SECRETION AND ANCHORING OF MYCOBACTERIUM TUBERCULOSIS ANTIGENS IN LACTOBACILLUS PLANTARUM

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Abstract

The aim of this study was to develop the LAB *Lactobacillus plantarum* WCFS1 as a delivery-vehicle for a human vaccine against infection by Mycobacterium tuberculosis. Because lactic acid bacteria have a long record of safe oral consumption of lactic acid bacteria and are natural inhabitants of the human gastrointestinal tract, these bacteria are being extensively studied as potential vectors for production and *in situ* delivery of heterologous proteins. *Lactobacillus plantarum* WCFS1is an interesting candidate for this purpose due to its ability to survive the passage of the stomach and to persist in the human gastrointestinal tract for up to seven days. Mucosal immunity plays a major role in the prevention of infectious diseases, and the use of live bacterial vectors for mucosal delivery of therapeutic molecules have been successful in several studies using animal models.

In the present study, intracellular and extracellular production of Ag85B-ESAT6, a fusion protein consisting of two major antigens from *Mycobacterium tuberculosis*, was achieved in *L. plantarum.* Secretion was accomplished using homologous signal peptides from the genome of *L. plantarum* WCFS1. The secretion efficiency was found to vary depending of the signal peptide, thereby emphasising the value of evaluating several signal peptides when selecting a signal peptide for secretion of a heterologous protein. The Ag85B-ESAT6 fusion protein was also targeted for covalent cell wall-anchoring using a LPXTG anchor from *L. plantarum*. In addition attempts were made to anchor Ag85B-ESAT6 to the cell membrane through the anchoring domain of a *L. plantarum* lipoprotein. Analysis of the presence of antigens on the cell surface showed that the putatively cell membrane-anchored, cell wall-anchored and secreted version of Ag85B-ESAT6 were present in nearly equal amounts, leaving the question whether the anchors contribute to cell wall localisation somewhat non-conclusive. *L. plantarum* harbouring the various plasmids for production of Ag85B-ESAT6 or the empty vector (pSIP-EV) was all able to elicit a cytokine response when co-incubated with dendritic cells, indicating the ability of these recombinant strains and *L. plantarum* itself to provoke an immune response in a future host.

This thesis shows that *L. plantarum* WCFS1 is able to produce, secrete and likely anchor the *M. tuberculosis* fusion protein Ag85B-ESAT6, by utilising homologous signal peptides and anchors. The present results are promising with respect to use of *L. plantarum* as a vaccine delivery vector.

Sammendrag

Målet med dette studiet var å utvikle melkesyrebakterien *Lactobacillus plantarum* WCFS1 som en vektor for levering av vaksineantigener mot infeksjon av *Mycobacterium tuberculosis*. Melkesyrebakterier blitt grundig undersøkt for potensiell bruk som vektorer for produksjon og *in situ* levering av heterologe proteiner , som en følge av den lange historien for bruk av trygge melkesyrebakterier i næringsmidler og deres naturlige tilstedeværelse i det humane mage-tarm systemet. *L. plantarum* WCFS1 er en interessant kandidat i dette henseende på grunn av denne stammens evne til å overleve ferden gjennom fordøyelsessystemet, samt evnen til å bli værende i det humane mage-tarm systemet i opptil syv dager. Mucosal immunitet spiller en stor rolle i forebyggingen av smittsomme sykdommer, og bruk av levende bakterievektorer for levering av terapeutiske molekyler til mucosa har vært vellykket i flere studier på dyremodeller.

I dette studiet ble intracellulær og ekstracellulær produksjon av Ag85B-ESAT6, et protein bestående av to viktige antigener fra *Mycobacterium tuberculosis*, oppnådd i *L. plantarum.* Homologe signalpeptider fra *L. plantarum* WCFS1 ble benyttet for sekresjon av Ag85B-ESAT6 og sekresjonseffektiviteten viste seg å variere for de ulike signalpeptidene. Å evaluere flere signalpeptider i letingen etter det optimale signalpeptid for sekresjon av heterologe proteiner kan derfor være av stor verdi. Muligheten for kovalent ankring av Ag85B-ESAT6 til celleveggen via ett LPXTG ankringsmotiv, samt ankring til cellemembranen via ett lipoprotein-anker, ble også undersøkt. En analyse over tilstedeværelsen av Ag85B-ESAT6 på celleoverflaten viste at de antatt cellemembran- eller celleveggankrede antigenene var til stede på celleoverflaten. Ankring av Ag85B-ESAT6 kunne ikke fullstendig bekreftes siden sekretert antigen også ble funnet på celleoverflaten. Stammer av *L. plantarum* med plasmider for produksjon av Ag85B-ESAT6 eller tom vektor (pSIP-EV) var alle i stand til å frembringe en cytokinrespons hos dendrittiske celler. Dette indikerer at disse re-kombinerte stammene, og *L. plantarum* i seg selv, har evne til å fremprovosere en immunrespons i en fremtidig vert.

Denne studien viser at *L. plantarum WCFS1* er i stand til å produsere, sekretere, og sannsynligvis også ankre Ag85B-ESAT6, ett antigen sammensatt av to *M. tuberculosis* proteiner, ved å benytte homologe signalpeptider og ankere. De nåværende resultatene er lovende med hensyn til bruk av *L. plantarum* som en vektor for levering av vaksiner.

Abbreviations

APC	Antigen presenting cell
BSA	Bovine Serum Albumin
BCG	Bacillus Calmette-Guérin
DC	Dendritic cell
dNTP	Deoxyribonucleotide triphosphate
ddNTP	Di- deoxyribonucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
GI tract	Gastrointestinal tract
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRAS	Generally Recognised As Safe
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL-4	Interleukin-4
IL-10	Interleukin-10
IL-12	Interleukin-12
LAB	Lactic acid bacteria
M cell	Microfold cell
MALT	Mucosa-associated lymphoid tissue
MCS	Multiple cloning site
MHC	Major Histocompatibility Complex
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
sIgA	Secreted immunoglobulin A
SP	Signal peptide
SPase	Signal peptidase
TB	Tuberculosis
TNF-alpha	Tumour necrosis factor-alpha

CONTENTS

1. INTRODUCTION

Lactic acid bacteria (LAB) are known for their widespread use in the food industry and have through ages been utilised to preserve and ferment food and beverages. LAB have a very long record of safe oral consumption and are recognised as natural inhabitants of the human gastrointestinal tract, where some strains are thought to possibly act as probiotics. Most LAB are rather acid resistance and certain strains have the ability to effectively survive the passage of the stomach. LAB are considered as potential live bacterial vectors for the delivery of antigens and other therapeutic proteins and several expressions systems for production of heterologous proteins have been developed. The goal of the research described in this thesis was to explore possibilities to develop the LAB *Lactobacillus plantarum* as a delivery-vehicle for a human vaccine against infection by *Mycobacterium tuberculosi*s.

1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are Gram-positive, non-sporeforming cocci, coccobacilli or rods that are able to grow both anaerobically and aerobically. Functionally LAB are described as a group of organisms able to ferment hexose sugars to produce primarily lactic acid. LAB include industrially important genera such as *Lactococcus, Enterococcus, Oenococcus, Pediococcus, Streptococcus, Leuconostoc,* and *Lactobacillus* species (Makarova et al. 2006). Most lactic acid bacteria are considered to be non-pathogenic and non-invasive bacteria with GRAS (Generally Recognised As Safe) status, and are among the most important group of microorganisms used in fermentation and preservation of food products. Acidification caused by production of lactic acid during sugar fermentation is the main reason for the preservative effect of LAB. The resulting low-pH conditions inhibit the growth of microorganisms that normally cause food spoilage (Todar 2008). LAB can also produce bacteriocins, ribosomally synthesised antimicrobial peptides able to inhibit growth of competing Gram-positive microorganisms (Eijsink et al. 2002).

The genus *Lactobacillus* encompasses high phylogenetic and functional diversity and its members have been recognized as potentially health promoting microorganism in the human gastrointestinal tract, also known as probiotics (Kleerebezem et al. 2010). The World Health

organization defines probiotics as “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO 2002). During the last decade a number of *Lactobacillus* genome sequences have been completed, including some of the genome sequences of the species and strains thought to be probiotic. It has been found that the extracellular characteristics of different species and strains of lactobacilli are of particular importance for their ability to interact with and respond to the conditions in the gastrointestinal (GI) tract (Kleerebezem et al. 2010). Due to their GRAS status and their potential to act as *in situ* delivery factors in humans, lactobacilli have in recent years been engineered to function as expression and secretion hosts of heterologous proteins, such as antigens, enzymes, therapeutic single-chain antibodies and pathogen receptors (Turner et al. 2004).

1.1.1 *Lactobacillus plantarum* WCFS1

Lactobacillus plantarum is a versatile and widespread microorganism found in a range of environments, including dairy, vegetable, and meat fermentations as well as in the human GI-tract (Kleerebezem et al. 2003). *L. plantarum* has been shown to survive the passage of the stomach, and is considered a natural inhabitant of the GI tract, where it is able to persist for up to seven days (de Vries et al. 2006; Vesa et al. 2000). Some strains of *L. plantarum* are marketed as probiotics, and claimed to have health promoting activities, through interactions with the human GI- tract (de Vries et al. 2006). Attachment of probiotic bacteria to intestinal mucosal cells in the human GI- tract might result in modulation of host cell responses toward a desired immune response, and exclude pathogens as a result of competition. Predictions of the exoproteome of *L. plantarum* WCFS1 revealed twelve proteins that putatively could be involved in adherence to host components such as collagen and mucin in the human GI-tract (Kleerebezem et al. 2010). In probiotic bacteria such adherence factors are believed to play an important role in their health stimulating interactions with the host, including their persistence in the GI tract and the exclusion of pathogens by competition (Marco et al. 2006). Some strains of *L. plantarum* have been shown to give strong adjuvant effect on the mucosal and systemic immune response (Krüger et al. 2002). Proteins exposed on the surface of the bacterial cell is thought to be of great importance to the immunogenicity of the bacteria (Boekhorst et al. 2006). This effect and the strains ability to survive for several days in the human gut have caused *L. plantarum* to be considered as carriers of oral vaccines.
L. plantarum WCFS1 was the first *Lactobacillus* strain to be sequenced(Kleerebezem et al. 2003). It originates from a single colony isolate of the strain *L. plantarum* NCIMB8826,

which was originally isolated from human saliva. *L. plantarum* WCFS1 contains a single, relatively large, circular chromosome (3,3 Mb) with an overall GC-content of 44,5 % (Kleerebezem et al. 2003). Predicted proteins in this strains show similarity with proteins predicted in other low- GC Gram positive bacteria, such as genomes from *Listeria*, *Streptococcus*, and *Lactococcus*. The species adaptability to a variety of environmental niches is reflected in the genome, harbouring relatively large numbers of regulatory and transport functions. The chromosome contains 3, 052 putative proteins-encoding genes, and more than 200 of the proteins are thought to be extracellular proteins. 144 proteins contain potential signal peptidase cleavage sites. Twenty-five of these 144 proteins are predicted to be covalently anchored to the cell wall through a C-terminal LPXTG- type motive, which in *L. plantarum* has the distinct consensus sequence, LPQTXE, found in twenty-two predicted proteins. Forty-eight of these 144 extracellular proteins were found to contain an N-terminal lipobox, a common mechanism for secretion and membrane attachment of proteins through covalent binding to lipids in the cell membrane (Boekhorst et al. 2006).

The last decade, several *Lactobacillus* species, such as *L. plantarum*, have been subjected to genetic studies to gain insight in the underlying mechanisms for their interactions with the environment and their probiotic effects. Also, efforts have been made to develop efficient and well-regulated systems for gene expression for the purpose of creating reliable systems for food-grade and potentially *in situ* production of heterologous proteins.

1.2 Inducible gene expression in *L. plantarum* using the pSIP-system

Vectors for high-level expression of target genes, the so called pSIP-system, have been developed for inducible gene expression in *L. plantarum* and *Lactobacillus sakei* (Mathiesen et al. 2004; Sørvig et al. 2005). The system uses regulated promoters of operons involved in the production of the class II bacteriocins sakacin A and sakacin P in strains of *L. sakei.* Promoter activity is controlled by a two-component signal transduction system that responds to a separately produced and secreted peptide pheromone (Sørvig et al. 2005). In nature, this unmodified peptide pheromone has no antimicrobial activity and is solely dedicated to induce production of class II bacteriocins in a cell-density dependent manner (Eijsink et al. 2002). In the pSIP vector system used in the present study expression of the gene of interest is controlled by the promoter of the Sakacin P structural gene (*sppA*) called P_{sppA} , whereas

expression of the two-component system needed to activate this promoter is controlled by its natural promoter P_{sppIP} (Figure 1.1)(Eijsink et al. 1996; Sørvig et al. 2005). The SppIP-pheromone activates the two-component system, which again activates both the promoters. The pheromone encoding gene, which is located upstream of and in the same operon as the two-component system in natural bacteriocins producers, is not included in the vector-system. Because of this and because expression of the gene of interest is controlled by the tightly controlled P_{sppA} promoter, the SIP-system permits strict regulation of gene expression, with low basal activity. Mathiesen et al. (2004) were among the first to show that the regulated promoters and regulatory genes from the sakacin P system allow controlled overproduction of heterologous proteins in *L. plantarum.*

Several pSIPvectors with different properties have been designed (Sørvig et al. 2003; Sørvig et al. 2005). Previous studies indicate that the sakacin P based vector pSIP401 is one of the most promising (Sørvig et al. 2005), and derivative vectors based on the pSIP401 plasmid (Figure 1.1) are currently used. In addition to the regulatory genes (*sppK* and *sppR*) and inducible promoters (P_{sppIP} and P_{sppA}), the vector contains two origins of replication (one for *E. coli* and one for lactobacilli), an erythromycin resistance gene (*ermB*) and a multiple cloning site.

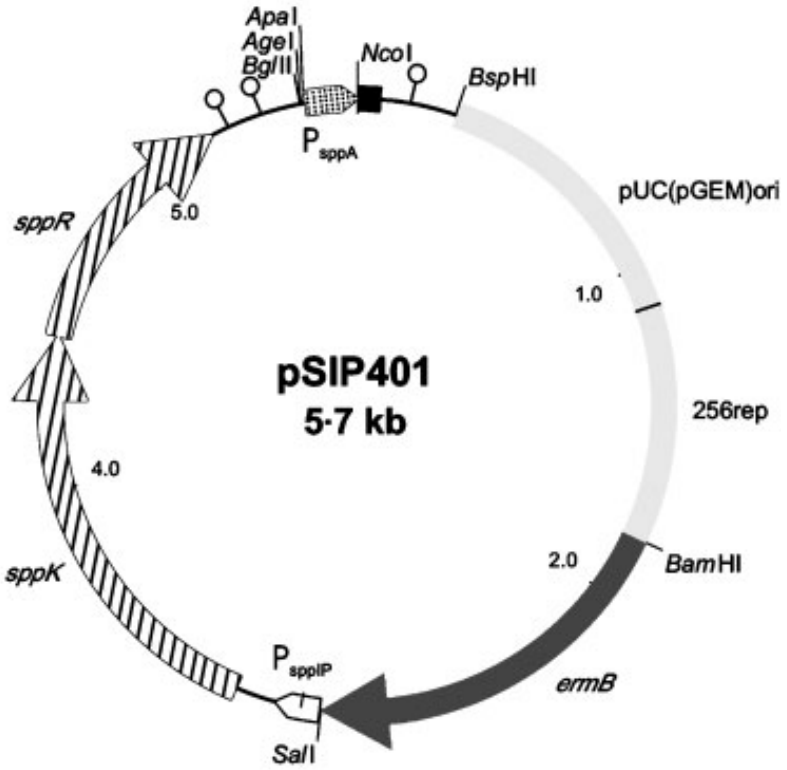

Figure 1.1: Graphic representation of pSIP401. The light-grey regions are replication determinants; dark-grey region, erythromycin resistance marker; vertically hatched regions, histidine protein kinase (*sppK*) and response regulator (*sppR*) genes; dotted regions, inducible P_{sppA} promoter; white region, inducible *sppIP* promoter; lollypop structures, transcriptional terminator; black box, multiple cloning site. The gene of interest may be translationally coupled to the P_{sppA} promoter by using the *Nco*I cloning site. The figure is taken from Sørvig et al. (2005)

The pSIP- system was adapted for expression of secreted proteins by Mathiesen et al. (2008). By incorporating a cassette (Figure 1.2) that allows easy exchange of signal peptides and target gene the pUsp45 vector was constructed, a derivative of pSIP401. The *NcoI* site of pSIP401 was replaced by an *NdeI* site for incorporation of the secretion cassette downstream of the P_{sppA} promoter. In addition a *SalI* restriction site found in the P_{sppIP} promoter of pSIP401 was removed to enable the use of a unique *SalI* site in the secretion cassette. Easy exchange of the various elements of the pUsp45 vector is achieved by utilising the *NdeI* and *SalI* restriction sites and the multiple cloning site (MCS) downstream of the target gene (Figure 1.2).

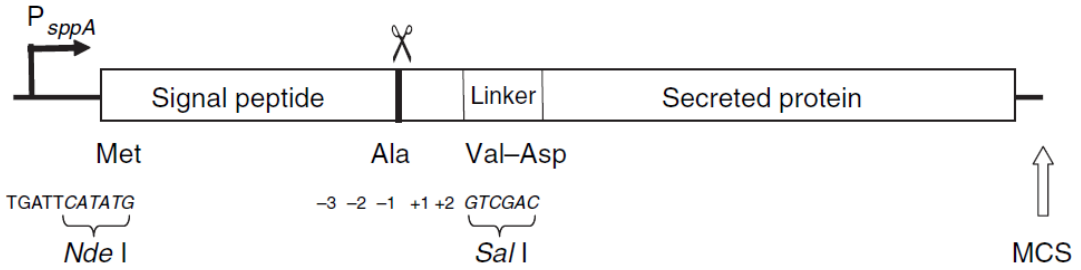

Figure 1.2: Schematic overview of the secretion cassette. The signal peptide (SP) is translationally fused to the P_{sppA} promoter and includes the first two amino acids of its native gene product. A linker encoding the amino acids valine (Val) and aspartic acid (Asp) was inserted to create a *SalI* restriction site. MCS indicates the multiple cloning site (the same as in Figure 1.1). The figure was taken from Mathiesen et al. (2008)

1.3 Secretion of proteins in Gram-positive bacteria

In Gram-positive bacteria, proteins can be transported across the cytoplasmic membrane and released directly into the extracellular environment due to the lack of an outer membrane. Gram-positive bacteria are therefore considered as promising host organism for the secretory production of heterologous proteins (Caspers et al. 2010). Several different mechanisms for protein transport are known. In Gram-positive bacteria, seven main mechanism for secretion of proteins have been characterized (Kleerebezem et al. 2010).

I. Secretion via the Sec pathway
II. Twin- arginine translocation (Tat pathway)
III. Flagella export apparatus (FEA pathway)
IV. Fimbrillin-protein exporter (FPE pathway)
V. Holin-system
VI. Peptide efflux ABC-transporters
VII. WXG100 secretion system (Wss pathway)

The Sec pathway (I) is the major protein secretion pathway in Gram positive bacteria and utilizes N-terminal signal peptides with conserved features (Kleerebezem et al. 2010) This pathway is utilised in the pSIP-system and discussed in more detail below. The Tat pathway (II) is used to transport fully folded proteins and employs signal peptides with a highly conserved twin-arginine motif. The signal peptides are generally longer than the signal peptides utilized in the Sec-pathway (Dilks et al. 2003). The FEA pathway (III) is involved in translocation of flagellar components in Gram positive bacteria, but is also used for secretion

of extracellular virulence factors in two known Gram positive bacterial species (Wooldridge 2009b). The FPE pathway (IV) is thought to be involved in the translocation of precursors involved in the development of bacterial competence allowing uptake of exogenous DNA over the cytoplasmic membrane (Kleerebezem et al. 2010). Holins (V) are integral transmembrane proteins that constitutes a specialized secretion system found only in Gram positive bacteria (Fagerlund et al. 2010). The holin system is involved in autolysis and consists of small integral membrane proteins that secrete murein hydrolases lacking signal peptides (Kleerebezem et al. 2010). The peptide efflux ABC transporters (VI) are a subfamily of the ATP-binding cassette transporters, which use ATP to drive export. They are primarily used to export antimicrobial peptides, such as bacteriocins. Another specialized secretion system found in Gram positive bacteria is the Wss pathway (VII) involved in transport of WXG100 (ESAT-6) family proteins.(Fagerlund et al. 2010).

Kleerebezem et al. (2010) evaluated the presence of these pathways in 13 published *Lactobacillus* genomes by performing searches for sequence homology and protein domains. This study revealed that these genomes do contain genes encoding the Sec, FPE, peptide-efflux ABC and holin systems. On the other hand, genes encoding the main factors involved in the Tat, FEA and Wss protein secretion pathways are not present.

1.3.1 The major secretion pathway: Sec

The major protein transfer system in Gram-positive bacteria is based on the Sec translocase. The Sec translocase is a protein complex found in the cytoplasmic membrane that translocates targeted precursor proteins across the membrane (Kleerebezem et al. 2010). The Sec-pathway utilises N-terminal signal peptides that are characterised by a positively charged N-terminal region, a hydrophobic core region, and the more polar C-terminal region containing the signal peptidase cleavage site (Driessen & Nouwen 2008). A model of the Sec-dependent secretion system is shown in Figure 1.3. Secretory proteins are synthesized on the ribosomes as a preprotein with an N-terminal signal peptide. The polypeptide is recognized by a signal recognition particle (SRP) that together with the FtsY-protein keeps the preprotein in a translocation competent state and facilitate targeting of the polypeptide to the membrane located translocase (Tjalsma et al. 2004). The ATP-dependent motor protein SecA recognises the preprotein indirectly through SecY. The translocation of the preprotein is driven by ATP hydrolysis at SecA and by the proton motif force (PMF) over the membrane. The stepwise

translocation process is initiated by the binding of ATP to SecA. The resulting conformational changes in SecA allow the insertion of a hairpin-like loop structure formed by the signal peptide and the N-terminal region of the protein into the translocation pore formed by SecYEG. The bound preprotein is released from SecA as a result of ATP hydrolysis. Further translocation is driven by cycles of ATP binding and hydrolysis by SecA and the PMF over the membrane (Driessen & Nouwen 2008).

