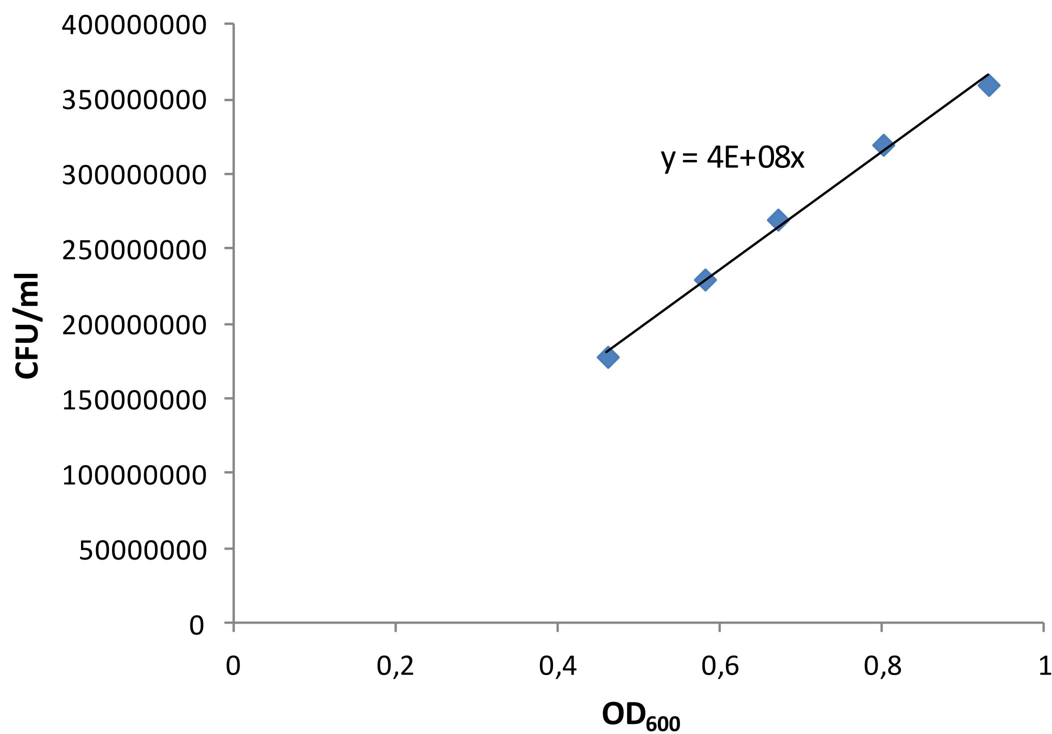

Figure A.1. The relationship between OD_{600} and CFU/ml for *L. plantarum* harbouring pEV. This graph was used to calculate approximately the same number of cells based on the OD_{600}-value at the time of harvest for *L. plantarum* harbouring constructs for production of antigen. This graph was provided by Geir Mathiesen.