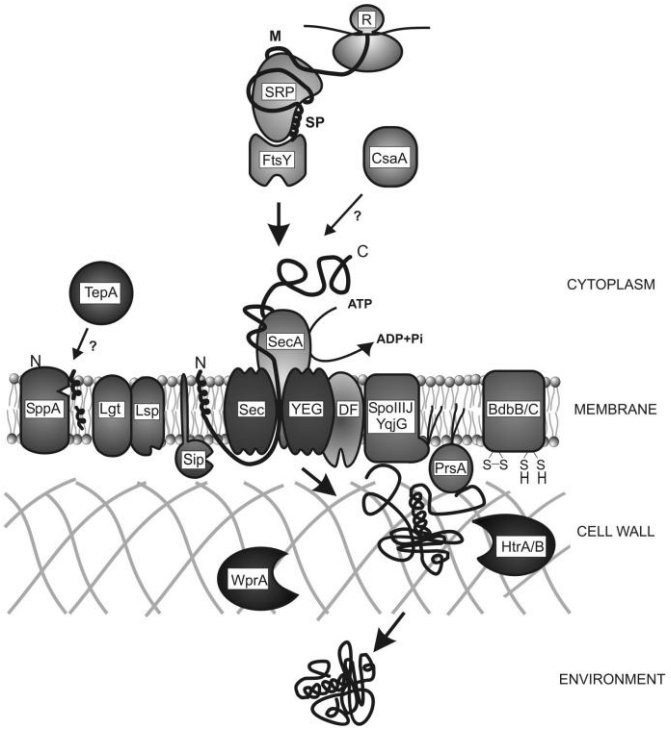

Figure 1.3: Main components of the Sec-dependent protein secretion system in the Gram positive bacterium *B. subtilis*. Targeted preprotein is translocated across the cytoplasmic membrane and secreted to the extracellular environment (further details in the text). The complex consists of a protein-conducting channel (SecYEG) embedded in the membrane and an ATPase motor protein (SecA). Genes encoding the SecDF protein appear to be absent in all *Lactobacillus* genomes, including *L. plantarum* WCFS1(Kleerebezem et al. 2010). The figure is taken from Tjalsma et al. (2004)

The signal peptide is cleaved off during or shortly after translocation by a membrane bound enzyme, signal peptidase (SPase, or Sip as in the Figure 1.3)(Tjalsma et al. 2004). Due to the hydrophobicity of the signal peptide and its charged N-terminal end, the preprotein is temporary arrested in the cell membrane (Figure 1.3). The cleavage site is thereby positioned in the proximity of the SPase located on the periplasmic face of the membrane, which then breaks the peptide bond between the signal peptide and the mature protein (Wooldridge 2009a). Type-I SPases generally recognize residues at the -1 and -3 positions relative to the

cleavage site. In most bacteria, the majority of extracellular proteins have been found to contain the consensus amino acid sequence $^{-3}$Ala-X-Ala^{-1} at the cleavage site of the mature protein (Tjalsma et al. 2004).

It has been shown that the secretion efficiency is highly dependent of the signal peptide used to target a specific protein for secretion, especially for heterologous proteins (Caspers et al. 2010; Mathiesen et al. 2009; Perez-Martinez et al. 1992; Slos et al. 1998). Changes in the hydrophobicity of the core region of the signal peptide and the length of the signal peptide have been shown to affect the secretion efficiency in Gram-positive bacteria (Brockmeier et al. 2006). In addition to the effect of the signal peptide, variation in the N-terminal region of the mature protein can affect secretion (Mathiesen et al. 2009). An optimal combination of signal peptide and desired target protein is important to reach high secretion efficiency. Several Gram-positive bacteria are capable of secreting proteins in high amounts. The Gram-positive model bacterium *Bacillus subtilis*, and related bacilli have been intensely studied with respect to their potential to secrete heterologous proteins (Bolhuis et al. 1999; Brockmeier et al. 2006; Caspers et al. 2010). The lactic acid bacteria *Lc. lactis* and *L. plantarum* is also receiving increasing interest as a host organism for production of recombinant proteins (Mercenier et al. 2000; Morello et al. 2008). Secretion of heterologous proteins in Gram positive bacteria is often inefficient (e.g. (Le Loir & Langella 1999; Nouaille et al. 2006)), even in organisms that secrete homologous proteins at a high capacity. Generally, high secretion capacity is linked to properties of both the secreted protein and the secretion machinery (Bolhuis et al. 1999).

1.4 Anchoring of proteins in Gram-positive bacteria

Gram-positive bacteria utilise several mechanisms to anchor proteins to the cell surface. Anchoring of proteins in Gram-positive bacteria is of great interest, not only because anchored proteins may play important roles in interactions with the environment but also because anchoring enables the display of antigens or other therapeutic compounds on the bacterial cell surface (Desvaux et al. 2006). One known strategy to obtain cell-surface display of heterologous proteins in Gram-positive bacteria is fusing the target protein to a homologous protein that holds an anchoring motif (Krüger et al. 2002; Lee et al. 2003; Moorthy & Ramasamy 2007).

Surface associated proteins are usually divided into four groups based on their interaction with the cell envelope (Figure 1.4).

I. Transmembrane proteins
II. Lipoproteins
III. LPXTG-like proteins
IV. Cell wall binding proteins

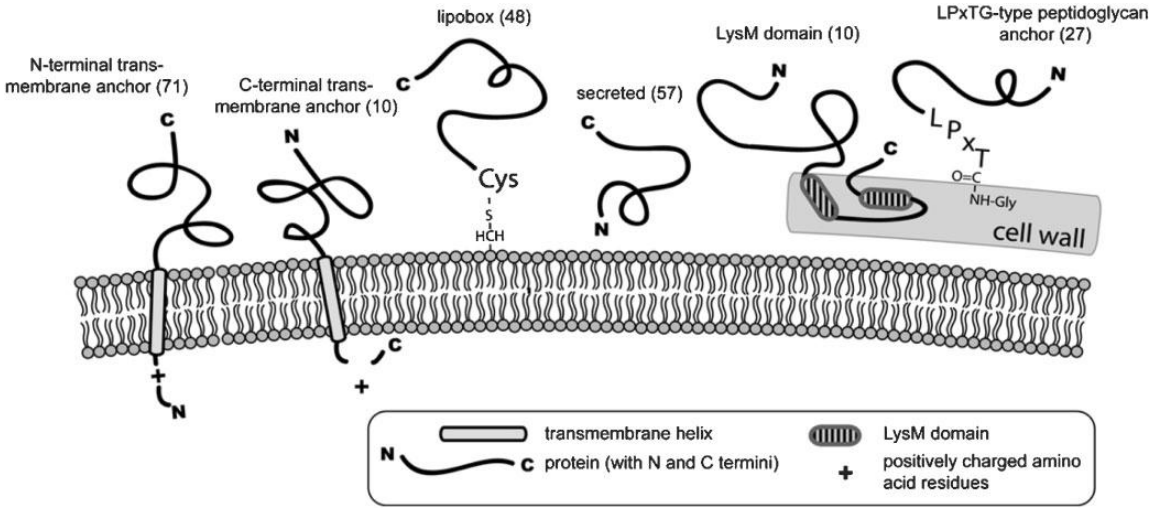

Figure 1.4: Schematic overview of the different types of surface proteins found in the Gram-positive bacterium *L. plantarum*. Membrane associated proteins can either possess transmembrane domains (left two scenarios) or they can be the lipoproteins, meaning that they are covalently linked to to a long chain fatty acid that is inserted into the cytoplasmic membrane. Cell wall associated proteins can be covalently attached to the cell wall via C-terminal LPXTG-like motives or non-covalently through LysM domains. See text for more details. The figure is taken from Boekhorst et al. (2006)

1.4.1 Transmembrane proteins

Transmembrane proteins are anchored to the cytoplasmic membrane through a single hydrophobic N- or C-terminal domain. The N-terminal signal peptide targeting the precursor protein to the Sec-translocase can be either cleaved by SPase I, leading to secretion, or left uncleaved to function as a membrane anchor domain. In other words the lack of a SPase cleavage site in the C-terminal region of the signal peptide causes the mature protein to be N-terminally anchored to the cell membrane. N-terminally anchored proteins often have functions related to extracellular processes such as transport, cell-envelope metabolism,

competence, signal transduction and protein turnover (Kleerebezem et al. 2010). Transmembrane proteins may also be anchored within the cytoplasmic membrane even though the N-terminal signal peptide is cleaved by a signal peptidase, due to the presence of a C-terminal anchor domain (Kleerebezem et al. 2010).

1.4.2 Lipoproteins

Lipoproteins are targeted to the Sec pathway by Type-II signal peptides harbouring the same distinctive (N-, H- and C) domains found in Type-I signal peptides. Type-II signal peptides differ from Type-I signal peptides by the somewhat shorter H-region and the lipobox motif found in the C-region (Kleerebezem et al. 2010). After translocation, the conserved lipobox sequence [L-(A/S)-(A/S)-C] directs the prelipoprotein to a prolipoprotein diacylglyceryl transferase (Lgt). The Lgt adds a diacylglyceryl group to the SH-group of a invariable cysteine residue located in the lipobox, generating a thioether linkage (Desvaux et al. 2006) . Following insertion of the diacylglyceryl group into the lipid bilayer of the cytoplasmic membrane, the signal peptide is cleaved by a SPase II, N-terminally of the invariable cysteine residue. Like the transmembrane proteins, cell surfaced displayed lipoproteins can be involved in a variety of functions, such as adhesion proteins, transporters, receptors, enzymes or virulence factors (Kleerebezem et al. 2010). Recently, the use of lipoprotein anchor domains to attach heterologous proteins to the cell surface of *L. plantarum* has been explored by Nygaard (2011), and this strategy also considered in this thesis.

1.4.3 LPXTG-like proteins

LPXTG anchored proteins are proteins that contain a C-terminal LPXTG-like motif that targets the protein to become covalently attached to the peptidoglycan by the activity of the sortase (SrtA) enzyme. The C-terminal LPXTG motif is followed by a hydrophobic domain and a charged tail which retains the protein in the membrane, thus permitting the LPXTG motif to be recognized by the sortase. The sortase is a membrane associated transpeptidase which cleaves the LPXTG motive between the T and G residues and then attaches the threonine carboxyl group to the peptidoglycan (Boekhorst et al. 2005). LPXTG-anchored proteins have a diverse range of functions and sortase-like proteins have been identified in almost all Gram-positive bacteria (Desvaux et al. 2006). Several studies have investigated the use of LPXTG-like motives to anchor heterologous proteins to the cell wall, e.g. to display antigens on the cell surface (Fredriksen et al. 2010; Turner et al. 2003).

1.4.4 Cell wall binding proteins

Proteins can be non-covalently attached to the cell surface by various cell wall binding domains or through protein-protein interactions with other cell wall proteins. These can be domains such as the choline-binding domains which anchor the protein to the cell surface by specifically recognising the choline groups of teichoic and lipoteichoic acids in the cell wall, or the lysine motif domain (LysM), which binds to the peptidoglycan. Other known domains are the Type-II cell wall binding domains, GW-modules or the S-layer homology domains (Kleerebezem et al. 2010).

1.5 The human mucosal immune system

The mucous membranes are moist tissue lining that comprises particular organ and body cavities, such as the gastrointestinal, the vaginal, the rectal and the aerodigestive tracts. The airways and the digestive tract are considered the main ports of entry of microorganisms to the body, and the mucosal lining represents the site of the first dynamic interactions between microbes and the human host. The mucosal membranes act as a barrier, with a highly specialised innate and adaptive mucosal immune system to protect the mucosal surfaces from microbial pathogens (Holmgren & Czerkinsky 2005). This system also plays a major role in promoting cohabitation with commensal microflora (Spreng et al. 2006). Delivery of therapeutic recombinant bacteria via these routes is considered an interesting strategy in the development of vaccination regimes (Mohamadzadeh et al. 2008). Mucosal vaccination has advantages compared to traditional systemic vaccination, due to the potential of eliciting a mucosal immune response in addition to a systemic response (Mercenier et al. 2000).

The mucosa-associated lymphoid tissue (MALT) plays an important role in regulating the mucosal immunity and this tissue functions essentially independent of the systemic immune system. Unlike the systemic immune system, which functions in a normally sterile environment, the MALT is constantly presented with foreign material, making it an important site for the launching of appropriate immune responses at the mucosal surfaces (Figure 1.5) (Mohamadzadeh et al. 2008). The MALT is a highly compartmentalized immunological system that consists of anatomically defined lymphoid micro-compartments such as the Peyer's patches, the mesenteric lymph nodes, the appendix and solitary follicles in the intestine, and the tonsils and adenoids of the aerodigestive tract. The MALT is populated with

phenotypically and functionally diverse T and B cells, macrophages and subsets of antigen-presenting dendritic cells (DCs). The nature of the compartmentalised mucosal immune system restricts the recirculation of lymphoid cells between mucosal sites and the main immune response to a mucosal vaccination route is expected to be local (Holmgren & Czerkinsky 2005). Despite this, both mucosal and systemic immune responses have often been reported after immunisation via mucosal routes (Bermúdez-Humarán et al. 2005; Huang et al. 2005; Mielcarek et al. 2001; Poo et al. 2006; Shanley & Wu 2005).

Mucosal vaccines can be administered via a number of routes such as oral, rectal, pulmonary, vaginal, and intranasal. The oral and nasal routes of vaccine delivery are seen as the most accessible and acceptable routes (Azizi et al. 2010; Jabbal-Gill 2010). The nasal route of immunization is often considered more advantageous than the oral route and has shown great potential in mice, monkey and human trials, where nasal immunisation induced specific mucosal immunoglobulin A (IgA) and IgG antibodies in non-local mucosal tissues, such as the salivary glands, upper and lower respiratory tracts, the male and female genital tracts, and the small and large intestines (Kozlowski et al. 2002; Rudin et al. 1999; Staats et al. 1997). The local production and secretion of immunoglobulin A (IgA) antibodies is an important characteristic of the mucosal adaptive immune response and secreted IgA (sIgA) has several roles in the mucosal defence system. Local antibodies such as sIgA play an important part in the defence against pathogens by preventing the binding of microorganisms and their toxins to the epithelium (Neutra & Kozlowski 2006).

Orally administered vaccines are processed and presented in the gut-associated lymphoid tissue (GALT) (Figure 1.5) and have been shown to induce local immune responses, as well as responses at distant mucosal sites as well and systemic immune responses (Wang & Coppel 2008). Concerns associated with oral administration of vaccines are the highly acidic conditions met in the stomach and the digestive enzymes in the gut that can degrade protein components. It has also been reported that the oral vaccination route gives an increased risk of developing tolerance, mainly because the immune system in the gastrointestinal tract is adjusted to keep it from reacting to the load of dietary antigens and commensal bacteria found in this environment. The development of oral tolerance is considered an active process, leading to the generation of antigen-specific T lymphocytes that suppress further immune

stimulation. Too low or too high dosages of antigenic material are believed to play an important part in the induction of oral tolerance (Kraal et al. 2006).

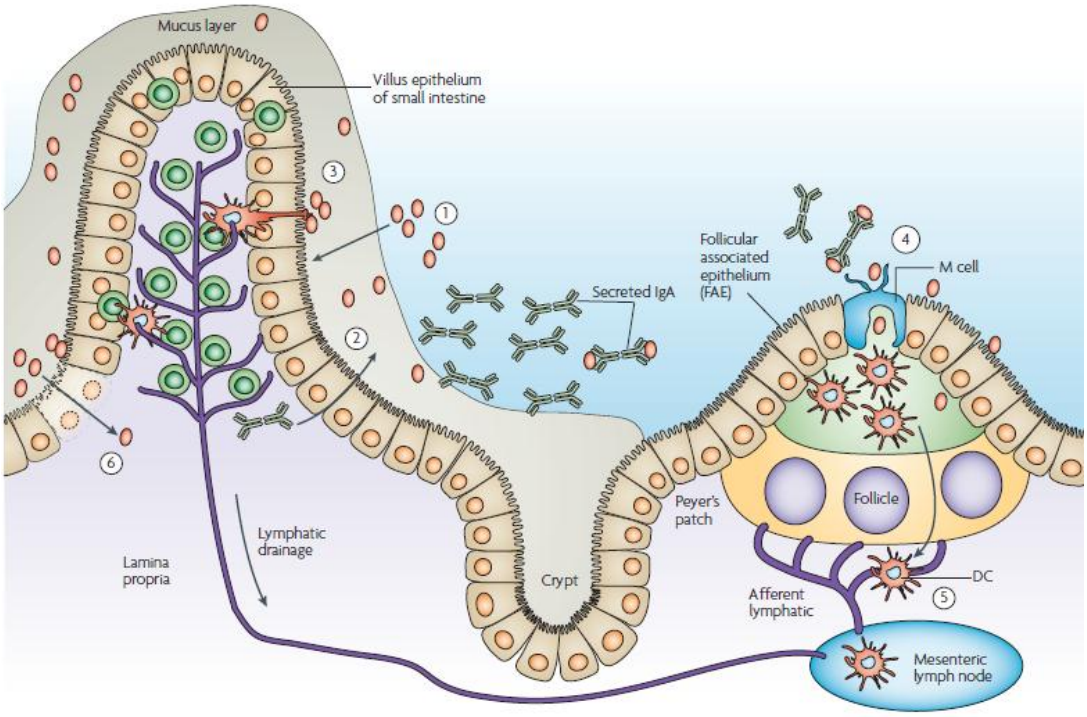

Figure 1.5: The mucosal surfaces of the gastrointestinal (GI) tract (the gut- associated lymphoid tissue (GALT)). Most of the bacteria that enter the GI-tract end up in the lumen or are trapped in the mucus layer covering the epithelium (1). IgA is secreted (sIgA) by mature plasma cells (2) and play an important part in the defence against pathogens by preventing the binding of microorganisms and their toxins to the epithelium (Neutra & Kozlowski 2006). Bacteria that come in contact with the surface of the epithelium may be sampled by dendritic cells (DCs) (3). The Peyer's patches are inductive sites in the small intestine where M cells (4), which are specialised cells located in the follicle associated epithelium, transport luminal antigens across the epithelium where they can induce specific immune responses (Wells & Mercenier 2008). DCs present in the Peyer's patches (5) are able to phagocytise bacteria and then migrate to the mesenteric lymph nodes where they present bacterial antigens to T-cells. The figure is taken from Wells & Mercenier (2008).

The use of live bacteria as vaccine vectors enables vaccination strategies where the vaccine can mimic the route of entry of many disease causing pathogens and stimulate the mucosal immune system to induce a sufficient immune response at the mucosal surfaces and in the MALT (Detmer & Glenting 2006). Antigens carried by the bacterial vector can be absorbed by microfold cells (M cells), which are specialised cells located in the follicle associated epithelium of the Peyer's patches (Figure 1.5) (Hase et al. 2009). M cells are easily accessible

for antigens because they do not secrete mucus or digestive enzymes like other epithelial cells. M cells transport antigens across the epithelial barrier in a process known as transcytosis. Unlike dendritic cells (DCs) and macrophages, where antigens are subjected to degradation in lysosomes, the M cells mainly shuttle antigens through the cell and delivers them to the underlying organised lymphoid follicles where the antigen is subsequently presented to professional antigen-presenting cells (APCs) such as dendritic cells, B cells and macrophages (Holmgren & Czerkinsky 2005). Transcytosis of antigens is considered essential for the initiation of antigen-specific mucosal immune responses, thereby making M cells promising targets of oral vaccines.

Antigen-presenting cells initiate primary immune responses by presenting peptides, derived from antigens degraded in the cytosol, via major histocompatibility complex (MHC) on their cell surface to naïve CD4+ and CD8+ T cells. CD4+ T cells are only stimulated by exogenous antigens (antigens sampled from the environment surrounding the APC) in association with MHC class II molecules, which are only present on the cell surface of professional APCs. CD8+ T cells are on the other hand stimulated by endogenous antigens (antigens from the cytosol of APCs, e.g. in cells infected by pathogens) presented on MHC class I molecules that are present on the cell surface of nearly every nucleated cell in the body (Ryan et al. 2001).

After encountering antigens at the inductive site the now activated B and T cells migrate through the lymph, enter the circulation and differentiate into effector and memory cells at specific mucosal sites (Holmgren & Czerkinsky 2005). Memory B cells starts generating high affinity antigen receptors against the antigen it encountered, which will ensure a more rapid immune response upon the next encounter with this antigen .Activated CD8+ cytotoxic T cells kills infected host cells, whereas CD4+ T cells mediate antibody production, activates phagocytic cells of the innate immune system, and regulates the immune response. CD4+ T cells can proliferate into T-helper cells, which are roughly divided into two groups that express different kind of cytokines, T_H1 and T_H2 cells. T_H1 and T_H2 cells are reciprocally regulated by the cytokines secreted by one another, as well as by cytokines secreted by cells of the innate immune system (Lea 2006). T cells of the T_H1 type typically mediate cellular immunity and stimulate increased bactericidal activity against intracellular bacteria infecting the cells of the host. T_H2 type cells mainly elicit a humoral immune response by stimulating B cell antibody production (such as sIgA), and are generally induced in response to helminthic

parasites, allergens and soluble antigens (Ryan et al. 2001). The typical immune reaction against antigens coming from non-pathogenic sources is the generation of T_H2 cell and various regulatory T cells responses, which usually results in active suppression of systemic immunity, and thereby oral tolerance. The type of immune response generated is dependent of several factors, such as the type of organism and its route of entry, the nature of the antigen and its interaction with the mucosal inductive sites, as well as the genetic background of the host (Holmgren & Czerkinsky 2005).

1.6 Bacteria as live vectors for mucosal delivery of microbial antigens

Mucosal immunity plays a major role in the prevention of infectious diseases and the successful use of live bacterial vectors for delivery of therapeutic molecules and immunisation in animal models has been reported in several studies (Bermúdez-Humarán 2009; Grangette et al. 2001; Grangette et al. 2004; Mannam et al. 2004). A phase I clinical trial has been described for oral administration of transgenic *Lc.* lactis, secreting interleukin-10, to patients with Crohns disease (Braat et al. 2006). Although not a vaccination study, this study showed that bacterial delivery of immunomodulatory proteins was a novel and promising approach in the battle against mucosal diseases.

Vaccination is considered one of the most cost effective strategies to eliminate the occurrence of infectious diseases in humans. Prophylactic vaccines have been administered to prevent or improve the outcome of future infections by pathogens for over two centuries, and great efforts have been made in developing therapeutic vaccines, such as vaccines against cancer (Mielcarek et al. 2001). A number of safe and efficient vaccines are commercially available, but improvement is still needed. Greater understanding of the immune system and the progress of biotechnology have made genetically engineered microorganisms a promising tool in the development of new and improved vaccine candidates. Use of recombinant microorganisms has several advantages compared to traditional systemic vaccination methods (Spreng et al. 2006). Their administration via mucosal routes does not require medically trained personnel and-, reduces the risk of blood-borne diseases and the costs of sterile needles and syringes. Mucosal administration also offers important advantages such as reduced secondary effects and the possibility to stimulate both systemic and mucosal immune responses. Bacterial vectors have an additional advantage over viral vectors in that their

genome can harbour many heterologous genes. While viral vectors are limited by their capacity to encapsulate foreign DNA, recombinant bacteria have the ability to produce different heterologous antigens, and possibly to function as multivalent vaccines (Bermúdez-Humarán 2009).

Two classes of live recombinant bacteria exist: attenuated strains of pathogenic bacteria and recombinant bacteria with GRAS status. Most attenuated strains of pathogenic bacteria are able to replicate in the host or enter through mucosal surfaces, thereby inducing strong and long-lasting immune responses, also against antigens that are only expressed in vivo (Spreng et al. 2006). Attenuated recombinant strains of pathogens have also been used successfully as delivery vectors for heterologous antigens. These attenuated vectors can elicit a strong immune response against a heterologous antigen, due to their natural inherent immunogenicity. For example, attenuated mutant strains of different *Salmonella* serotypes have been used successfully as delivery vectors for heterologous antigens in mice (Pasetti et al. 2011). The drawback of using attenuated pathogenic bacteria is the possibility of these bacteria to cause undesired reactogenicity in weak or immunocompromised hosts, thereby making the vaccine unsuitable for application in an already vulnerable part of the human population.

Bacteria with GRAS status are often used in the food industry and/or found as a part of the normal commensal microbiota in humans. The microbiota is an essential part of human health contributing, not only to food digestion, but also to the development and optimal functioning of the immune system (Lebeer et al. 2010; Mielcarek et al. 2001). Bacterial vectors with GRAS status represent a good alternative to the use of attenuated pathogenic bacterial carriers because of their safe and extensively documented association with humans. Lactic acid bacteria (LAB) are the most commonly used GRAS bacteria for vaccine purposes, and their potential as recombinant vectors for *in situ* delivery, has been described in several publications (Norton et al. 1996; Robinson et al. 1997; Robinson et al. 2004; Seegers 2002). The non-invasive, non-colonising *Lc. lactis* is one of the most widely studied LAB in this context, and recombinant strains have been clinically tested in humans (Braat et al. 2006). Many beneficial effects have been attributed to LAB, especially to the lactobacilli (see section 1.1). Certain *Lactobacillus* strains have therefore received great attention as potential recombinant vectors due to their probiotic effects, natural adjuvanticity and ability to colonize

in the gut. A major advantage of utilising LAB as delivery vectors for vaccines is the potential of several LAB to elicit antigen-specific sIgA responses at mucosal surfaces (Mielcarek et al. 2001; Wells & Mercenier 2008). sIgA play an important part in the defence against pathogens by preventing binding of microorganisms and bacterial toxins to the epithelium (Neutra & Kozlowski 2006). The genetic tools available for manipulation of LAB offer a variety of strategies for genetic engineering of live bacterial vector systems. These tools include efficient and easy-to-handle expression vectors for production of heterologous gene products and tools for integration of heterologous genes into the bacterial chromosomes.

The potential of *Lactobacillus plantarum* as a bacterial delivery vector has been the subject of several studies over the last years (Cortes-Perez et al. 2005; Fredriksen et al. 2010; Grangette et al. 2004) *L. plantarum* are able to persist in the gastrointestinal tract of humans for up to seven days. Generally, longer residence times have the potential advantage that the bacteria get the chance to produce more antigen during their transport through the body; this may reduce the necessary frequency of administration. It should be noted that no difference in the ability to elicit an antibody response against tetanus toxin fragment C (TTFC) was found in a comparison between *L. plantarum*, *Lactobacillus casei* and *L. lactis*, when mice were immunised repeatedly by the intragastric route, despite the different residence times of these bacteria (unpublished result by J.W., A.M., M.C. Geoffroy and C. Rush, presented in a review by Wells & Mercenier (2008)). This suggests that persistence of the bacteria at mucosal sites isn't the only important factor affecting the induction of a systemic response to an expressed antigen.

1.7 *Mycobacterium tuberculosis*, the causative agent of tuberculosis

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is one of the world's most devastating human pathogens, leaving an estimated one-third of the global population infected. TB is a re-emerging disease, and remains one of the leading causes of morbidity and mortality in humans, primarily affecting the poorest regions of the world. *M. tuberculosis* ability to persist for years in a clinically latent state within the human host is fundamental to its successful pathogenicity. About 95 % of infected individuals develop a latent infection. In most cases TB can be cured with a low-cost treatment with antibiotics. Unfortunately, difficulties with timely diagnosis of the infection, socioeconomic factors in endemic areas and

the requirement for a durable antibiotic treatment to ensure bacterial clearance, have prevented a successful global TB control. Development of multidrug-resistant and extremely drug-resistant variants of the pathogen underpins the importance of an increased effort against TB (Dietrich & Doherty 2009).

Vaccination is considered the most important tool in fighting TB on a global scale, with Bacillus Calmette-Guérin (BCG) as the most widely used vaccine today. BCG is prepared from a strain of live, attenuated *Mycobacterium bovis* and is thought to provide effective protection against TB in vaccinated children (Dietrich et al. 2006b). Problematically, the duration of BCG-induced protection is limited in highly epidemic areas, worldwide estimates of protection against pulmonary TB in in vaccinated individuals vary from 0 to 80 % (Dietrich & Doherty 2009) . Additionally, BCG cannot be used as a booster vaccine in already sensitised individuals, making it difficult to counteract the waning protective effect of BCG vaccination. Development of a more efficient vaccine is therefore of great importance.

M. tuberculosis is an intracellular pathogen with the ability to survive in the phagosomal compartment of macrophages (Dietrich et al. 2006b). Induction of a T_H1 dominated immune response is considered essential to control *M. tuberculosis* infection because this stimulates towards cellular immune responses and the eradication of intracellular pathogens (Takatsu & Kariyone 2003). *M. tuberculosis* usually invades the host through mucosal surfaces of the respiratory tract after inhalation of infectious droplets (Hall et al. 2009). Several studies have shown that vaccine delivery via mucosal routes may elicit local respiratory immunity, which in the case of *M. tuberculosis* infection might lead to increased protection (Andersen et al. 2007; Badell et al. 2009; Dietrich et al. 2006a). Delivery systems based on live bacterial vectors could thus be promising candidates. In one study mice were subjected to oral vaccination with an attenuated *Salmonella* vector expressing a fusion protein of the two *M. tuberculosis* antigens Ag85B and ESAT-6 (Hall et al. 2009). Live oral vaccination with such a recombinant vector, in addition to boosting with purified Ag85B-ESAT6 protein and an adjuvant, was found to represent an effective mucosal vaccination strategy.

1.8 *Mycobacterium tuberculosis* antigens used in this study

A 41 kDa fusion protein consisting of antigen 85B (Ag85B) and ESAT-6, two important antigens secreted by *M. tuberculosis* during infection, was utilised in this study.

The Ag85-family consist of three closely related mycolyl transferases (antigen 85A, 85B and 85C) secreted by *M. tuberculosis* (Harth et al. 1996). The most abundant protein of *M. tuberculosis* is Ag85B, a 30 kDa major secretory protein. Ag85B is involved in the synthesis of mycolic acid in the cell wall. The antigen Ag85B was early on considered a promising candidate for a vaccine against TB, and is among the most potent antigens discovered so far, together with Ag85A. Ag85A and Ag85B are major targets of human T-cell responses against *M. tuberculosis* (Takatsu & Kariyone 2003). In a guinea pig model of pulmonary tuberculosis, immunization experiments with purified Ag85B induced protective immunity *M. tuberculosis*, as demonstrated in an aerosol challenge test (Horwitz et al. 1995; Olsen et al. 2004). In a similar trial, guinea pigs were immunised with a recombinant strain of BCG, expressing and secreting Ag85B (Horwitz et al. 2000). The recombinant BCG strain induced stronger protective immunity than the ordinary BCG vaccine, again demonstrated after an aerosol challenge with *M. tuberculosis*.

Early secreted antigen target of 6 kDa (ESAT-6) is a member of a multigene family that encodes several immunogenic proteins that are strongly recognised by T cells from *M. tuberculosis* infected humans or in animal models of TB (Dietrich et al. 2006b).The 95 amino acid ESAT-6 protein is one of several culture filtrate proteins found in the culture supernatant of *M. tuberculosis,* in the early phase of growth (Brodin et al. 2004). ESAT-6 was found to be the most potent antigen among some of the lower molecular mass culture filtrate proteins. Studies with mice have shown that the ESAT-6 subunit vaccine induce protective immunity against *M. tuberculosis*, comparable to that induced by BCG (Brandt et al. 2000). It was reported though that a strong adjuvant was needed, and that ESAT-6 alone had low inherent immunogenicity. In a survey of the presence of the gene encoding ESAT-6 in *Mycobacteria* it was found to exist only in fully virulent strains (Wang et al. 2009).
Several studies have shown that vaccination with a fusion protein consisting of Ag85B and ESAT-6 promotes strong protective immune responses against *M. tuberculosis* in mouse, guinea pig and non-human primate models (Langermans et al. 2005; Olsen et al. 2004; Olsen et al. 2001). It has been suggested that Ag85B may amplify the immune responses to the low-

immunogenic ESAT-6. The fusion of Ag85B-ESAT6 was shown to give higher levels of protection, compared to vaccination with the individual antigens, in mouse and guinea pig models (Doherty et al. 2004). An additional advantage of such a subunit vaccine containing multiple epitopes is a potentially broader coverage of a genetically heterogeneous human population.

1.9 The goals of this study

The aim of this study was to investigate the production, secretion and anchoring of a fusion protein consisting of the *Mycobacterium tuberculosis* antigens Ag85B and ESAT-6, in *Lactobacillus plantarum* by using an inducible gene expression system (pSIP) originally developed for efficient intracellular production of proteins(Sørvig et al. 2003).

The final cellular localisation of the heterologous antigen (cytoplasmic, secreted or anchored in/to the cell wall) is expected to influence immunogenicity, the optimal localisation is debated in the literature, although evidence suggests that antigens localised on the cell surface are more immunogenic than cytoplasmic antigen (Norton et al. 1996; Scavone et al. 2007). Therefore all three locations were addressed in this study. Immune responses were evaluated by co-incubation of recombinant bacteria with dendritic cells derived from human peripheral blood. As host strain the sequenced strain *L. plantarum* WCFS1 (Kleerebezem et al. 2003) was used.

The work was carried out in the following five steps:

1. Initially, the ability of *L. plantarum* WCFS1 to express an intracellular version of the Ag85B-ESAT-6 fusion protein was evaluated. Expression of the Ag85B-ESAT-6 fusion protein *L. plantarum* had not previously been studied.
2. In the next step the possibility to secrete the Ag85B-ESAT6 fusion protein was investigated using expression vectors carrying homologous signal peptides from *L. plantarum*. The most promising signal peptide was selected for further studies.
3. In addition to the use of inducible pSIP-vectors, attempts were made to express and secrete Ag85B-ESAT-6 constitutively. The inducible pSIP-promoter P_{sppA} (see section 1.2) was therefore replaced with a constitutive promoter for high-level (p11) or lower-

level (p27) protein expression. Both promoters were from a *L. plantarum* promoter library generated by Rud et al. (2006).

4. Subsequently two LPXTG cell wall-anchors and one lipoprotein-anchor, all from *L. plantarum* were added to the expression constructs in order to anchor Ag85B-ESAT6 to /in the cell wall.
5. The most promising expression systems coming out of the previous steps were tested in more detail: the localisation of Ag85B-ESAT6 was evaluated by immunological imaging methods, while the immunogenicity of Ag85B-ESAT6 expressing strains was evaluated by analysing levels of TNF-alpha and IL-10, after co-incubation with dendritic cells.

2. MATERIALS

2.1 Laboratory equipment

Laboratory equipment	Supplier
ART® aerosol resistant pipette tips	Promega
CL-Xposure™ Film	Thermo Scientific
Corex tubes	Corning Inc.
Cuvettes	
Disposable cuvettes, 1.5 ml	Brand
Electroporation cuvettes, Gene pulser®, 0.2 cm	Bio-Rad
Eppendorf tubes	
Regular 1,5 ml	Axygen
Sequencing tubes	
Falcon 2059 Polypropylene Round Bottom tubes	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
Nunc tubes, 15 ml and 50 ml	Nunc
Steri-Cycle CO_2 Incubator	Thermo-electron Corporation
Sterile filters, 0,22 µn pore size	Millex GP
Syringes, 10ml - 50 ml	Plastipak
Various glass equipment	Labsystems
Waterbaths	

Instruments	Supplier
ABI PRISM™ 3100 DNA sequencer	AME Bioscience
Bio-Rad GenePulser® II	Bio-Rad
Bio-Rad Pulse controller plus	Bio-Rad
Centrifuges	
Eppendorf centrifuge 5415R	Eppendorf
Table centrifuge	Biofuge Pico, Heraeu
Cooling centrifuge	Avanti J-25 Beckman Coulter
Eppendorf Centrifuge 5430R	Eppendorf
Megafuge 1.0	Heraens
Vacuum centrifuge	Maxi dry lyo, Heto
Coulter Counter® Z1	
Coulter® Particle Count Z1. Beckment Coulter ™	Nerliens Meszansky
Electrophoresis electricity supplier	
MacsQuant®Analyzer	Miltenyi Biotec
pH-meter	Metrohm
SNAP i.d. Protein Detection System	Millipore
Tecan Sunrise™ Absorbance Microplate Reader	Tecan
Photo equipment	
GelDoc machine	Bio-Rad
Qubit™ Fluorometer	Invitrogen

Software	Supplier
CLC DNA Workbench 5	CLC bio
MacsQuantify™ Software	Miltenyi Biotec
pDRAW32	www.acaclone.com
Prosite	expasy.org/prosite

2.2 Chemicals

Chemicals	Supplier
Acetone	Merck
Agar	Difco
Agarose	
SeaKem® LE agarose	Cambrex
NuSieve® GTG® agarose	Cambrex
Ampicillin	Sigma
Calcium chloride, $CaCl_2$	Merck
Chloroform, $CHCl_3$	Sigma
Disodium hydrogen phosphate, Na_2HPO_4	Merck
EDTA, $C_{10}H_{16}N_2O_8$	Sigma
Erythromycin, $C_{37}H_{67}NO_{13}$	Sigma
Ethanol, C_2H_5OH	Arcus
Ethidium Bromide, EtBr	Sigma
Glucose, $C_6H_{12}O_6$	Merck
Glycerol, $C_3H_8O_2$	Merck
Glycine, $C_2H_5NO_2$	Merck
Kanamycin	Sigma
Magnesium chloride, $MgCl_2$	Qiagen
Magnesium sulfate, $MgSO_4$	Merck
Methanol, CH_3OH	Merck
Polyethylen Glycol, PEG_{1450}	Sigma
Potassium chloride, KCl	Qiagen
Potassium dihydrogen phosphate, KH_2PO_4	Qiagen
Skimmed milk powder	Difco
Sodium acetate, $NaC_2H_3O_2x3H_2O$	Merck
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, $C_4H_{11}NO_3$ xHCl	Sigma
Tween-20	Sigma

2.3 Proteins and enzymes

Protein/enzyme	Supplier
Antibodies	
Ag85B Rabbit pAb (ab43019)	abcam
EsaT-6 Rabbit pAb (APO03011PU-N)	Acris
ESAT6 Mouse mcAb (ab26246)	abcam

HRP-Goat Anti-Rabbit IgG (65-6120)	Invitrogen
HRP-Rabbit Anti-Mouse IgG	Invitrogen
Anti-Mouse IgG FITC	Sigma
Bovine Serum Albumin (BSA)	Sigma
Fetal Calf Serum (FCS)	Sigma
GM-CSF	Immunotools
Interleukin -4	Immunotools
Lysozyme	Sigma
Mutanolysine	
Inducer peptide (SppIP)	Sigma
Protein standards	
BenchMark™ Protein Ladder	Invitrogen
MagicMark™	Invitrogen
Phusion High Fidelity DNA polymerase	Finnzymes
Restriciton buffers	
NEBuffer 2	NEB
NEBuffer 3	NEB
NEBuffer 4	NEB
Restriction enzymes	
BamHI	NEB
BglII	NEB
EcoRI	NEB
HindIII	NEB
MluI	NEB
NdeI	NEB
SalI	NEB
T4 DNA ligase	NEB
Taq DNA polymerase (with 10x reaction buffer)	Finnzymes
Quick T4 DNA ligase (with 2x Quick ligation buffer)	NEB

2.4 DNA

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

2.5 Primers

Table 2.1 Primer sequences

Name	Sequence	Restriction site in sequence
pAgESATCyt-F	CATATGTTTAGTCGTCCAGGTTTGC	NdeI
pAgESATCyt-R	GGAAACAGCTATGACCATGATTAC	HindIII
AgSalF	GTCGACTTTAGTCGTCCAGGTTTGCC	SalI
AgMluR	CCTTAACGCGTTGCAAACATGCCGGT	MluI
pGus1F(pSIP1F)	GCGCCTTTAGATTACATTCTCAA	
PB11-R	CATATGTAAAATCTCCTTGTAATAGTATTT	
PB27-R	CATATGTAAAATCTCCTTGTAATAGTAT	
pSekF	GGCTTTTATAATATGAGATAATGCCGAC	
PSeqR	CCGCCCTTATGGGATTTATCT	

Table 2.2 Primer descriptions

Name	Description
pAgESATCyt-F	Forward primer for Ag85B-ESAT6 sequence from pUC57-Ag85B-E6
pAgESATCyt-R	Reverse primer for Ag85B-ESAT6 sequence from pUC57-Ag85B-E6
AgSalF	Forward primer for Ag85B-ESAT6 sequence from pUsp45 based vectors
AgMluR	Reverse primer for Ag85B-ESAT6 sequence from pUsp45 based vectors
pGus1F(pSIP1F)	Forward primer for sequencing of promoter/signal peptide sequences in pUSP45 based vectors
PB11-R	Reverse primer for promoter 11
PB27-R	Reverse primer for promoter 27
pSekF	Forward primer for the sequencing of signal sequences in pUsp45 based vector
PSeqR	Reverse primer for the sequencing of inserts in pUsp45 based vector

2.6 Bacterial strains and plasmids

Table 2.1. Bacterial strains

Strain	Source of reference
Escherichia coli TOP10	Invitrogen
Lactobacillus plantarum WCFS1	(Kleerebezem et al, 2003)

Table 2.4 Plasmids

Plasmid	Description	Source of reference
puc57-Ag85B-ESAT6	Vector containing Ag85B-ESAT6, the antigen is codon optimised for *L. plantarum*	GenScript, USA
pCR®-Blunt II-TOPO®	Vector for cloning of PCR fragments, Kan^r	Invitrogen™
pSIP-EV	pSIP401 derivative lacking any target gene	L. Fredriksen, unpublished
tp-Ag85B-E6	Vector containing Ag85B-ESAT6	This work
tp-pb11	Vector containing promoter p11	L. Fredriksen, unpublished
tp-pb27	Vector containing promoter p27	L. Fredriksen, unpublished
pCytAg85B-E6	pUSP45A derivative with Ag85B-ESAT6 instead of NucA	This work
pCyt-p11Ag85B-E6	pCytAg85B-E6 derivative with constitutive promoter, p11	This work
pCyt-p27Ag85B-E6	pCytAg85B-E6 derivative with constitutive promoter, p27	This work
pLp_0141sNucA	pUsp45D-derivative with a short version of Lp_0141 instead of Usp45	A. Sveen (2007)
pLp_0297sNucA	pUsp45D-derivative with a short version of Lp_0297 instead of Usp45	A. Sveen (2007)
pLp_0373sNucA	pUsp45D-derivative with a short version of Lp_0373 instead of Usp45	A. Sveen (2007)
pLp_0469sNucA	pUsp45D-derivative with a short version of Lp_0469 instead of Usp45	A. Sveen (2007)
pLp_0600sNucA	pUsp45D-derivative with a short version of Lp_0600 instead of Usp45	A. Sveen (2007)
pLp_1448sNucA	pUsp45D-derivative with a short version of Lp_1448 instead of Usp45	A. Sveen (2007)
pLp_2174sNucA	pUsp45D-derivative with a short version of Lp_2174 instead of Usp45	A. Sveen (2007)
pLp_3050sNucA	pUsp45D-derivative with a short version of Lp_3050 instead of Usp45	A. Sveen (2007)
pLp_3117sNucA	pUsp45D-derivative with a short version of Lp_3117 instead of Usp45	A. Sveen (2007)
pLp_3189sNucA	pUsp45D-derivative with a short version of Lp_3189 instead of Usp45	A. Sveen (2007)
pLp_3676sNucA	pUsp45D-derivative with a short version of Lp_3676 instead of Usp45	A. Sveen (2007)
pLp_0141Ag85B-E6	pLp_0141sNucA derivative with Ag85B-ESAT6 instead of NucA	This work
pLp_0297Ag85B-E6	pLp_0297sNucA derivative with Ag85B-ESAT6 instead of NucA	This work
pLp_0373Ag85B-E6	pLp_0373sNucA derivative with Ag85B-ESAT6 instead of NucA	This work
pLp_0469Ag85B-E6	pLp_0469sNucA derivative with Ag85B-ESAT6 instead of NucA	This work
pLp_0600Ag85B-E6	pLp_0600sNucA derivative with Ag85B-ESAT6 instead of NucA	This work
pLp_1448Ag85B-E6	pLp_1448sNucA derivative with Ag85B-ESAT6 instead of NucA	This work
pLp_2174Ag85B-E6	pLp_2174sNucA derivative with Ag85B-ESAT6 instead of NucA	This work
pLp_3050Ag85B-E6	pLp_3050sNucA derivative with Ag85B-ESAT6 instead of NucA	This work
pLp_3117Ag85B-E6	pLp_3117sNucA derivative with Ag85B-ESAT6 instead of NucA	This work
pLp_3189Ag85B-E6	pLp_3189sNucA derivative with Ag85B-ESAT6 instead of NucA	This work
pLp_3676Ag85B-E6	pLp_3676sNucA derivative with Ag85B-ESAT6 instead of NucA	This work
pLp_0373sOFAcwa1	p037sOFAm-derivative with long version of Lp_2578 anchor sequence downstream of OFA	L. Fredriksen (2007)
pLp_0373sOFAcwa2	p037sOFAm-derivative with medium version of Lp_2578 anchor sequence downstream of OFA	L. Fredriksen (2007)
pLp_0373sOFAcwa3	p037sOFAm-derivative with short version of Lp_2578 anchor sequence downstream of OFA	L. Fredriksen (2007)
pLp_1261InvL	pLp_2588sAmy derivative harbouring Lp_1261 lipo- anchor signal sequence	C.S. Nygaard (2011)
pLp_0373Ag85B-E6cwa2	pLp_0373sOFAcwa2 derivative harbouring Ag85B-ESAT6 instead of OFA	This work
pLp_0373Ag85B-E6cwa3	pLp_0373sOFAcwa3 derivative harbouring Ag85B-ESAT6 instead of OFA	This work
pLp_3050Ag85B-E6cwa2	pLp_0373Ag85B-E6cwa2 derivative harbouring Lp_3050 as a signal sequence	This work
pLp_3050Ag85B-E6cwa3	pLp_0373Ag85B-E6cwa3 derivative harbouring Lp_3050 as a signal sequence	This work
pLp_1261Ag85B-E6	pLp_1261InvL derivative harbouring Ag85B-ESAT6 instead of InvL	This work

2.7 Kits

Kit	Supplier
BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
BigDye® Terminator v3.1 Ready Reaction Premix	
BigDye® Terminator v3.1 Sequencing Buffer (5x)	
Human IL-12 (p70) ELISA MAX™ Standard set	BioLegend
Human IL-12 (p70) ELISA MAX™ Capture Antibody (200X)	
Human IL-12 (p70) ELISA MAX™ Detection antibody (200X)	
Human IL-12 (p70) Standard	
Streptavidin-HRP (1000X)	
iBlot™ Dry Blotting system	Invitrogen
Blotting Roller	
iBlot™ Transfer Stack, Regular and Mini	
iBlot™ Cathode Stack, top	
iBlot™ Anode, bottom	
iBlot™ Disposable sponge	
iBlot™ Filter Paper	
iBlot™ Gel Transfer Device	
Interleukin 10, high sensitivity human ELISA Set	Immunotools
and	
TNF alpha, high sensitivity human ELISA Set	Immunotools
Coating antibody	
Biotinylated detector	
Standard	
NucleoSpin® Plasmid miniprep Kit	Macherey-Nagel
NucleoSpin® Plasmid Columns with 2 ml collection tubes	
Resuspension Buffer A1	
Lysis Buffer A2	
Wash Buffer AW	
Wash Buffer A4	
Elution Buffer AE	
RNase A	
NucleoSpin® Extract II	
NucleoSpin® Extract II Columns with 2 ml collection tubes	Macherey-Nagel
Binding Buffer NT	
Wash Buffer NT3	
Elution Buffer NE	
NuPAGE® Novex Bis-Tris Gels	Invitrogen

10% Bis-Tris, 8 cm x 8 cm x1 mm, 10 wells NuPAGE® LDS Sample Buffer (4x) NuPAGE® Reducing Agent (10x)	
MOPS Running Buffer	Invitrogen
Pellet Paint® Co- Precipitant Pellet Paint® Co- Precipitant 3 M sodium acetate, pH 5,2	Merck
Qubit™ dsDNA BR Assay Qubit™ dsDNA BR reagent (Component A) Qubit™ dsDNA BR buffer (Component B) Qubit™ dsDNA BR standard #1(Component C) Qubit™ dsDNA BR standard #2 (Component D)	Invitrogen
Restore™ Western Blot Stripping Buffer Restore Western Blot Stripping Buffer	Pierce
SilverSNAP® Stain for Mass Spectrometry SilverSNAP® Sensitizer SilverSNAP® Enhancer SilverSNAP® Developer SilverSNAP® Stain Silver Destain reagent A Silver Destain reagent B	Pierce
SNAP i.d. Protein Detection System SNAP i.d. Single Well Blot Holder SNAP i.d. Spacer SNAP i.d. Blot Roller	Millipore
SuperSignal® West Pico Chemiluminescent Substrate Luminol/Enhancer Solution Stable Peroxide Solution	Pierce
Zero Blunt® TOPO® PCR Cloning Kit pCR®-Blunt II-TOPO® vector dNTP mix Salt solution M13 Reverse Primer M13 Forward Primer OneShot TOP10 chemically Competent *E. coli* cells	Invitrogen

2.8 Agars and media

Medium	Supplier

BHI (Brain-Heart-Infusion) — Oxoid

Medium: 37 g BHI
dH_2O to 1 litre
Sterilized in a Certoclav for 15 min at 115°C
Agar: BHI medium with 1,5 % (v/v) agar
Appropriate antibiotic added after cooling of the media to ~60°C, and transferred to petri dishes.

MRS (de Man, Rogosa, Sharpe) — Oxoid

Medium: 52 g BHI
dH_2O to 1 litre
Sterilized in a Certoclav for 15 min at 115°C
Agar: MRS medium with 1,5 % (v/v) agar
Appropriate antibiotic added after cooling of the media to ~60°C, and transferred to petri dishes.

MRSSM medium

52 g MRS
171 g sucrose (0,5 M)
2,0 g $MgCl_2x6H_2O$ (0,1 M)
dH_2O to 1 litre, filter sterilized (0,22 µm pore size)

RPMI-1640 — PAA

S.O.C. medium

2 g Bacto™ Tryptone
0,5 g Bacto™ yeast extract
0,057 g NaCl (1 M)
0,019 g KCl (1 M)
0,247 $MgSO_4$
60 ml dH_2O
Sterilized in a Certoclav for 15 min. at 115°C.
2 ml glucose (1 M) (added after autoclaving)
Sterilized dH_2O to 100 ml.

2xYT medium

16 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.

3. METHODS

3.1 Buffers and solutions

Buffers/ solutions	Content
Coomassie Brilliant Blue staining for SDS- PAGE	0.1 % (v/v) Coomassie Brilliant Blue R250 50 % (v/v) methanol 10 % (v/v) acetic acid
Destaining solution for SDS-PAGE	10 % (v/v) ethanol 10 % (v/v) acetic acid
Ethanol wash for silverstain	10 % (v/v) ethanol
Fixing solution for silverstain	30 % (v/v) ethanol 10 % (v/v) acetic acid
PBS (Phosphate Buffered Saline), pH 7,4	8 g/l Nacl 0. 2 g/l KCL 1. 44 g/l Na2HPO4 0.24 g/l KH2PO4
Stop solution for silverstain	5 % (v/v) acetic acid
TAE, 50 x	242 g Tris Base 57.1 ml acetic acid 100 ml 0.5 M EDTA, pH 8 Final volume 1 l
TBS (Tris Buffered Saline), pH 7.4	0.150 M NaCl 0.010 M Tris- HCl
TEN- buffer	10 mM Tris- HCl, pH 8 1 mM EDTA, pH 8 100 mM NaCl
TTBS	TBS 0.1 % (v/v)Tween-20

3.2 General methods in molecular biology

3.2.1 Growing of bacterial cultures

Bacterial cultures were grown in suitable liquid media. Single colonies were isolated by spreading bacteria on plates containing medium solidified 1.5 % (w/v) with agar. Antibiotics were added, to select for recombinant bacterial cells containing plasmids encoding an antibiotic resistance genes. Further details are given below.

Cultivation of *Escherichia coli*

E. coli cells were grown overnight at 37 º C in Brain-Heart-infusion (BHI) - medium. Cells in liquid culture were grown in a shaking incubator. Cells grown on solid media were incubated without shaking. The following antibiotic concentrations were used for *E. coli*.

- TOPO-vector: Kanamycin, 100 µg/ml in liquid and 50 µg/ml in solid medium.
- pUC57-Amp-vector: Ampicillin, 100 µg/ml in liquid and 200µg/ml in solid medium.
- pSIP-derivatives: Erythromycin, 200 µg/ml in liquid and solid medium.

Cultivation of *Lactobacillus plantarum*

L. plantarum cells were grown overnight in liquid de Man, Rogosa, Shrape (MRS)-medium without shaking, at either 30 or 37º C. On solid medium, cells were incubated without shaking for two days at 30 º C. The following antibiotic concentrations were used for *L. plantarum*:

- pSIP-derivatives: Erythromycin, 5-10 µg/ml in both liquid and solid medium.

3.2.2 Long-term storage of bacteria

Bacterial cultures were stored as glycerol stocks at – 80 º C. 300 µl 87 % (v/v) glycerol was added to 1 ml of bacterial culture to prevent cell disruption during long-term storage at low temperatures. To grow bacteria from glycerol stocks, a small amount of frozen culture was picked with a sterile toothpick and inoculated in a suitable growth medium.

3.2.3 Plasmid isolation from *Escherichia coli*

In order to isolate plasmid DNA from *E. coli*, the Nucleospin® Plasmid miniprep Kit from Macherey-Nagel was used.

Materials:

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

Procedure:

The procedure was performed according to the protocol for isolation included in the kit. All centrifugation steps were performed at room temperature.

1. 1-5 ml overnight culture was centrifuged at 11 000 x g for 30 sec to pellet the cells in an eppendorf tube.
2. The resulting pellet was completely resuspended in 250 µl Buffer A1/RNAse A by pipetting up and down.
3. SDS/alkaline lysis of *E. coli* cells were achieved by adding 250 µl Buffer A2. The solution was mixed gently by inverting the tube 6-8 times. The solution was incubated for 5 min in order to ensure complete cell lysis.
4. To remove cell debris, 300 µl of Buffer A3 was added to the clear lysate and the tube was inverted 6-8 times. To pellet the resulting precipitate, the tube was centrifuged at 11.000 x g for 5 min.
5. The supernatant was transferred to a NucleoSpin® Plasmid Column assembled in a collection tube. The column was then centrifuged at 11.000 x g for 1 min in order to bind the plasmid DNA to the silica column.
6. The flow-through was discarded and the column washed with 500 µl Buffer AW pre-heated to 50º C. The column was centrifuged for 1 min. at 11.000 x g.
7. To ensure the removal of contaminants like salts and metabolites, 600 µl of Buffer A4, supplemented with ethanol, was loaded to the column. The column was centrifuged at 11.000 x g for 1 min. Residual ethanol was removed by a second centrifugation for 2 min at 11.000 x g.
8. The dry column was placed in a clean eppendorf tube. 50 µl of elution buffer, Buffer AE, was added and the column incubated for 1 min at room temperature. The column was then centrifuged for 1 min. at 11.000 x g. The eluted plasmid was stored at – 20 º C.

3.2.4 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a technique that exponentially amplifies DNA by *in vitro* enzymatic replication. The desired DNA sequence can be amplified in a three-step process involving specific oligonucleotide primers, dNTPs and DNA polymerase. Primer annealing to complementary parts of DNA sequence allows the polymerase to initiate DNA replication of DNA fragments located between primers. In a PCR machine, thermal cycles comprising the three steps, denaturation, annealing and elongation, lead to repeated synthesis of the desired DNA fragments. In this study PCR was used for amplification of DNA fragments for cloning purposes and for DNA sequencing.

Materials:

Phusion™HotStart High-Fidelity DNA polymerase

5 x Phusion HF buffer (containing $MgCl_2$)

dNTP-mix

Primers (see materials, section 2.5)

Procedure:

1. The PCR reactions were performed essentially as suggested by the supplier of the Phusion™HotStart High-Fidelity DNA polymerase (NEB). A typical reaction setup is shown in Table 3.1.

Table 3.1 PCR-reagents

Reagent	Volume	Final conc.
H_2O	To 50 µl	
5 x Phusion HF buffer	10 µl	1x
10mM dNTP	1 µl	200 µM
Forward primer (50 pmol)	1 µl	1 pmol
Reverse primer (50 pmol)	1 µl	1pmol
Template DNA	1 µl	~ 200 ng
Phusion HotStart DNA polymerase	0.5 µl	

2. The reagents were mixed and the reaction mixture placed in a thermal cycler. Typical setting for the cycler are shown in Table 3.2

Table 3.2 PCR-settings

Temperature	Action	Time	Cycles
98 º C	Initial denaturation	30 seconds	1
98 º C	Denaturation	5 seconds	
60 º C*	Annealing	10 seconds	25
72 º C	Extension	20 seconds	
72 º C	Final extension	5 minutes	1
4 º C	Storage	∞	

***** This temperature was varied and adapted to the primers used.

The temperature was set to be about 5 ºC below the average melting point of the primers.

3.2.5 Agarose gel electrophoresis

Agarose gel electrophoresis is a method used to separate DNA fragments according to size, by applying an electric current. DNA can be visualised by the addition of ethidium bromide prior to casting of the gel. Regular gels were 1.2 % whereas a 3.5 % agarose gel was utilized for separation of DNA fragments between 100-450 bp.

Preparation of 1.2 % agarose gels

Materials:

SeaKem® LE agarose

1 x TAE buffer (section 3.1)

10 mg/ml ethidium bromide

Procedure:

1. A 1 l stock solution of 1.2 % agarose gel was prepared by dissolving 12 g of SeaKem® LE agarose in 1 liter 1 xTAE buffer. The agarose solution was autoclaved at 115 ºC for 15 min in a Certoclave. The agarose solution was then stored at 60 ºC.
2. Prior to casting, 1 µl of 10 mg/ml ethidium bromide was added to 60 ml agarose solution. The solution was mixed well and poured into the gel rack, where combs were inserted to make the wells.

3. The gel was left to solidify for about 20 min. Then the combs were removed and the gel placed in an electrophoresis chamber filled with 1 x TAE buffer.

Preparation of 3.5 % agarose gels

Materials

NuSieve® GTG® agarose

1 x TAE buffer (section 3.1)

10 mg/ml ethidium bromide

Procedure:

1. 3.1 g NuSieve® GTG® agarose was added to a 250 ml Erlenmeyer flask with 60 ml chilled 1x TAE-buffer, while the solution was rapidly stirred.
2. The agarose was soaked in the buffer for 15 min. to reduce the tendency of the agarose solution to foam during heating.
3. A cap was fitted to the flask to reduce evaporation during heating. The solution was heated in a microwave oven on medium power for two minutes. The flask was then gently swirled to resuspend any settled particles in the solution.
4. The solution was reheated in the microwave oven on high power until the solution reached the boiling point; it was kept boiling, until all particles were dissolved.
5. Evaporated liquid was replaced by adding an equal amount of TAE buffer. The agarose solution was then left to cool down to about 60ºC prior to the addition of 1 µl of 10 mg/ml ethidium bromide.
6. The solution was poured into the gel rack, combs were inserted to make the wells, and the gel left to solidify for about 30 min.
7. The solidified gel was transferred to an electrophoresis chamber filled with enough TAE buffer to completely cover the gel.

3.2.6 Purification of DNA from agarose gels

DNA fragments were separated by agarose gel electrophoresis after treatment with restriction enzymes or amplification by PCR. The DNA fragments were extracted from the gel by the use of the NucleoSpin® Extract II kit from Macherey-Nagel. A clean scalpel was used to excise the desired DNA bands from the gel. The gel pieces exact weight was determined and DNA was isolated according to the recommended protocol.

Materials:

NucleoSpin® Extract II, Purification of nucleic acids (Macherey-Nagel).

Procedure:

1. Excised gel pieces containing DNA fragments were solubilized in the following volumes of buffer NT
    - For gels containing less than 2 % agarose: 200 µl buffer per 100 mg agarose.
    - For gels containing more than 2 % agarose: 400 µl buffer per 100 mg agarose.
2. The gel-buffer solution was incubated in a 50 ºC water bath for 5-10 min. The sample was vortexed every 2-3 min to ensure complete solubilisation of the gel piece.
3. A NucleoSpin® Extract II column was assembled in a 2 ml collection tube and the dissolved gel sample was loaded onto the column. The column was centrifuged at 11.000 x g for 1 min in order to bind the DNA to the matrix of the column.
4. The flow-through was discarded and the column was washed with 700 µl NT3 buffer, (NT3 buffer was diluted with 96 % ethanol according to protocol). The column was centrifuged at 11.000 x g for 1 min.
5. The flow-through was discarded and the column was centrifuged at 11.000 x g for 2 min. in order to remove all buffer NT3 residues from the silica membrane of the column.
6. The DNA was eluted from the column and into a clean eppendorf tube by the addition of 40 µl of buffer NE. After 1 min incubation at room temperature, the column was centrifuged at 11.000 x g for 1 min.

3.3 TOPO-cloning and transformation

PCR-products purified by agarose electrophoresis and subsequent gel extraction (sections 3.2.5 and 3.2.6) were cloned using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen). TOPO-cloning was the method of choice because a PCR-product located in a vector is easier to digest with restrictions enzymes, than the PCR-product itself. The TOPO-vector also provides as a direct source to the PCR-product, and eliminates the need to repeatedly amplify DNA-fragments. TOPO-cloning utilises the enzyme DNA topoisomerase I, which functions both as a restrictions enzyme and as a ligase as described in Figure 3. 1.

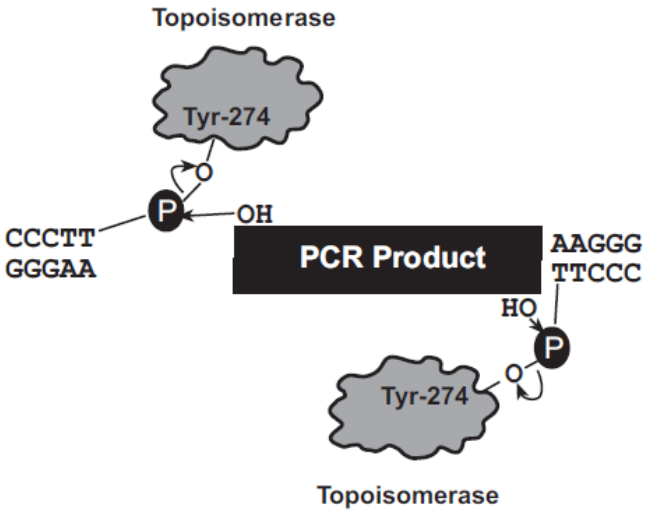

Figure 3. 1: The principle of Zero Blunt® TOPO cloning. The pCR®-Blunt II-TOPO® vector is supplied with DNA topoisomerase I covalently bound to the 3´end of each DNA strand. DNA topoisomerase I was originally isolated from *Vaccinia* virus and functions by binding to duplex DNA at specific sites. (Shuman 1991). Bound DNA topoisomerase I cleaves the phosphodiester backbone after 5´-CCCTT in one strand and the energy from the broken phosphodiester backbone is conserved by the formation of a covalent bond between the 3´phosfate of the cleaved strand and a tyrosyl residue of topoisomerase I. This phosphor-tyrosil bond can subsequently be attacked by the 5´hydroxyl group of the original cleaved strand, resulting in a reversing of the reaction and release of the topoisomerase (Shuman 1994). In the Zero Blunt® TOPO® cloning system this reaction is exploited to efficiently clone PCR products. The figure is taken from the Zero Blunt® TOPO® PCR Cloning Kit protocol (Invitrogen™).

Materials:

Zero Blunt® TOPO® PCR Cloning Kit

pCR®-Blunt II-TOPO® vector

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

OneShot TOP10 chemically Competent *E. coli* cells

S.O.C medium (see section 2.8)

BHI agar plates with kanamycin (50 µg/ml)

Procedure:

- The TOPO® cloning reaction was set up as described in Table 3.3.

Table 3.3. Reagents in TOPO-reaction

Reagent	Volume
PCR-product	2 µl
Salt solution	1 µl
dH2O	2 µl
pCR®-Blunt II-TOPO® vector	1 µl

1. The reagents were mixed carefully and incubated at room temperature for 5 min.
2. Aliquots of TOP 10 *E. coli* cells were transferred to a chilled Falcon tube and 2 µl of the TOPO® Cloning reaction was transferred to the cells. The Falcon tube was then placed on ice for 30 minutes.
3. The Falcon tube was transferred directly from ice to a 42 ºC water bath for 30 seconds to heat shock the cells.
4. The Falcon tubes was instantly transferred to ice for 2 minutes.
5. 250 µl of room tempered S.O.C. medium was added to the *E. coli* cells and the Falcon tube was incubated at 37 ºC with shaking for one hour.
6. 50-100 µl of the TOP 10 *E.* coli cell suspension was spread on prewarmed BHI plates containing 50 µg/ml kanamycin and the plates were incubated at 37 ºC overnight.

3.4 Preparation of chemically competent *Escherichia coli* TOP10 cells

Chemically competent *E. coli* TOP10 was prepared in order to have a supply of competent cells, in addition to the competent cells supplied in the Zero Blunt® TOPO® PCR Cloning Kit.

Materials:

0.05M $CaCl_2$

0.05M $CaCl_2$ w/15% glycerol

2xTY medium (Section 2.8)

Procedure:

1. A single *E. coli* TOP 10 colony was picked and cultivated in 5 ml 2xTY, overnight in a shaking incubator at 37 º C.
2. The culture was transferred to 200 ml 2xTY and grown at 37 º C, with shaking, until OD_{600} ~ 0.5.
3. The culture was transferred to 50 ml Nunc tubes and placed on ice for 10 min. The culture was centrifuged at 4 º C at 4000 rpm for 10 min.
4. The supernatant was discarded and the pellet re-suspended in 10 ml ice-cold 0.05M $CaCl_2$. The cell suspension was placed on ice for 15-30 min and then re-centrifuged.
5. The supernatant was discarded and the pellet re-suspended in 10 ml ice-cold 0.05M $CaCl_2$ w/15% glycerol. The cell suspension was placed on ice for 5-10 min before aliquots of 200 µl cell suspension were dispensed in eppendorf tubes. The competent cells were stored at – 80 ºC for up to 6 months.

3.5 Preparation of electro-competent *Lactobacillus plantarum* WCFS1 cells

Lactobacillus plantarum cells must be made electro-competent to permit transformation of plasmids into the cells by electroporation. Transformability was increased by growing *Lactobacillus* cells on media containing high amounts of glycine (Aukrust et al. 1995). The addition of glycine increases cell wall permeability by replacing L-alanine in the cell wall. Plasmid DNA is thereby easier taken up by the cells. The procedure was performed according to the protocol by Josson et al (1989).

Materials:

MRS medium

20% Glycine (w/v)

30% PEG-1450(w/v)

Corex tubes

Procedure:

All buffers, tubes and centrifuges were kept at 4 º C during harvesting of cells.

1. *L. plantarum* WCFS1 was inoculated in MRS overnight at 37 º C.
2. A serial dilution of the overnight culture was made in MRS + 1% glycine. The diluted cultures were incubated overnight at 37 º C.
3. The culture with an OD_{600} = 2.5 ±0.5 was diluted 1:20 in 50 ml MRS+1% glycine. The culture was grown until the OD_{600} reached = 0.7 ±0.07 (logarithmic growth phase) and then placed on ice for ten minutes.
4. The culture was centrifuged at 4500 rpm for 10 min. The supernatant was discarded and the cells re-suspended in 15 ml ice-cold 30 % PEG-1450. The suspension was transferred to a chilled corex-tube and kept on ice for 10 min. The suspension was re-centrifuged and the supernatant discarded.
5. The pellet was re-suspended in 400 µl ice-cold 30 % PEG-1450. While the cells were kept on ice, aliquots of 40 µl cell suspension were transferred to sterile eppendorf tubes. The eppendorf tubes were immediately placed on ice and stored at – 80 º C.

3.6 Digestion with restriction endonucleases and ligation of DNA fragments

Restriction endonucleases are enzymes that cut double-stranded DNA at specific nucleotide sequences. To cut the DNA, a restriction enzyme makes an incision through each sugar-phosphate backbone of the DNA double helix. Cohesive ends are created if the restriction enzyme cuts at different positions in each DNA strand. If the enzyme cuts at the same position in both strands, blunt ends are generated. Several factors have to be considered when setting up a restriction endonuclease digestion reaction. The reaction must be carried out in a buffer compatible with the restriction enzyme. The enzymes show optimal activity at various temperatures. Some restriction endonucleases need an additional adjuvant, such as the commonly used BSA, to have 100 % activity. Often compromises must be made to ensure

satisfying conditions for both restriction enzymes when performing a double-digest. Alternatively the reaction can be performed in two separate steps.

3.6.1 Restriction endonuclease digestion

A standard restriction enzyme digestion was performed according to the following protocol. The type of restriction buffer used was selected according to supplier recommendations.

Materials:

Restriction enzyme/enzymes	Listed in section 2.3
10x restriction buffer	Listed in section 2.3
100x BSA (bovine serum albumin)	Used in some reactions

Procedure:

An appropriate amount of DNA (depending on the experiment) was mixed with sterile water, 10x restriction buffer and 100x BSA as recommended by the supplier. Suitable restriction enzymes were added in volumes that were always less than 10 % of total reaction volume. After brief mixing the restriction mixture was incubated at the recommended temperature (usually at 37 ºC) for 1-2 hours.

In cases where a double-digest had to be performed in two steps, enzyme and buffer from the first restriction digest reaction were removed to ensure proper conditions for the second reaction. The method for removal of reaction residues is described in section 3.6.2, precipitation with Pellet Paint® Co- Precipitant.

The resulting DNA fragments were separated on agarose gel as described in section 3.2.5. The desired fragments were excised from the gel and purified according to the protocol described in section 3.2.6.

3.6.2 Nucleic acid precipitation with Pellet Paint® Co- Precipitant

Incomplete DNA digestions with restriction enzymes occasionally resulted in poor yield of digested fragments. In order to increase DNA concentration, a precipitation step was performed after agarose purification of the digested sample. A higher DNA concentration can increase the success rates of the following ligation. Pellet Paint® Co- Precipitant can also be

used as a clean-up step, removing undesired reactants, such as primers. Precipitation with Pellet Paint® Co- Precipitant is also recommended prior to digestion with certain restriction enzymes in order to increase reaction yield.

Materials:

Pellet Paint® Co- Precipitant

3 M sodium acetate, pH 5.2

Procedure:

1. The Pellet Paint® Co- Precipitant reagent was brought to room temperature and carefully mixed to ensure a homogeneous solution.
2. 2 µl Pellet Paint and 0.1 x volume 3 M sodium acetate was added to the sample and the solution was mixed briefly.
3. After addition of two volumes of 96 % (v/v) ethanol the reaction mixture was incubated at room temperature for 2 minutes.
4. The sample was centrifuged in a microcentrifuge at 16.000 x g for five minutes at 4 ºC. A pink DNA pellet appeared and the supernatant was aspirated.
5. The pellet was washed with 100 µl 96 % (v/v) ethanol, followed by centrifugation at 16.000 x g for five minutes at 4 ºC.
6. After removal of the supernatant the pellet was vacuum dried for five minutes.
7. The pellet was dissolved in an appropriate volume of sterile dH_2O and using a vortex for mixing.

3.6.3 DNA ligation

DNA ligase is an enzyme that repairs single-stranded breaks in the sugar-phosphate backbone of double stranded DNA at the expense of ATP. The enzyme catalyses the formation of a phosphodiester bond between 5´phosphate and 3´hydroxyl termini in double stranded DNA, thereby ligating the two DNA molecules ends together. The commercially available T4 DNA ligase and Quick T4 DNA ligase may be used to join DNA fragments with both cohesive and blunt ends.

T4 DNA ligation

Materials:

T4 DNA ligase (NEB)

1 x T4 DNA ligase reaction buffer (NEB)

Procedure:

The reaction was carried out according to the protocol provided by the supplier. A typical ligation reaction set-up is shown in Table 3.4.

Table 3.4. T4 DNA ligation reagents

Component	Amount
DNA	50 ng vector + 3-fold molar excess of insert
dH_2O	To 18 µl
10 x T4 DNA ligase reaction buffer	2 µl
T4 DNA ligase	1 µl

The reaction mixture was vortexed briefly and incubated at room temperature for 2-3 hours. Over-night ligation at 16 ºC was sometimes performed to increase success rate of difficult ligations (normally in cases with a low concentration of insert DNA).

Quick T4 DNA ligation

Materials:

Quick ligation™ kit

- Quick T4 DNA ligase
- 2 x Quick ligation buffer

Procedure:

The reaction was carried out according to the protocol provided by the supplier. A typical ligation reaction set-up is shown in Table 3.5.

Table 3.5. Quick ligation reagents

Component	Amount
DNA	50 ng vector + 3-fold molar excess of insert
dH_2O	To 10 µl
2 x Quick ligation buffer	10 µl
Quick T4 DNA ligase	1 µl

The reaction mixture was vortexed briefly and incubated at room temperature for 5 minutes. The reaction was then kept on ice until transformation.

3.7 Transformation of chemically competent Escherichia *coli* TOP10

Escherichia coli TOP10 cells were used as host cells for plasmids resulting from the ligation procedures described in section 3.6.3.

Materials:

One Shot® *E. coli* cells (Invitrogen) or home-made chemically competent *E. coli* TOP10 cells (section 3.4).

Plasmid DNA

S.O.C. medium

Falcon 2059 Polypropylene Round Bottom tubes (14 ml)

Procedure:

1. The competent cells were thawed on ice and 25 -50 µl of cell suspension transferred to a Falcon tube. 1-5 µl of plasmid DNA was added to the cells followed by gentle mixing.
2. The cell suspension was incubated on ice for about 30 min and then subjected to heat-shock for 30 seconds at 42 ºC.
3. 250 ml of room tempered S.O.C. medium was added to the cells and the culture was incubated for about one hour in a shaking incubator at 37 ºC.
4. 50-200 µl culture was spread on pre-warmed BHI agar plates with the appropriate antibiotics. The plates were incubated overnight at 37 ºC.

3.8 Electroporation of *Lactobacillus plantarum* WCFS1

The electroporation of electro-competent *L. plantarum* WCFS1 was conducted according to the protocol of Aukrust et al.(1995).

Materials:

Bio-Rad GenePulser® II

Bio-Rad Pulse controller plus

Electro-competent *Lactobacillus plantarum* WCFS1

Electroporation cuvettes

MRSSM medium

MRS agar plates with appropriate antibiotics

Procedure:

1. 40 µl electro-competent *L. plantarum* WCFS1 cells where thawed on ice prior to the addition of 5 µl of plasmid DNA. The mixture was transferred to a chilled electroporation cuvette, and the cuvette carefully tapped to mix the solutions and to remove any air bubbles in the solution.
2. The electroporation parameters were adjusted to the following:
    - Voltage: 1.5 kV
    - Capacitance: 25 mF
    - Resistance: 400 W
3. The cuvette was placed in the electroporation apparatus and the cells were given the electric pulse.
4. 950 µl of room tempered MRSSM medium was added to the cuvette; the cell suspension was then transferred to sterile eppendorf tubes and incubated for 2-3 hours at 30 º C.
5. About 100 µl of the cell suspension was spread on MRS agar plates with the appropriate antibiotics, and the plates were incubated at 30 º C for 48 hours.

3.9 DNA sequencing

DNA sequencing is a method for determination of the nucleotide sequence of a DNA fragment. A commonly used technique is the chain termination method developed by Frederick Sanger. This method uses a DNA polymerase and a limited concentration of dideoxsyribonucleotides (ddNTPs) in addition to the regular deoxyribonucleotides (dNTPs).

Incorporation of a ddNTP will terminate the DNA synthesis reaction, and ultimately result in a series of related DNA fragments, terminated at random positions. In dye terminator sequencing, the four unique ddNTPs are labelled in different fluorescent dyes. The latter incorporated fragment in a chain can thereby be identified as either A, T, G or C. The dNTPs and ddNTPs are added in a relationship that facilitates the production of fragments that are separated in length by only one nucleotide, making it possible to determine the sequence of the template DNA.

To ensure that no mutations had occurred during PCR amplification, all cloned fragments were sequenced using the ABI Prism 3100 BigDye® Terminator v3.1 Cycle Sequencing Kit for chain terminator sequencing.

Materials:

BigDye® Terminator v3.1 Cycle Sequencing Kit

BigDye® Terminator v3.1 Ready Reaction Premix
BigDye® Terminator v3.1 Sequencing Buffer (5x)

Procedure:

1. The sequencing reaction was set up as shown in Table 3.6.

Table 3.6. DNA sequencing reaction reagents

Reagent	Amount
Premix*	2.0 µl
Plasmid DNA	25-30 ng
Primer	3.2 pmol
Seq. buffer, 5x	3.0 µl
Sterile dH_2O	x µl
Total volume	20 µl

* Contains DNA polymerase, dNTPs, ddNTPs and buffer.

2. The sequence reaction mixture was placed in a thermal cycler and the settings shown in Table 3.7 applied.

Table 3.7. PCR-settings

Temperature	Time	Cycles
96 º C	1 minute	
95 º C	10 seconds	
50 º C	5 seconds	25
60 º C	4 minutes	
4 º C	∞	

Ethanol/EDTA precipitation of amplified DNA:

3. The sequencing reaction solution (total volume 20 µl) was transferred to 1.5 ml sequencing eppendorf-tubes To each sample 2 µl of 125 mM EDTA and 62.5 µl of 96 % ethanol were added and the solution mixed by inverting the tubes 4-5 times.
4. The samples were incubated at room temperature for 15 minutes prior to centrifugation at 4 º C at 16.100 x g for 30 minutes.
5. The supernatant was immediately aspirated and 60 µl of 70 % ethanol was added to each tube.
6. The samples were centrifuged at 4 º C and 16.100 x g for 10 min, and the supernatant immediately aspirated. The samples were left on the bench to dry for 10 – 30 minutes.
7. The samples were stored at - 20 º C prior to sequencing using the ABI Prism™ 3100 Genetic Analyzer. The resulting data were analysed using CLC DNA Workbench 5.

3.10 Cultivation and harvesting of *Lactobacillus plantarum* WCFS1 for analysis of gene products

L. plantarum WCFS1 harbouring the plasmid of interest was cultivated on media containing appropriate antibiotics (see section 3.2.1) and gene expression was induced by the addition of an inducer peptide (SppIP, see section 2.3). Cells harbouring plasmids for constitutive production of protein was not induced, but otherwise treated similarly. After harvesting by centrifugation, cells were mechanically disrupted by glass beads to release the intracellular protein fraction. If different cell fractions were to be analysed from the same culture, enzymatic cell lysis were performed. Culture supernatant were sterilized by filtration and used to analyse secreted proteins.

Materials:

MRS medium

Erythromycin (10 mg/ml)

Inducing peptide SppIP (0.1 mg/ml)

TEN- buffer (section 3.1)

50 mM PMSF (Phenylmethylsulphonyl fluoride) dissolved in isopropanol

Tris-buffered sucrose (10 mM Tris, 250 m-m sucrose, pH 7)

2x osmotic digestion buffer (20 % sucrose in 20 mM Tris-HCl, pH 7)

100 % TCA

Acetone

Glass beads (106 microns and finer)

3.10.1 Analysis of intracellular protein production by *L. plantarum* WCFS1

All incubation steps were carried out at either 30 ºC or 37 ºC depending on the purpose of the individual experiment.

Procedure:

1. *L. plantarum* WCFS1 harbouring the desired plasmid construct was grown overnight at the desired temperature in MRS containing 10 µg/ml erythromycin
2. The overnight culture was diluted in MRS, containing 10 µg/ml erythromycin, until the culture reached an absorbance of 0.11-0.15 at OD_{600}. The diluted culture was then incubated at 30 or 37 ºC until an absorbance of 0.27-0.33 at OD_{600} was reached.
3. When the culture reached an absorbance of 0.27-0.33 at $OD_{600,}$ induction was carried out by adding SppIP. The concentration of SppIP added varied from 0.1-25 ng/ml depending on the individual experiments. Induced culture was then incubated until the desired OD-value or incubation time was reached. The desired incubation time or OD-value at the time of harvest, varied between experiments.
4. 14-45 ml of cell culture (depending on the individual experiment) was harvested in an Eppendorf centrifuge 5430R by centrifugation at 3000 x g for 10 minutes at 4 ºC. After addition of PMSF to a final concentration of 1 mM PMSF, the supernatant was sterilized by filtration (0.2 µm filter) and stored at -20 ºC for further analysis.

5. The resulting cell pellet was washed with 10 ml TEN-Base buffer and re-centrifuged. The washing buffer was discarded and the cell pellet was then either kept on ice prior to subsequent cell disruption or stored at -20 º C.
6. The cell pellet was resuspended in 1 ml TEN-Base buffer and 1 mM PMSF and transferred to a pre-chilled FastPrep tube filled with 1.5 grams of glass beads.
7. The FastPrep tube was placed in a FastPrep® FP120 Cell Disrupter and shaken at a speed of 6.5 m/s for 45 seconds.
8. The FastPrep tube was centrifuged at 16.100 x g for 5 min at 4 º C. The supernatant was transferred to a sterile eppendorf tube and recentrifuged.
9. The supernatant was transferred to a new eppendorf tube in order to remove glass bead remnants. The resulting protein extract was kept on ice for immediate analysis or stored at -20 º C until use.

3.10.2 Analysis of different cell fractions in *L. plantarum* WCFS1

Procedure:

Cultivation and incubation of *L. plantarum* WCFS1 was performed according to steps 1-3 described in section 3.10.1. Protein production was induced by addition of 10 ng/ml SppIP.

4 45 ml of cell culture was harvested in an Eppendorf centrifuge 5430R by centrifugation at 3000 x g for 10 minutes at 4 ºC.

5 After addition of PMSF to a final concentration of 1 mM PMSF, the supernatant was sterilized by filtration (0.2 µm filter) and subsequently transferred to a sterile Nunc tube and stored at -20 ºC until further analysis. The cell pellet and supernatant were processed separately from this point onward.

Supernatant fraction:

A desired volume of supernatant was precipitated by one of the following two precipitation methods. TCA precipitation was most often used in this study.

TCA precipitation of protein

Procedure:

1. The supernatant was adjusted to pH 7 with 6M NaOH, and 0.2 mg/ml of Sodium Deoxycholate was added. The sample was kept on ice for 30 minutes before proceeding with TCA precipitation.
2. One volume of 100 % TCA was added to four volumes of adjusted supernatant. The reaction mixture was then vortexed and incubated on ice for 20 minutes.
3. The sample was centrifuged at 16.100 x g for 5 minutes at 4 ºC to collect the precipitated proteins.
4. The supernatant was discarded and 200 µl ice-cold acetone added to the protein pellet to remove remaining TCA. The mixture was vortexed before the sample was re-centrifuged.
5. The pellet was dried in a vacuum centrifuge for 5 minutes.

Methanol-Chloroform precipitation of protein

Procedure:

1. To a sample volume of 200 µl, 600 µl methanol and 150 µl chloroform were added and the solution was mixed thoroughly by vortexing. For bigger volumes of sample the volume of all additives was increased accordingly.
2. The sample was centrifuged at room temperature at 16.100 x g for 1 min. For bigger volumes the sample was centrifuged at 5000 x g for 10 min.
3. The upper liquid fraction was aspirated; care was taken not to disrupt the precipitate at the protein-chloroform interface.
4. 450 µl of methanol was added to the sample and the solution was mixed thoroughly by vortexing (for larger sample volume a corresponding amount of methanol was added).
5. The samples were re-centrifuged for 3 minutes at 16.100 x g, or at 5000 x g for 15 minutes for bigger volumes.
6. The liquid fraction was aspirated and the pellet dried by exposing to air.

Cell wall fraction:

1. A fresh cell pellet from harvested culture was resuspended in 1.5 ml ice-cold Tris-buffered sucrose (pH 7) and transferred to an eppendorf tube. The tube was centrifuged for 5 minutes at 3000 x g and 4 ºC.
2. The supernatant was discarded and the pellet was resuspended in 500 µl 2x osmotic digestion buffer, 1 mM PMSF, 1 µM Pepstatin A, 10 mM 1.10-phenanthroline and 500 U mutanolysin. dH_2O was added to a final volume of 1 ml.
3. The cell suspension was mixed carefully and incubated in a 37 ºC water bath for 2 hours. The tube was regularly inverted during the incubation period.
4. The eppendorf tube was centrifuged at 3000 x g, 4 ºC for 30 minutes to remove the cells (now protoplasts).
5. The supernatant was transferred to a new eppendorf tube and centrifuged at 16.100 x g for 15 min., at 4ºC.
6. The supernatant was transferred to a new eppendorf tube; care was taken to make sure no cell debris followed.
7. The proteins in the supernatant (containing the digested cell wall) were precipitated using the TCA-precipitation protocol describes above (section 3.10.2).

3.11 Gel electrophoresis of proteins using the NuPAGE Electrophoresis system

Proteins can be separated according to their electrophoretic mobility in a sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). SDS-PAGE is a widely used technique in biotechnology to separate denatured proteins by size. The SDS-PAGE system used in this study was the NuPAGE® Novex Bis-Tris Electrophoresis System (Invitrogen™) with pre-casted Bis-Tris-HCl buffered polyacrylamide mini gels. Optimal protein sample preparation for SDS-PAGE requires denaturing, introduction of a uniform protein charge and reducing of protein disulphide bonds. The sample buffer contains lithium dodecyl sulphate (LDS), an anionic detergent that denatures proteins by disrupting non-covalent bonding, leaving the protein more negatively charged, with an approximately fixed charge per residue ratio. The addition of the reducing agent dithiothreitol (DTT) removes the disulphide bonds of the proteins. The size of sample proteins is determined by comparing with suitable protein

standards. Resulting protein bands may be visualized by Coomassie Brilliant Blue-staining, Silver staining or Western blotting.

3.11.1 Protein gel electrophoresis

Materials:

NuPAGE® Novex Bis-Tris Gels-kit (section 2.7)

1 x MOPS SDS Running buffer

Procedure:

In order to apply equal amounts of protein sample from each initial cell culture, the amount of sample loaded to the gel was calculated according to the measured OD_{600} at the time of harvest.

1. The protein sample was prepared by adding 1x NuPAGE® LDS Sample Buffer (4x), NuPAGE® Reducing Agent (10x concentrated) and dH_2O. If the protein sample had previously been precipitated, the resulting protein pellet was dissolved in 4M Urea, 1,1M Thiourea and 1 % ASB-14 prior to this step. The sample was mixed thoroughly by vortexing.
2. The sample was heated at 90 ºC for 10 minutes in order to denature the protein.
3. The electrophoresis container was set up and the gel placed in the buffer core chamber. The inner and outer buffer chambers were filled with 1x NuPAge® MOPS Running Buffer.
4. The samples and protein standard were carefully loaded onto the gel. Electrophoresis was carried out by applying 200 V for 50 minutes.
5. The gel was removed from the electrophoresis chamber and the plastic casing surrounding the gel was removed with a gel spatula.

3.11.2 Comassie Brilliant Blue-staining and destaining of NuPAGE® Novex Bis-Tris Gels

Materials:

Coomassie Brilliant Blue staining for SDS- PAGE (section 3.1)

Destaining solution for SDS-PAGE (section 3.1)

Procedure:

1. The gel was placed in a plastic tray and washed twice with dH_2O for 5 min., with shaking.
2. After removal of dH_2O, Coomassie Brilliant Blue staining solution was added to the gel and the gel was incubated for a minimum of 30 minutes with shaking.
3. The colour solution was removed and destaining solution added to the gel. After a few minutes of incubation with shaking, this was removed and fresh destaining solution was added.
4. The gel was incubated in destaining solution with shaking, until the appropriate removal of staining was achieved (approximately 15 minutes).

3.11.3 Silver staining of NuPAGE® Novex Bis-Tris Gels

Silver staining represents a more sensitive alternative to Coomassie Brilliant Blue-staining of polyacrylamide gels. The silver staining method is able to detect proteins in the nanogram range. The procedure was performed according to the protocol included in the SilverSNAP® Stain for Mass Spectrometry- kit.

Materials:

SilverSNAP® Stain for Mass Spectrometry- kit (Section 2.7)

Ethanol wash (section 3.1)

Fixing solution (section 3.1)

Stop solution (section 3.1)

Procedure:

1. The gel was washed by shaking the gel in a plastic tray filled with dH_2O for 5 min. The water was replaced and a second identical wash performed.

2. The water was decanted and a fixing solution was added to the gel. The gel was incubated for 15 min with shaking at room temperature. The solution was replaced and the gel fixed for another 15 min.
3. The gel was washed twice with ethanol wash for 5 min.
4. The ethanol was replaced with dH_2O and the gel was washed for 5 min. The water was decanted and a second wash with water was performed.
5. The sensitizer working solution was prepared by mixing one part SilverSNAP® Sensitizer with 500 parts of dH_2O.
6. The gel was incubated in sensitizer working solution for precisely one minute. Two washes with dH_2O, each for one min, were then performed.
7. One part of SilverSNAP® Enhancer was mixed with 100 parts of SilverSNAP® Stain and immediately added to the gel. The gel was incubated with shaking for 5 min.
8. The developer working solution was prepared by mixing one part SilverSNAP® Enhancer with 100 parts SilverSNAP® Developer.
9. The gel was washed twice for 20 seconds in dH_2O. The developer working solution was added immediately to the gel tray and the gel was incubated until protein bands appeared.
10. The developer solution was replaced with stop solution when the desired band intensity was reached. The gel was washed briefly before the solution was replaced with fresh stop solution and the gel was then incubated for 10 min.
11. The stop solution was replaced with water and the gel was analysed and documented.

3.12 Western blot analysis

Western blot analysis is a widely used technique that utilises antibodies for very sensitive detection of specific proteins. Proteins separated by gel electrophoresis are transferred from the gel to a membrane (usually nitrocellulose or Polyvinylidene fluoride) were they are available for antibody hybridisation. The blotting procedure is performed by applying an electric current that pulls the proteins from the gel and onto the membrane. After blotting, Coomassie- staining (section 3.11.2) of the gel was performed to confirm successful transfer of proteins. Because of the membrane's high affinity for proteins it must be blocked prior to antibody hybridization to prevent unspecific binding of the antibody to the membrane. During the detection process, the protein of interest is probed with a primary antibody that binds to a specific epitope on the protein (Figure 3. 2). After rinsing of the membrane to remove unbound primary antibody, the membrane is exposed to a secondary antibody that will bind to the primary antibody. The secondary antibody is linked to a reporter enzyme that upon addition of a proper substrate produces a detectable signal. In this study chemiluminescent detection was used.

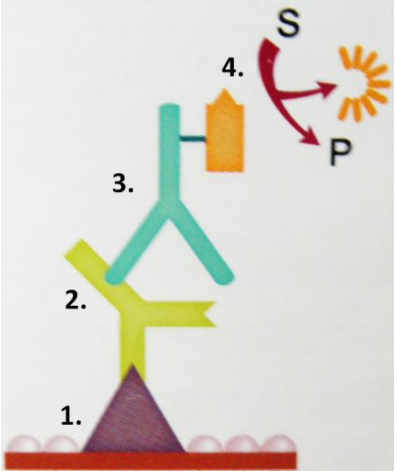

Figure 3. 2: The principle of Western blotting. The protein (1) on the membrane is hybridized to the primary antibody (2) by its binding to a specific epitope on the protein. The secondary antibody (3) binds to a specific part of the primary antibody. The secondary antibody is linked to an enzyme that catalyses the the added substrate (S) to a product (P), which give a detectable signal, for example a chemilumenescent signal. The figure is taken from the BioRad Protein Blotting guide (3rd edition).

3.12.1 Blotting with the iBlot™ Dry Blotting system

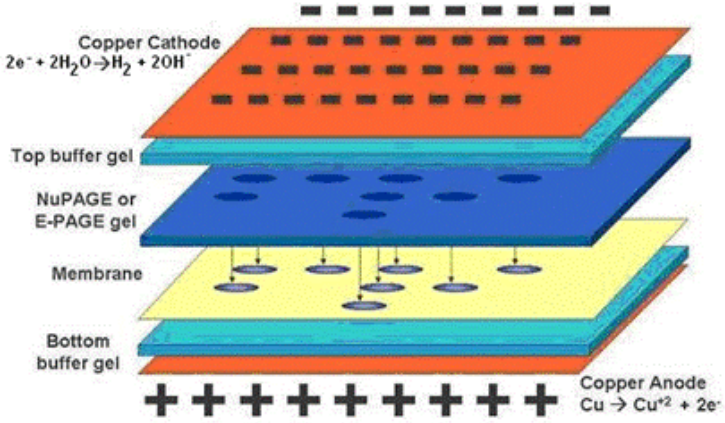

Figure 3. 3: Assembly of the iBlot ™ Gel Transfer stack. The transfer stack contain the appropriate anode and cathode buffers in addition to the copper electrodes (anode and cathode) required for electrophoresis. The iBlot™ Anode Stack, holding the nitrocellulose membrane, is placed on the bottom, followed by the NuPAGE gel, a wet filter paper and finally the iBlot™ Cathode Stack. The figure is taken from the iBlot® Dry Blotting System user manual (Invitrogen™).

Materials:

iBlot™ Dry Blotting system (section 2.8)

- 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 SDS-PAGE gel (see section 3.11.1) was washed with dH_2O for 5 minutes.
2. The anode stack was put in its aligned place in the transfer device. A blotting roller was used to remove air-bubbles visible between the nitrocellulose membrane and the transfer stack gel below the nitrocellulose membrane.
3. The gel was placed on the nitrocellulose membrane in the anode stack (Figure 3. 3). A wet filter paper was placed on top of the gel. A blotting roller was used to remove any air-

bubbles between the nitrocellulose membrane and gel to ensure proper blotting of the proteins.

4. The cathode stack was placed on top of the wet filter paper, with the copper-electrode side facing up and a blotting roller was used to remove any air-bubbles.
5. The disposable sponge was placed inside the lid of the transfer device. The lid was closed and the blotting carried out by applying 20 V for 7 minutes (program 3).

3.12.2 Antibody hybridization

Two different approaches for antibody hybridization were used, the traditional immunodetection method and the SNAP i.d. immunoblotting system. The advantage of the SNAP i.d. immunoblotting system was a far less time consuming protocol than the traditional method. In this study the SNAP i.d. immunoblotting system was used to probe most of the western blots.

Traditional immunodetection

Materials:

Blocking buffer (TBS w/3% BSA)
TBS (section 3.1)
TTBS (TBS w/0.05 % TWEEN-20)
TBS w/ 10 % skimmed milk
Primary antibody (section 2.3)
Secondary antibody (section 2.3)

Procedure:

1. After blotting, the membrane (nitrocellulose membrane) was removed from the blotting device and transferred to TBS buffer.
2. The membrane was washed 2 x 10 min with shaking in TBS. A volume of at least 25 ml was used every time.
3. The membrane was incubated for one hour in blocking buffer with shaking.
4. After blocking the membrane was washed 2 x 10 min in TTBS, with shaking.
5. The membrane was then washed once in 25 ml TBS for 10 min.

6. The appropriate amount of primary antibody was added to 15 ml of blocking buffer and poured onto the blot membrane. Care was taken to cover every part of the blot with antibody solution.
7. The blot membrane was incubated with primary antibody for one hour at room temperature. Alternatively the blot could be incubated first for 15 min at room temperature, then overnight at 4 ºC, and finally for 30 min in room temperature the day after.
8. The blot membrane was washed 2 x 10 min with TTBS, then for 10 min in TBS.
9. Secondary antibody was diluted in TBS w/ 10 % skimmed milk and incubated with the blot for one hour.
10. A final washing sequence of 4 x 10 min of 25 ml of TTBS was performed.
11. The blotted membrane was now ready for incubation with the appropriate detection agent (see section 3.12.3).

Immunodetection using the SNAP i.d. immunoblotting system:

The SNAP i.d. Protein Detection System is a vacuum driven system for high quality protein blotting (Figure 3. 4). The system allows for detection of proteins in less than 25 minutes.

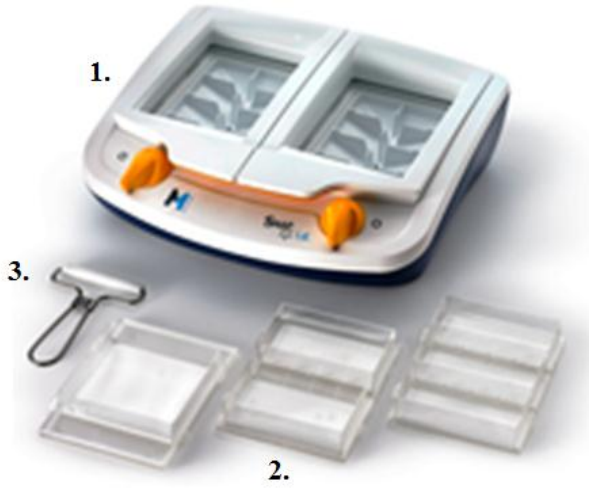

Figure 3. 4: The SNAP i.d. Protein Detection System. The SNAP i.d. system (1) consists of two independent blot holder stations**.** The system allows for blotting of up to six individual blots, using the differetn blot holders (2). Seperately controlled blotting is possible by adjusting the vacuum of each station. The blot roller (3) is used for removal of air bubbles when placing the membrane in the blot holder. The illustrative figure is taken from the SNAP i.d Protein Detection System- protocol (Millipore)

Materials:

TTBS (section 3.1)

TTBS w/ 0.5 % BSA

Blocking buffer (TTBS w/1 % BSA)

Primary antibody (section 2.3)

Secondary antibody (section 2.3)

SNAP i.d. Protein Detection System

Mouse monoclonal antibody to ESAT6 (HYB 076-08) was used as primary antibody at a concentration of 0.1 µg/ml for all blots shown in this thesis. The secondary antibody, HRP-Rabbit Anti-Mouse IgG, was used at a concentration of 0.02 µg/ml.

Procedure:

1. The inner face of the blot holder was wetted with dH_2O until it turned grey. Excess water was removed by using the blot roller.
2. The wetted membrane was placed in the blot holder with the protein side facing down. Air-bubbles were removed by using the blot roller.
3. The spacer was placed on top of the blot membrane and the blot roller used to ensure complete contact between the two.
4. The lid was closed and the blot holder placed, with the well side up, in the chamber of the SNAP i.d.® Protein Detection System device (Figure 3. 4).
5. 30 ml of blocking solution (TTBS w/1% BSA) was added under continuous vacuum.
6. When the well had emptied completely, the vacuum was turned off and 3 ml of TTBS containing 0.5 % BSA and primary antibody was added. Care was taken to ensure that the antibody solution completely covered the blot. The antibody solution was allowed to incubate for 10 min with the vacuum off.
7. The blot was washed 3 x 10 of TTBS with the vacuum running continuously.
8. The vacuum was turned off and 3 ml of TTBS containing 0.5 % BSA and secondary antibody was added.
9. After a 10 minute incubation of secondary antibody, the vacuum was turned on and three sequential washes were performed. Again with 3 x 10 ml TTBS.
10. The blot was removed from the blot holder and incubated with the appropriate detection agent.

3.12.3 Chemiluminescent detection of proteins

Materials:

CL-Xposure™ Film

SuperSignal® West Pico Chemiluminescent Substrate (section 2.7)

Luminol/enhancer Solution

Stable Peroxide Solution

Procedure:

1. A 1:1 working solution of Luminol/Enhancer Solution and Stable Peroxide Solution was made and incubated with the antibody treated membrane with shaking for 5 minutes at room temperature. About 0.125 ml of working solution was used per cm^2 of membrane.
2. The membrane was removed from the working solution and covered with plastic foil. The covered membrane was placed in a film-cassette with the protein side of the membrane pointing upwards. The rest of the procedure was carried out in a dark room.
3. CL-Xposure™ Film was placed on the membrane in the film-cassette. The film-cassette was closed and the film was exposed for ~1 min. (or less) and all up to 30 min, depending on the strength of the chemiluminescent signal.
4. The exposed film was transferred to a tray containing developer solution and incubated~ up to 2 min.
5. The film was then transferred to a tray containing fixing solution and incubated for at least 2 min.
6. Finally the film was washed in water and subsequently air-dried.

3.12.4 Stripping and reprobing of nitrocellulose membrane probed by Western blotting

The Western blotting procedure contains many different parameters that have to be optimised, especially when using new primary and/or secondary antibodies. Nitrocellulose membranes analysed using the techniques described above (in sections 3.12.2 and 3.12.3) can be stripped for reprobing by using a Western blot stripping buffer. This allows for optimising of Western blot parameters by stripping secondary and primary antibodies from membranes and enables repeated reprobing of the same membrane. It should be noted that reprobing can cause decreased signals due to incomplete removal of antibody and wearing of the membrane.

Materials:

Restore™ Western Blot Stripping Buffer

TTBS (section 3.1)

Procedure:

The Restore™ Western Blot Stripping Buffer was brought to room temperature prior to use.

1. The previously blocked, probed and developed Western blot membrane was stored in TBS at 4ºC until the stripping procedure could be performed.
2. The blot was incubated in the Restore™ Western Blot Stripping Buffer for 30 min at room temperature.
3. The blot was washed with TTBS and a new antibody hybridization and detection procedure could be performed (see sections 3.12.1 to 3.12.3).

3.13 Detection of cell surface antigens using FITC-labelled secondary antibody

Fluorescein is a fluorochrome that is widely used in fluorescent assays. Linking a fluorochrome to an antibody allow for direct visualization of target antigen by a variety of methods, such as flow cytometry and fluorescent microscopy. In this study a fluorescein isothiocyanate (FITC) -labelled secondary antibody was used to detect anchoring of a specific antigen (via a primary antibody) to the cell surface of *L. plantarum* WCFS1 cells harbouring different plasmid constructs. The fluorescent signal was analysed by fluorescent microscopy and flow cytometry.

Flow cytometry enables the measurement of cellular and fluorescent properties of individual cells in a heterogeneous suspension as they pass by a laser or other light source in a flow cytometer. The flow cytometer can count and measure the individual properties of thousands of cell by detecting the changes in light scatter, absorbed light and the light emitted as they passes by certain detectors as shown in Figure 3. 5. The data gathered from these measurements can be used to quantify specific cell populations and subsets within a given cell population. Cells labelled with FITC give distinct green emission spectra at 530 nm, making it possible to evaluate a population of cells for the existence of distinct cell surface antigens (Rahman 2006)

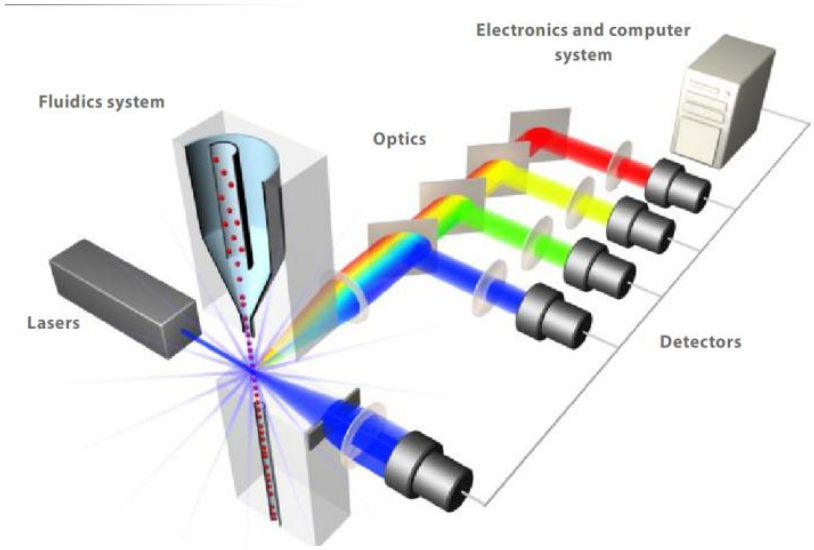

Figure 3. 5: A schematic illustration of the primary systems of a flow cytometer. The fluidic system carries the samples to the interrogation point and removes the waste; the lasers are the light source for scatter and fluorescence; the optics gathers and directs the light; the detectors receive the light; the electronics and the peripheral computer system convert the signal from the detectors into digital data and perform the requested analysis. The figure is taken from "Introduction to flow cytometry" by Invitrogen ™

3.13.1 Staining surface antigens with FITC-labelled secondary antibody

Materials:

PBS (section 3.1)

PBS containing 2% (w/v) BSA

Anti-ESAT6 monoclonal primary antibody (section 2.3)

FITC- labelled secondary antibody (anti-mouse) (section 2.3)

Procedure:

All centrifugation steps were performed at 4 ºC.

1. *L. plantarum* WCFS1 harbouring the desired plasmid was cultivated, and protein production was induced according to steps 1-3 in section 3.10.1.
2. The cell culture was incubated for 1-2 hours after induction (depending on the individual experiment) and approximately 1 ml of cell culture was then transferred to an eppendorf tube and centrifuged at 5000 x g for 3 minutes to pellet the cells. The amount of cell culture used was adjusted according to the measured OD_{600}. In general one ml of a bacterial culture was used for cultures with an OD_{600} of 0.5

3. The supernatant was discarded and the cell pellet was washed twice with 1 ml of PBS. The sample was centrifuged at 5000 x g for 2 min between each wash. After the final wash the pellet was recentrifuged and the supernatant carefully aspirated.
4. The pellet was resuspended in 50 µl PBS/2% BSA and 0.4 µl of primary antibody was added to the sample. The cell solution was mixed gently and incubated on ice for 20 min.
5. The cell solution was washed 3 times with 1 ml PBS/2% BSA. The sample was centrifuged at 5000 x g for 2 min between each wash. After the final wash the pellet was recentrifuged and the supernatant was carefully aspirated.
6. The pellet was resuspended in 50 µl PBS/2% BSA and 0.4 µl of FITC-labelled secondary antibody. Care was taken not to expose the FITC-labelled secondary antibody directly to light. The sample was incubated on ice in the dark.
7. The sample was washed 3 x 1 ml PBS/2% BSA.
8. The pellet was resuspended in 50 µl PBS/2% BSA and kept on ice, protected from light, until further analysis.

Flow cytometry-analysis was performed using the MacsQuant® Analyzer (Miltenyi Biotec) and the MacsQuantify™ Software. The appropriate setting for detection of FITC-signals on bacterial cells were used and the sample uptake volume was set to 10 µl and 100 000 events.

Fluorescent microscopy was performed on a Zeiss LSM 700 Confocal Microscope (Carl Zeiss) and the images were viewed and recorded using Zen software (Carl Zeiss).

3.14 Effects of the Ag85B-ESAT6 expressing strains on dendritic cells

Induced cells of *L. plantarum* WCFS1 harbouring the desired plasmid were incubated with dendritic cells isolated from human blood. The cytokine profile of the dendritic cells was subsequently evaluated by enzyme-linked immunosorbent assay (ELISA).

3.14.1 Isolation of CD14 + cells (positive selection) from human blood cells

CD14 + cells are monocytes that can differentiate into a variety of different cells depending on the presence of cytokines. Differentiation into dendritic cells is stimulated by the addition of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4). CD14 is a protein found on the surface of CD14 + cells and functions as a marker to select for these specific monocytes from human peripheral blood leukocytes. For isolation, the CD14 + cells are first labelled with magnetic beads conjugated to monoclonal anti-human CD14 antibodies. By loading the cell suspension onto a column placed in a magnetic field, the CD14 + cells are retained in the column, while unlabelled cells run through. When the column is removed from the magnetic field the CD14 + cells can be eluted as the positively selected cell fraction. In this study CD14 + cells were isolated from human peripheral blood leukocytes. Human peripheral blood leukocytes isolated from buffy coat and stored in liquid nitrogen were used for this purpose.

Materials:

Dulbecco's PBS 1(x)
Macs CD14 MicroBeads (human)
Macs Column
Macs Separator
RPMI containing 1% (v/v) Fetal Calf Serum (FCS) and 2mM EDTA.
RPMI containing 1% (v/v) Fetal Calf Serum (FCS).

Procedure:

Pre-cooled solutions were used and the cells were kept cold at all times.

1. Ampules containing human peripheral blood leucocytes were thawed in a 37 º C water bath and the cells were transferred to a 50 ml Nunc tube. Care was taken not to completely thaw the frozen blood leukocytes suspension. The number of ampules used

depended on the amount of cells in each ampule and the number of cells needed in the individual experiment.

2. The Nunc tube was filled with PBS to a total of 50 ml, and the solution was centrifuged at 300 x g for 10 minutes at 4 ºC in a Megafuge 1.0.
3. The supernatant was aspirated and after resuspending the cells in PBS their number was estimated by counting cells ranging in size from 3 µm -9 µm in a Coulter Counter® Z1.
4. The cell suspension was centrifuged at 300 x g for 10 min at 4 ºC. The supernatant was aspirated and the cells were resuspended in 1600 µl of RPMI/1% FCS and 2mM EDTA.
5. The cell suspension was passed through a pre-separating filter to remove cell aggregates. Removal of cell aggregates ensures optimum flow in the separation column and more effective labelling of the cells. The pre-separating filter was first wetted with 500 µl of RPMI/1% FCS. The cell suspension was subsequently passed through and collected in a 15 ml Nunc tube. The pre-separating filter was then washed with 500µl RPMI/1% FCS.
6. 20 µl of Macs CD14 MicroBeads was added pr. 10 million cells. The suspension was mixed well and incubated on ice for 15 min.
7. The cell suspension was washed with 10 ml RPMI/1% FCS and centrifuged at 300 x g for 10 min at 4 ºC. The supernatant was aspirated and the cells were resuspended in 1000 µl of RPMI/1% FCS.
8. A column was placed in the magnetic field of a magnetic separator and rinsed with 500 µl of RPM/1% FCS. 500 µl of cell suspension was added to the column. The unlabelled cells were collected and the column was washed three times with 500 µl of RPMI/1% FCS.
9. To collect the CD14 + cells, the column was removed from the magnetic field and placed in a collection tube. 1000 µl of RPMI/1% FCS was added onto the column and the plunger pushed in to release the magnetically labelled cells.

Step 8 and 9 was repeated with a second column for the remaining 500 µl of the in total 1000 µl cell solution.

10. The positively selected cells were washed by adding 10 ml of RPM/1% FCS and centrifuged at 300 x g for 10 min. The supernatant was aspirated and the cells resuspended in 10 ml of RPMI/1% FCS.
11. After counting the number of cells (see step 3), the cell solution was diluted in RPM/1% FCS and seeded as 0.6 million cells/500 µl solution into microplates (48 wells). Prior to seeding of cells, differentiation factors 25 ng/ml IL-4 and 50 ng/ml GM-CSF were added

to the cell suspension. The microplates were incubated in a Steri-Cycle CO_2 Incubator at 37 º C during the whole process.

12. On day 4 after seeding of cells (the day of seeding = day 0), media was aspirated from the wells and 500 µl of fresh RPMI with 25 ng/ml IL-4 and 50 ng/ml GM-CSF was added to each well.
13. On day 6 the cells was checked for differentiation under a Leica microscope and approved ready to be used in further experiments (see section 3.14.2.2).

3.14.2 Incubation of *L. plantarum* WCFS1 with dendritic cells.

L. plantarum cells carrying various plasmids was cultured and induced as described below. The induced *L. plantarum* cells were incubated with dendritic cells derived from CD-14 + cells isolated from human blood peripheral leukocytes. Six different plasmid constructs were tested.

Preparation of bacterial cultures

Plasmids (see section 2.6):

pCytAg85B-E6

pLp_3050Ag85B-E6

pLp_3050Ag85B-E6cwa2

pLp_3050Ag85B-E6cwa3

pLp_1261Ag85B-E6

pSIP-EV

Procedure:

1. An overnight culture of *L. plantarum* WCFS1 harbouring the desired plasmid was prepared in MRS containing 10 µg/ml erythromycin, by incubating at 37 º C.
2. The overnight bacterial culture was diluted in MRS containing 10µg/ml erythromycin, until an OD_{600} of 0.11 – 0.15 was reached. Two parallels were prepared of each strain and incubated at 37 º C.
3. At OD_{600} 0.27-0.33, protein production was induced by adding 10 ng/ml of inducer peptide (SppIP) to the bacterial culture.

4. The culture was incubated for another two hours at 37 º C. In the case of *L. plantarum* harbouring the pLp_3050Ag85B-E6 plasmid (for antigen secretion), the bacteria were only incubated for 30 minutes, after which the cells were pelleted by centrifugation at 5000 x g for 2 min, dissolved in RPMI- medium/1% FCS, 10µg/ml erythromycin and 10 ng/ml SppIP and incubated for another 1.5 hours. This was done to obtain secreted antigen in the RPMI-medium added directly to the dendritic cells.
5. After incubation, 1 ml of bacterial culture was pelleted by centrifuging at 5000 x g for 2 min. The bacterial cell pellet was resuspended in RPMI w/1% FCS, 10µg/ml erythromycin and 10 ng/ml SppIP to a final absorbance of 0.25 at OD_{600}. The culture of *L. plantarum* harbouring the pLp_3050Ag85B-E6 plasmid (see step 5) was directly diluted to an OD_{600} of 0.25

Co-incubation of bacteria and dendritic cells

Procedure:

1. The medium was aspirated from the wells of microplates seeded with dendritic cells (see section 3.14.1, step 13)
2. 600 µl of bacterial culture, prepared in section 3.14.2.1, step 5, was carefully added to each well, with two replicas of each biological parallel.
3. The microplates were incubated for 24 hours in a Steri-Cycle CO_2 Incubator at 37 º C.
4. After 24 hours the supernatant of each well was harvested in individual eppendorf tubes and centrifuged at 5000 x g for 5 minutes to remove any cell debris.
5. The supernatant was transferred to a new eppendorf tube and kept on ice, for immediate analysis by enzyme-linked immunosorbent assay **(**ELISA).

3.14.3 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a widely used technique to detect the presence of specific molecules in a sample with high sensitivity. The two antibody- "sandwich" ELISA is used to determine the concentration of specific antigens in unknown samples (Figure 3. 6). This assay requires two antibodies that bind to non-overlapping epitopes on the antigen. A capture antibody is bound to the bottom of a plate well. When the sample is added, the antigen will bind to the plate-bound antibody. Unbound molecules are removed by a washing procedure and a detection antibody that also binds the antigen is added. An enzyme-linked secondary antibody that will bind to the detection antibody is added to the well, enabling quantification by conversion of a colorimetric substrate.

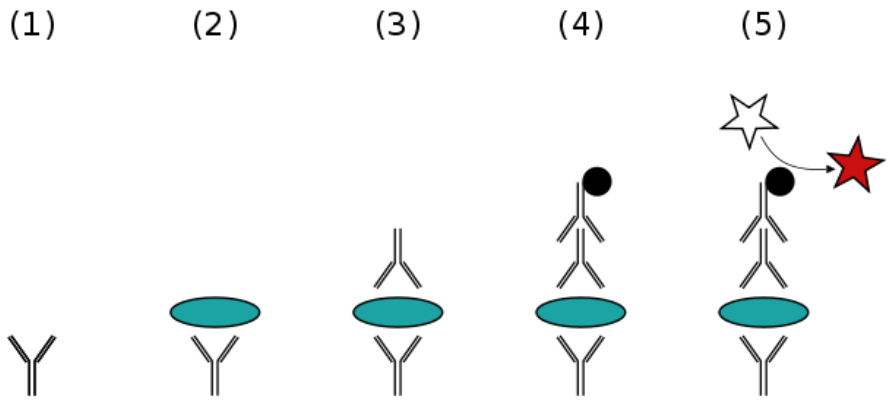

Figure 3. 6: Outline of the different steps of "sandwich" ELISA. 1) A microwell plate is covered in antigen; 2) sample is added and any antigen binds the "capture" antibody; 3) a detecting antibody is added and binds to antigen; 4) an enzyme- linked secondary antibody is added and binds to detecting antibody; 5) substrate is added and the enzyme converts it to a detectable form. The figure was designed by Vinocur (2006)

Enzyme-linked immunosorbent assay (ELISA) of TNF-alpha and IL-10

Materials:

TNF alpha; high sensitivity human ELISA Set (ImmunoTools) (section 2.7)

or

Interleukin 10; high sensitivity human ELISA Set (ImmunoTools) (section 2.7)

Blocking buffer: PBS containing 1% (w/v) BSA

Coating buffer: 100 mM Sodium carbonate buffer, pH 9.6

Dilution buffer: PBS containing 1% (w/v) BSA and 0.05 % (v/v) TWEEN-20

Stop solution: 2M H_2SO_4

Substrate solution: TMB (3,5,3',5'-tetramethylbenzidine)

Washing buffer: PBS containing 0.01% (v/v) TWEEN-20

Microwell plates: 96-well Nunc MaxiSorp™

Procedure:

1. The microwell plate was coated with appropriate coating antibody (TNF-alpha or IL-10). The coating antibody was diluted 1:200 in coating buffer and a 100 µl were transferred to each well. The plate was covered with a lid, and incubated overnight at 4 º C.
2. The next day the plate was washed five times with 200 µl washing buffer per well. Care was taken to dry the microwell plate between each wash.
3. The plate was blocked by adding 200 µl blocking buffer pr. well, followed by incubation for one hour at room temperature.
4. The plate was washed five times with washing buffer.
5. The standards were diluted in dilution buffer to make up the standard curve. The samples were diluted 1:100 in dilution buffer. 100 µl of standards and samples were transferred to the wells. The plate was covered and incubated for 1 hour at room temperature.
6. The plate was washed five times with washing buffer.
7. The plate was incubated with a biotin conjugate, the detection antibody (see Figure 3. 6, step 3). Biotinylated antibody was diluted 1:200 times in dilution buffer and a 100 µl was transferred to each well. The plate was incubated for 1 hour at room temperature.
8. The plate was washed five times with washing buffer.
9. Avidin-HRP conjugate, the enzyme- linked secondary antibody (see Figure 3. 6, step 4) was diluted 1:10000 in dilution buffer. 100 µl of diluted solution was transferred to each well and the plate was incubated for 30 minutes at room temperature.
10. The plate was washed five times with washing buffer.
11. Substrate solution with TMB was prepared by dissolving 0.01 g of TMB in 100 µl DMSO. 10 µl of this stock-solution was then dissolved in 9.9 ml of phosphate-citrate buffer and 25 µl 30 % hydrogen peroxide. 100 µl of the substrate solution was transferred to each well and the plate was incubated in the dark until the desired colour had developed.

12. To stop the reaction, 100 µl of 2M H2SO4 was added to each well Then, within 30 minutes the absorbance was read at 450 nm in a Tecan Sunrise™ Absorbance Microplate Reader.

Enzyme-linked immunosorbent assay (ELISA) of interleukin-12 (Il-12)

Materials:

ELISA MAX™ Standard set for human IL-12 (p70) (section 2.7).

Blocking buffer: PBS Containing 1% (w/v) BSA

Coating buffer: 100 mM Sodium carbonate buffer, pH 9.6

Dilution buffer: PBS containing 1% (w/v) BSA

Stop solution: 2M H_2SO_4

Substrate solution: TMB (3,5,3',5'-tetramethylbenzidine)

Washing buffer: PBS containing 0.05% (v/v) TWEEN-20

Microwell plates: 96-well Nunc MaxiSorp™

Procedure:

1. The IL-12 capture antibody was diluted 1:200 in coating buffer and 100 µl were transferred to each well of a microwell plate. The plate was sealed, and incubated overnight at 4 º C.
2. The next day the plate was washed four times with 300 µl washing buffer. Care was taken to dry the microwell plate between each wash.
3. The plate was blocked by adding 200 µl blocking buffer pr. well, followed by incubation for one hour with shaking at 200 rpm, at room temperature.
4. The plate was washed four times with washing buffer.
5. The standards were diluted in dilution buffer to make up the standard curve.100 µl of standards and samples were transferred to the wells. The plate was sealed and incubated at RT for two hours with shaking at 200 rpm.
6. The plate was washed four times with washing buffer.
7. Biotinylated detection antibody was diluted 1:200 times in dilution buffer and a 100 µl was transferred to each well. The plate was incubated for one hour at RT, with shaking.
8. The plate was washed five times with washing buffer.

9. Streptavidin-HRP was diluted 1:1000 in dilution buffer. 100 µl of the diluted solution was transferred to each well and the plate incubated with shaking for 30 minutes at RT.
10. The plate was washed five times with washing buffer.
11. The substrate solution with TMB was prepared according to section 3.14.1, step 11. 100 µl of the substrate solution was transferred to each well and the plate was incubated in the dark for about 25 minutes until the desired blue colour had appeared in positive wells.
12. To stop the reaction, 100 µl of 2M H_2SO_4 was added to each well, and the absorbance was read at 450 nm in a Tecan Sunrise™ Absorbance Microplate Reader.

4 RESULTS

The Ag85B-ESAT6 fusion protein was selected to create a vaccine delivery vector using the pSIP-system (Sørvig et al. 2003) and *L. plantarum* WCFS1. The Ag85B-ESAT6 fusion protein is a promising antigen in the development of effective vaccination strategies against tuberculosis and has been shown to promote protective immune responses against *M. tuberculosis* in animal models (Hall et al. 2009; Kamath et al. 2008; Olsen et al. 2001). *L. plantarum* WCFS1 is considered a promising carrier for antigen delivery to the mucosal surfaces due its stimulating effect on the immune system and possible adjuvant effect. Use of *Lactobacillus* vectors for production of Ag85B-ESAT6 had not been explored prior to this study.

4.1 Intracellular production of Ag85B-ESAT6 in *Lactobacillus plantarum*

4.1.1 Construction of plasmid for intracellular production of Ag85B-ESAT-6

A plasmid for production of non-secreted Ag85B-ESAT6 was constructed as described in Figure 4. 1. (all plasmids used in this study are listed in Table 2.4). The *Ag85B-ESAT6* gene was PCR-amplified from the template pUC57-Ag85B-E6 (with the antigen sequence codon usage optimised for *L.* plantarum) using primers pAgESAT-CytF and pAgESAT-CytR, which contains restriction sites for NdeI and HindIII, respectively (all primers used in this study are described in Table 2.1 and 2.2). The resulting 1191 bp Ag85B-ESAT6 fragment was subcloned into a TOPO-vector. All PCR-amplified gene fragments used in this study was subcloned via TOPO-vectors as described in section 3.3. The Ag85B-ESAT6 fragment were excised from the TOPO-vector with restriction enzymes NdeI and HindIII and ligated into the 5544 bp fragment of NdeI and HindIII-digested pLp_0373sNucA plasmid. Like all plasmids in this study the resulting pCytAg85B-E6 plasmid was transformed into *Escherichia coli* TOP 10 cells prior to transformation into electro-competent *L. plantarum* WCFS1.

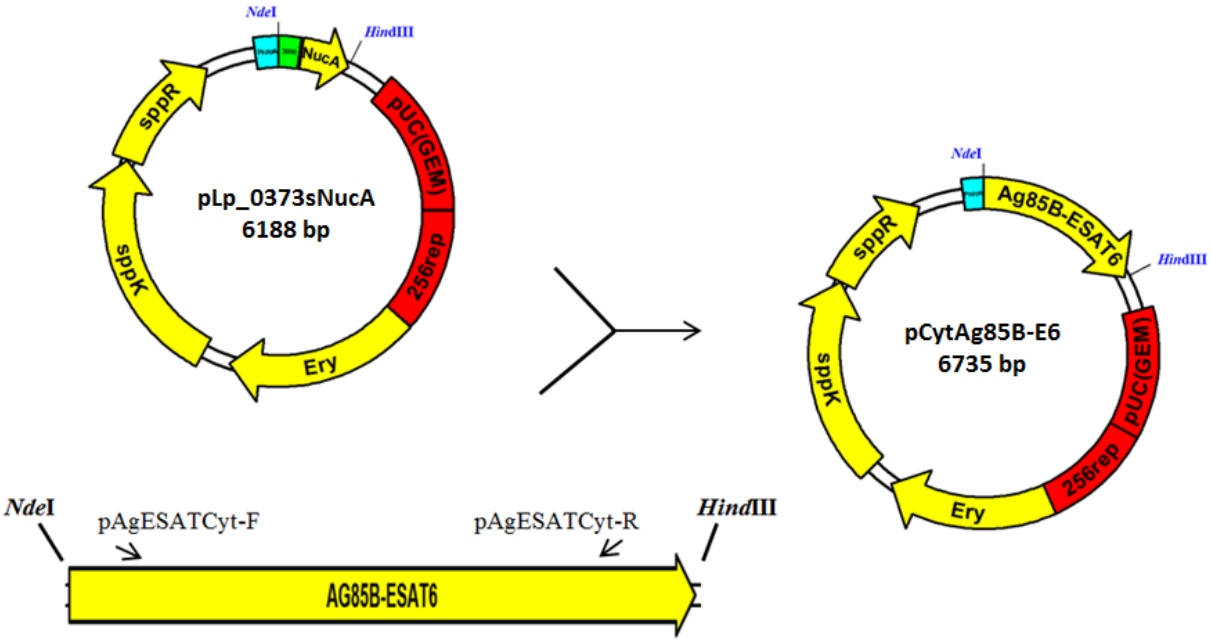

Figure 4. 1: Cloning strategy for the construction of pCytAg85B-E6 for intracellular production of Ag85B-ESAT6. The following colour scheme depicts the different gene fragments of the plasmids constructed in this study. The erythromicen resistance gene (*Ery*), the histidine protein kinase gene (*sppK*), the response regulator (*sppR*) and the target gene (*Ag85B-ESAT6*) are shown in yellow. The signal peptide is shown in green, while the P_{sppA} promoter (upstream of the signal peptide and/or target gene) is shown in blue. The red fragments depict the *Lactobacillus* replicon (256rep) and the *E. coli* replicon (pUC(GEM) respectively. Use of the *Nde*I site for cloning genes of interest yields constructs were this gene is translationally fused to the P_{sppA} promoter.

4.1.2 Construction of plasmid for constitutive intracellular production of Ag85B-ESAT6

Constitutive production of therapeutic proteins in the pSIP-system could be advantageous in a vaccine delivery system. The inducible pSIP-promoter P_{sppA} (see section 1.2) was therefore replaced with a constitutive promoter for high-level (p11) or lower-level (p27) production of Ag85B-ESAT6. Both promoters were from a *L. plantarum* promoter library generated by Rud et al. (2006). Plasmids coding for constitutive production of non-secreted Ag85B-ESAT6 protein were constructed as described in Figure 4. 2. The 117 bp promoter *p11* was excised from TOPO-vector tp-pb11 with restriction enzymes BglII and NdeI, and subsequently ligated into the 6576 bp fragment of BglII and NdeI -digested pCytAg85B-E6 plasmid, resulting in the pCyt-p11Ag85B-E6 plasmid. The same procedure was followed for the construction of pCyt-p27Ag85B-E6, utilizing the 117 bp promoter gene *p27*.

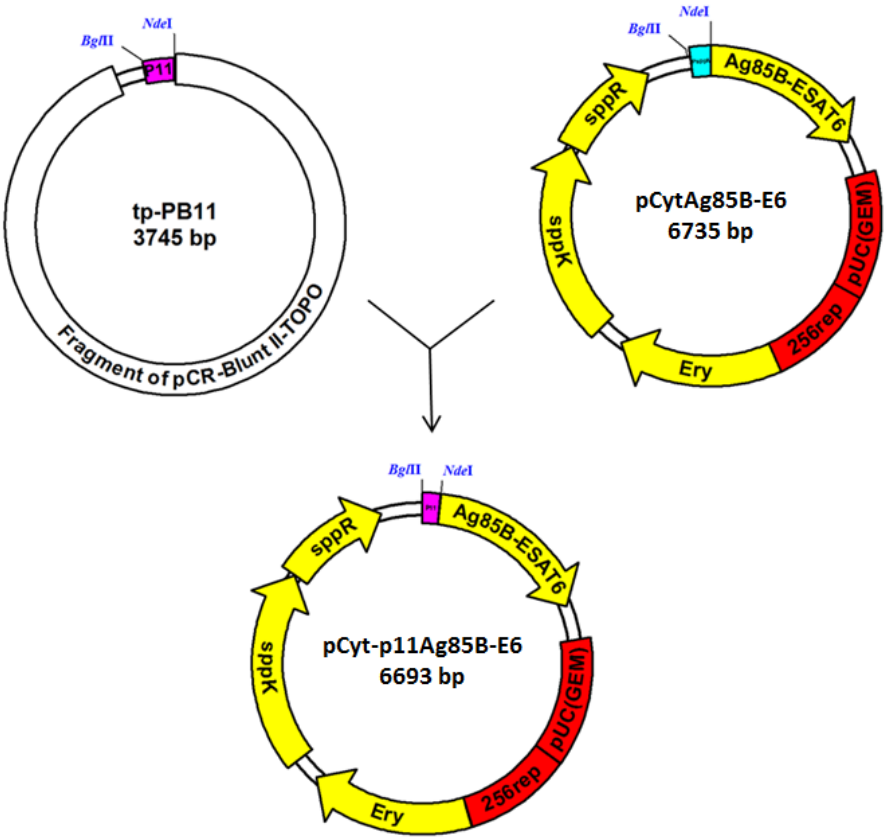

Figure 4. 2: Construction of pCyt-p11Ag85B-E6. The inducible P_{sppA} promoter (indicated in blue) is replaced by a constitutive promoter for high-level or lower-level production of Ag85B-ESAT6 (p11 or p27 respectively). The constitutive promoter is shown in pink.

4.1.3 Intracellular production of Ag85B-ESAT6 in *L. plantarum* WCFS1.

To determine inducible and constitutive production of Ag85B-ESAT6, overnight cultures of *L. plantarum* WCFS1 harbouring the pCytAg85B-E6, pCyt-p11Ag85B-E6 and pCyt-p27Ag85B-E6 plasmids, respectively, were diluted in fresh MRS medium with erythromycin and grown to OD_{600} ~0.3 at 37 ºC. Gene expression from the pCytAg85B-E6 plasmid was induced by adding 25 ng/ml of SppIP. Cell cultures of strains harbouring plasmids for induced and constitutive expression were harvested in time intervals from time 0 (at OD_{600} ~0.3) and then every 30 minutes for a total of 4 hours. The growth rates observed for all three Ag85B-ESAT6 producing *L. plantarum* strains were similar to that of a non-induced strain (Appendix, Figure A1) indicating that the Ag85B-ESAT6 protein is tolerated by the bacteria. The cells were harvested as described in section 3.10 and a crude cell- free protein extract was obtained by disrupting cells using glass beads (described in section 3.10.1). The intracellular protein extract was analysed by Western blotting as described in section 3.12, using the SNAP

i.d. immunoblotting system. In order to apply equal amounts of sample to the gels, the sample amount was calculated according to the cultures OD_{600} at the time of harvest. The resulting Western blots are shown in Figure 4. 3- Figure 4. 5.

Figure 4. 3 shows the intracellular production of Ag85B-E6 in induced *L. plantarum* cultures. Possible breakdown product of the Ag85B-ESAT6 protein appeared two hours after induction. The theoretical molecular weight of Ag85B-ESAT6 was calculated to about 41 kDa, which is consistent with the Western blot analysis. The about 75 kDa protein band (indicated by the arrow) seen in this blot and in Figure 4. 4 and Figure 4. 5 have also been identified in the negative control (pSIP-EV) as shown in Figure 4. 10 and Figure 4. 11.

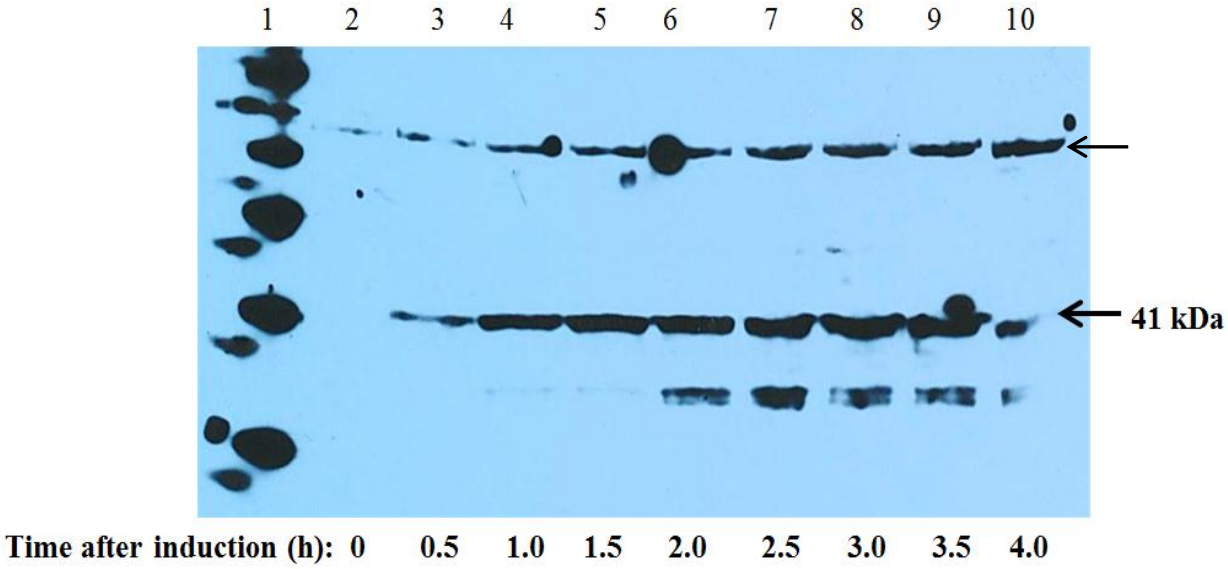

Figure 4. 3: Analysis of intracellular Ag85B-ESAT6 by Western blotting. The blot shows the intracellular protein samples from *L. plantarum* harbouring the pCytAg85B-E6 plasmid for induced production of the 41 kDa, non-secreted Ag85B-ESAT6 protein (indicated by the arrow). Lane 1, MagicMark protein ladder; lane 2-10, crude cell-free protein extracts froman induced culture of *L. plantarum* harbouring the pCytAg85B-E6 plasmid, 0-4 hours after induction (time points are given below the lanes). The negative control (pSIP-EV) is not shown.

Figure 4. 4 and Figure 4. 5 show constitutive production of Ag85B-ESAT6 in *L. plantarum* from promoters' p11 and p27, respectively. The strongest protein bands were observed for the high level constitutive promoter p11 (Figure 4. 4). In addition, a protein band of about 50 kDa, located straight above the 41kDa Ag85B-ESAT6 band, was observed in these blots (Figure 4. 4 and Figure 4. 5). This protein band was not apparent in cell extracts of induced *L. plantarum* cultures (Figure 4. 3) and could be a protein produced in detectable amounts only in the strains with constitutive Ag85B-ESAT6 expression. The appearance of possible breakdown products of Ag85B-ESAT6 was also evident in the case of constitutive expression.

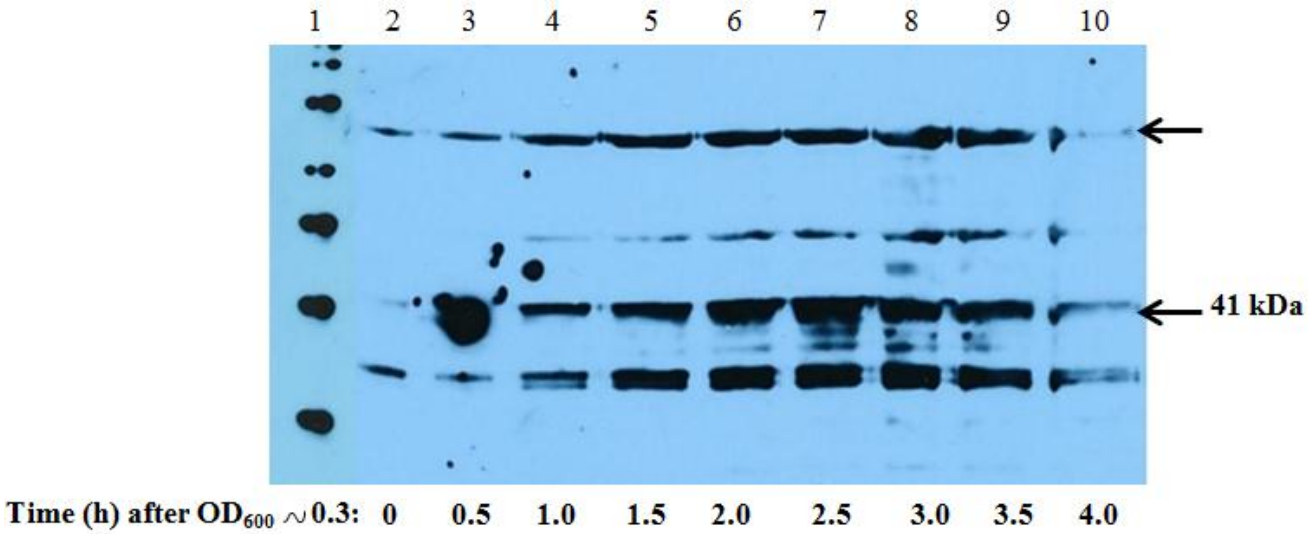

Figure 4. 4: Analysis of high-level constitutive production of Ag85B-ESAT6 by Western blotting. The blot shows the intracellular protein samples from *L. plantarum* harbouring the pCyt-p11Ag85B-E6 plasmid for high-level constitutive production of the non-secreted, 41 kDa Ag85B-ESAT6 protein (indicated by the arrow). Lane 1, MagicMark protein ladder.; lane 2-10, crude cell-free protein extracts from a non-induced culture of *L. plantarum* harbouring the pCyt-p11Ag85B-E6 plasmid, 0-4 hours after OD_{600} ~0.3. (time points are given below the lanes). The negative control (pSIP-EV) is not shown.

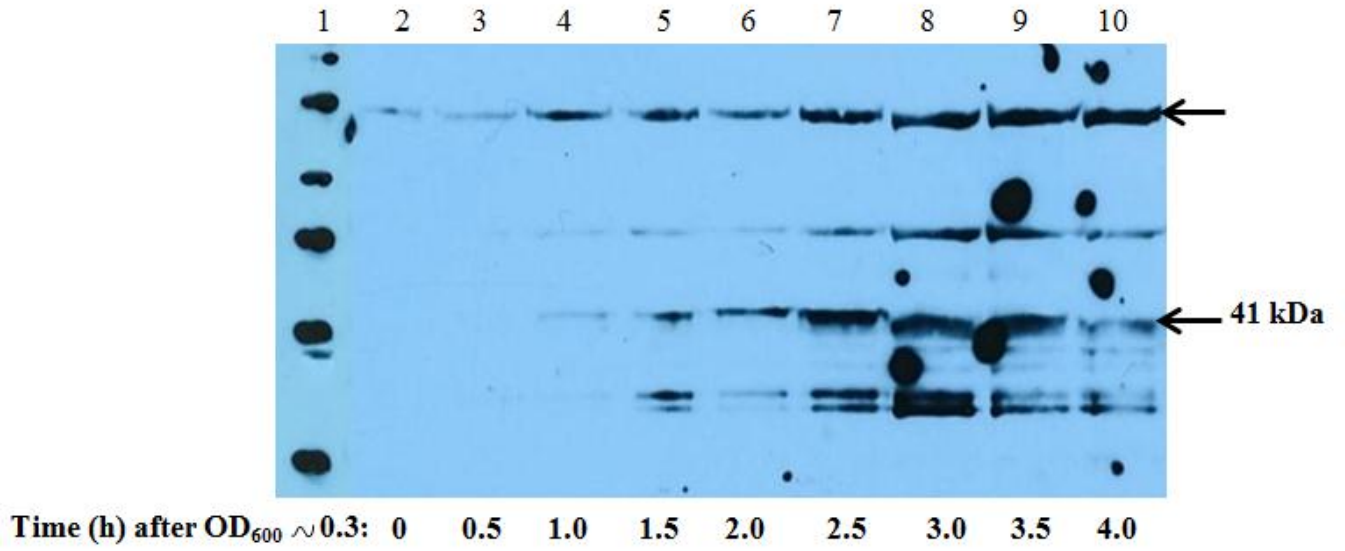

Figure 4. 5: Analysis of low-level constitutive production of Ag85B-ESAT6 by Western blotting. The blot shows the intracellular protein samples from *L. plantarum* harbouring the pCyt-p27Ag85B-E6 plasmid for low-level constitutive production of the 41 kDa, non-secreted Ag85B-ESAT6 protein (indicated by the arrow). Lane 1, MagicMark protein ladder; lane 2-10, crude cell-free protein extracts from a non-induced culture of *L. plantarum* harbouring the pCyt-p27Ag85B-E6 plasmid, 0-4 hours after OD_{600} ~0.3. (time points are given below the lanes). The negative control (pSIP-EV) is not shown.

From these initial studies it was concluded that *L. plantarum* was able to produce intracellular Ag85B-ESAT6 using induced and constitutive promoters. Ag85B-ESAT6 could not be detected by Coomassie staining or Silver staining of gels (results not shown), indicating that lower amounts of antigen were produced. The next step was to investigate secretion of Ag85B-ESAT6, using homologous signal peptides.

4.2 Secretion of Ag85B-ESAT6 in *L. plantarum* WCFS1

The secretion capacity of heterologous proteins in *L. plantarum* WCFS1 depend on both the signal peptide and the secreted protein, hence an optimal signal peptide should be determined for each individual target protein (Mathiesen et al. 2008; Mathiesen et al. 2009). Eleven different signal peptides from *L. plantarum* WCFS1 were tested for the secretion of the heterologous fusion protein Ag85B-ESAT6 in *L. plantarum* WCFS1. These signal peptides were chosen based on their properties and performance when targeting the reporter proteins Nuclease A (NucA) and Amylase A (AmyA) for secretion (Mathiesen et al. 2008; Mathiesen et al. 2009).

4.2.1 Construction of plasmids for secretion of Ag85B-ESAT6

The pLp_3050sNucA plasmid, containing SP from Lp_3050, and ten other analogous plasmids from a library of signal peptides (Mathiesen et al. 2009) (see Table. 2.4) were used for the construction of secreted versions of Ag85B-ESAT6. The *Ag85B-ESAT6* gene fragment was excised from pUC57-Ag85B-E6 by digesting with restriction enzymes SalI and HindIII and ligated into the SalI-HindIII digested pLp_3050sNucA plasmid, or one of the other ten secretion plasmids. All plasmids were created according to the cloning strategy described in Figure 4. 6.

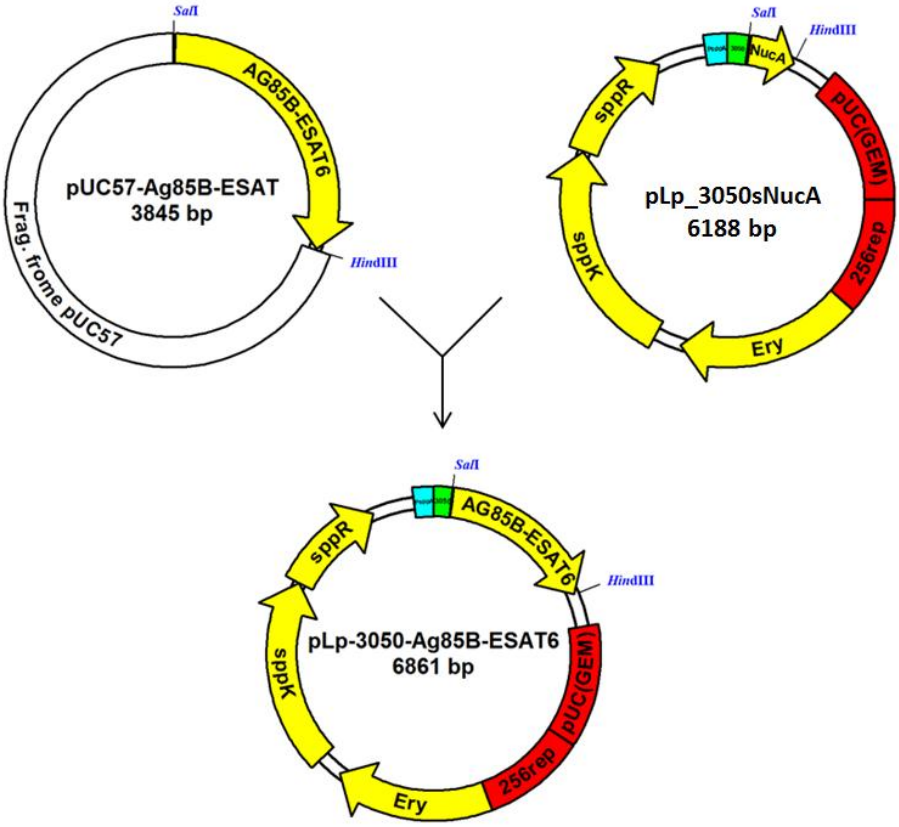

Figure 4. 6: Cloning strategy for the construction of pLp_3050Ag85B for inducible production and secretion of Ag85B-ESAT6. All plasmids that direct secretion of Ag85B-ESAT6 were created according to the cloning strategy shown in the present figure. Colour coding as in Figure 4. 1.

4.2.2 Secretion of Ag85B-ESAT6 in *L. plantarum* using *L. plantarum* signal peptides

SDS-PAGE was used to investigate secretion of Ag85B-ESAT6 in *L. plantarum.* Overnight cultures of *L. plantarum* harbouring the various Ag85B-ESAT6 secretion plasmids were diluted in fresh MRS medium and the cultures were grown to OD_{600} ~0.30. After induction (addition of SppIP) the cultures were incubated for four hours at 30 ºC. Figure 4. 7 shows

growth curves for *L. plantarum* strains harbouring various plasmids for secretion of Ag85B-ESAT6. The figure shows considerable variation between the strains and all strains grew slower than the induced control strain (harbouring the empty vector, pSIP-EV).

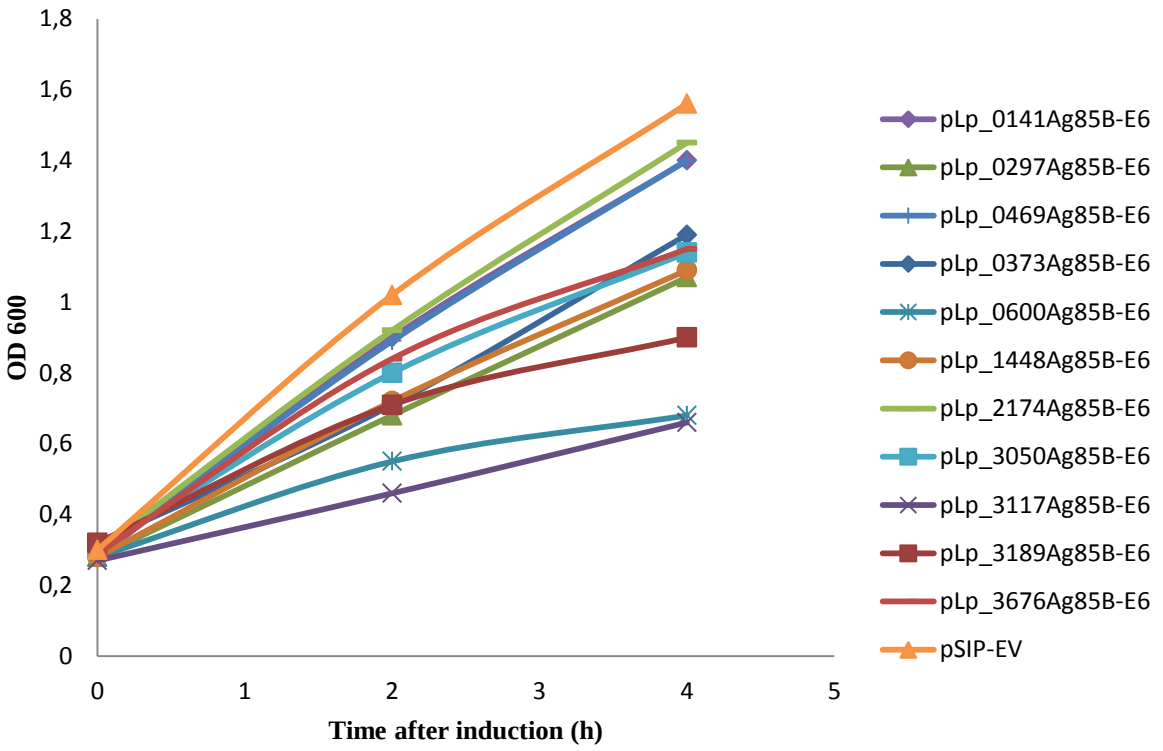

Figure 4. 7: Growth curves of induced *L. plantarum* strains harbouring plasmids for secretion of Ag85B-ESAT6. All cultures were induced by 25 ng/ml SppIP and incubated for a total of 4 hours at 30 ºC after induction. pSIP-EV is the empty vector.

Supernatants from these cultures (harvested after four hours) were concentrated by TCA precipitation and subjected to SDS-PAGE (described in section 3.11), after which proteins were visualised by Western blotting. All blots were produced using the SNAP i.d. immunoblotting system (section 3.12.2). Figure 4. 8 shows a Western blot of cell free supernatant of induced L. plantarum strains harbouring plasmids with different signal peptides for secretion of Ag85B-ESAT6. Figure 4. 8 demonstrates that all signal peptides were able to direct Ag85B-ESAT6 secretion, but to a varying degree. The protein band in the sample from SP Lp_600 (Figure 4. 8, lane 10) differs from the 41 kDa protein band in the other samples, and the strain carrying the Lp 600 construct was also one of the slowest growing strains. The size of the Lp_0600 protein band was comparable to the theoretical molecular weight of the unprocessed intracellular protein, i.e. about 45 kDa. In order to check for lysis of cells, which could lead to apparent secretion of unprocessed Ag85B-ESAT6, SDS-PAGE analysis of

supernatants was performed and proteins were visualized by silver- staining (Appendix, Figure A2). This analysis did not reveal cytoplasmic proteins in any of the strains carrying the signal peptide constructs. Thus, it seems that the presence of a 45 kDa band in the Lp_0600 sample is not due to cell lysis.

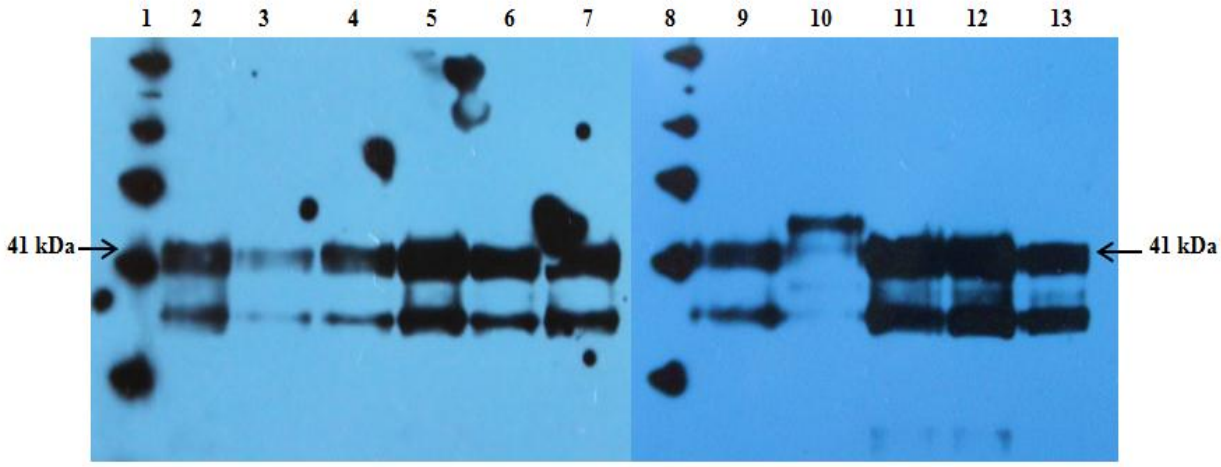

Figure 4. 8: Analysis of extracellular Ag85B-ESAT6 by Western blotting. The blot shows TCA-precipitated protein from culture supernatants from induced *L. plantarum* strains harbouring various plasmids for secretion of Ag85B-ESAT6, four hours after induction. The arrows indicate the 41 kDa secreted Ag85B-ESAT6 protein. The amount of supernatant loaded to the gel equals that of 1.6 ml cell culture. The OD_{600} at the time of harvest are noted in brackets. Lane s: (1) MagicMark protein ladder; (2) pLp_0373Ag85B-E6 [1.19]; (3) pLp_3189Ag85B-E6 [0.90]; (4) pLp_0297Ag85B-E6 [1.07]; (5) pLp_3050Ag85B-E6 [1.14]; (6) pLp_2174Ag85B-E6 [1,43]; (7) pLp_3676Ag85B-E6 [1.15]; (8) MagicMark protein ladder; (9) pLp_3117Ag85B-E6 [0.66]; (10) pLp_0600Ag85B-E6 [0.68]; (11) pLp_1448Ag85B-E6 [1.09]; (12) pLp_0469Ag85B-E6 [1.40]; (13) pLp_0141Ag85B-E6 [1.40].

Figure 4. 9 show a comparative analysis of the amounts of Ag85B-ESAT6 in crude cell-free protein extracts and supernatants for four different signal peptide constructs. These four signal peptides were chosen based on the observed amounts of secreted protein (Figure 4. 8). Lp_3050, Lp_3676 and Lp_1448 were selected because Figure 4. 8 (lanes 5, 7 & 11) indicated that these SPs leads to the largest amounts of secreted Ag85B-ESAT6, although it should be noted that these amounts did not appear to be significantly larger. In addition Lp_0373 (Figure 4. 8, lane 2) was selected as a representative of an SP giving lower levels of secretion. Figure 4. 9 give a semi-quantitative impression of the secretion efficiency, i.e. the ratio between intra- and extracellular Ag85B-ESAT6 levels. The figure shows that these efficiencies are low for all four constructs. The lower levels of secreted Ag85B-ESAT6

obtained with the Lp_0373 SP are accompanied by lower levels of intracellular protein and may thus not (only) be due to lower secretion efficiency. Interestingly the strain with the Lp_0373 construct grew slightly faster than the other three strains, which showed similar but slightly reduced growth rates (Figure 4. 7). The construct with the Lp_3050 SP showed the highest levels of secreted Ag85B-ESAT6, while this strain grew equally well as the other strains yielding high levels of secreted protein. Therefore, Lp_3050 (lane 8 and 9) was selected as a signal peptide for further studies.

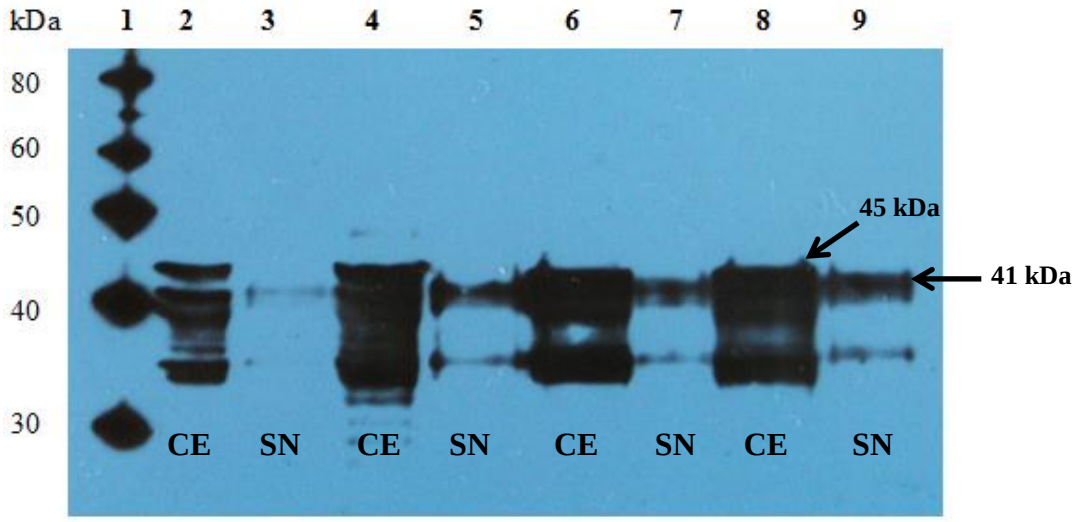

Figure 4. 9: Analysis of intracellular and ectracellular Ag85B-ESAT6 by Western blotting. The blot shows crude cell-free protein extracts (CE) and TCA-precipitated protein from the supernatants (SN) of induced *L. plantarum* strains harbouring various plasmids for secretion of the 41 kDa Ag85B-ESAT6. The cultures were harvested four hours after induction. The cell ectracts show both the processed protein (41 kDa) and the unprocessed precursor (45 kDa) as indicated by the arrows. (1) MagicMark protein ladder; (2) and (3) pLp_0373Ag85B-E6; (4) and (5) pLp_1448Ag85B-E6; (6) and (7) pLp_3676Ag85B-E6; (8) and (9) pLp_3050Ag85B-E6. The amount of supernatant (SN) loaded equals 0.8 ml cell culture.; the amount of cell extract (CE) loaded equals 1/3 of the amount of supernatant.

4.2.3 Dose-response experiment: the effects of inducer peptide on production and secretion of Ag85B-ESAT6 in *L. plantarum*.

Dose-response experiments evaluating the effect of the inducer peptide dosage on the production and secretion of Ag85B-ESAT6 were performed at two different incubation temperatures, 30 and 37 ºC. The optimal temperature for heterologous protein production by the pSIP-system had previously been found to be 30 ºC (Mathiesen et al. 2004). Since the aim of this study was the development of a vaccination strategy using recombinant *L. plantarum* for *in vivo* presentation of antigens in humans, it was important to investigate the induction of

the pSIP-system, and the following production and secretion of Ag85B-ESAT6 at 37 ºC. An overnight culture of *L. plantarum* WCFS1 harbouring the plasmid pLp_3050Ag85B-E6 (for secretion of Ag85B-ESAT6) was diluted and incubated according to the protocol described in section 3.10. Cells were induced at OD_{600} of ~0.3 using the following concentrations of SppIP: 0.1 ng/ml, 0.5 ng/ml, 1.0 ng/ml, 3.0 ng/ml, 6.0 ng/ml, 12.5 ng/ml or 25 ng/ml. The cultures were harvested 4 hours after induction and Western blot analyses of cell extracts and precipitated supernatants were performed.

In the cultures grown at 30 ºC, Ag85B-ESAT6 was detected both intracellularly and in the supernatant at an inducer dosage of 1.0 ng/ml SppIP. The maximal production of Ag85B-ESAT6 appears to be reached at 3 ng/ml SppIP (Figure 4. 10), and a clear dose-response effect is observed at induction dosages between 0 and 3 ng/ml SppIP. The Western blot analysis further reveals that no drastic changes in secretion efficiency are caused by the different inducer dosages. Interestingly it appears that most of the protein remains intracellularly, even at a low-level production. On the other hand the processing of protein appears more complete at inducer dosages of 0.1 and 0.5, where only the 41 kDa band (and smaller bands) is visible intracellularly, and not the 45 kDa precursor protein. These results are even more evident at 37 ºC.

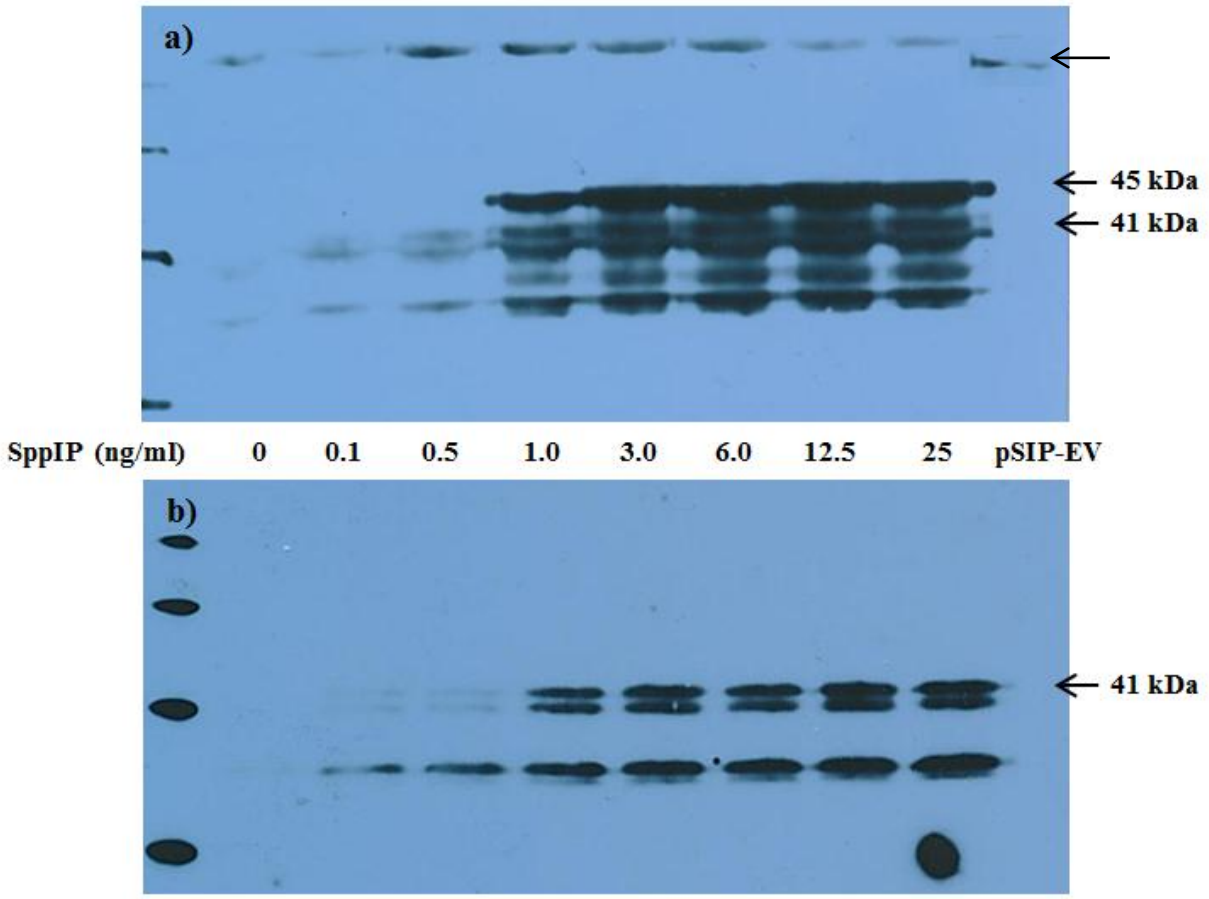

Figure 4. 10: Dose-response effects at 30 ° C. Western blots showing cell extract (a) and methanol/chloroform precipitated protein from the supernatant (b) of *L. plantarum* harbouring the pLp_3050Ag85B-E6 construct for secretion of Ag85B-ESAT6. The following concentrations of inducer peptide (SppIP) were added to the cultures; 0,1 ng/ml, 0,5 ng/ml, 1,0 ng/ml, 3,0 ng/ml, 6,0 ng/ml, 12,5 ng/ml and 25 ng/ml, as indicated, whereas one culture was left non-induced ("0"). The control culture (*L. plantarum* carrying pSIP-EV) was induced with 25 ng/ml SppIP. The amount of cell extract (a) loaded onto the gel corresponds to 1/3 of the amount of supernatant (b) loaded onto the gel.

In the cultures grown at 37 ºC, Ag85B-ESAT6 was detected both intracellularly and in the supernatant at an inducer dosage of 3.0 ng/ml SppIP (Figure 4. 11 b). Similar to the results at 30 ºC, the maximal production of Ag85B-ESAT6 appears to be reached at this inducer dosage (3 ng/ml SppIP), and likewise a clear dose-response effect is observed at induction dosages between 0 and 3 ng/ml SppIP. In the cell extract production of Ag85B-ESAT6 was detected at all inducer dosages, also, at very low level in the non-induced *L. plantarum* culture (Figure 4. 11a). Interestingly Ag85B-ESAT6 was barely detected in the supernatant at inducer dosages lower than 3 ng/ml SppIP, although production and processing of the protein is clearly observed intracellularly.

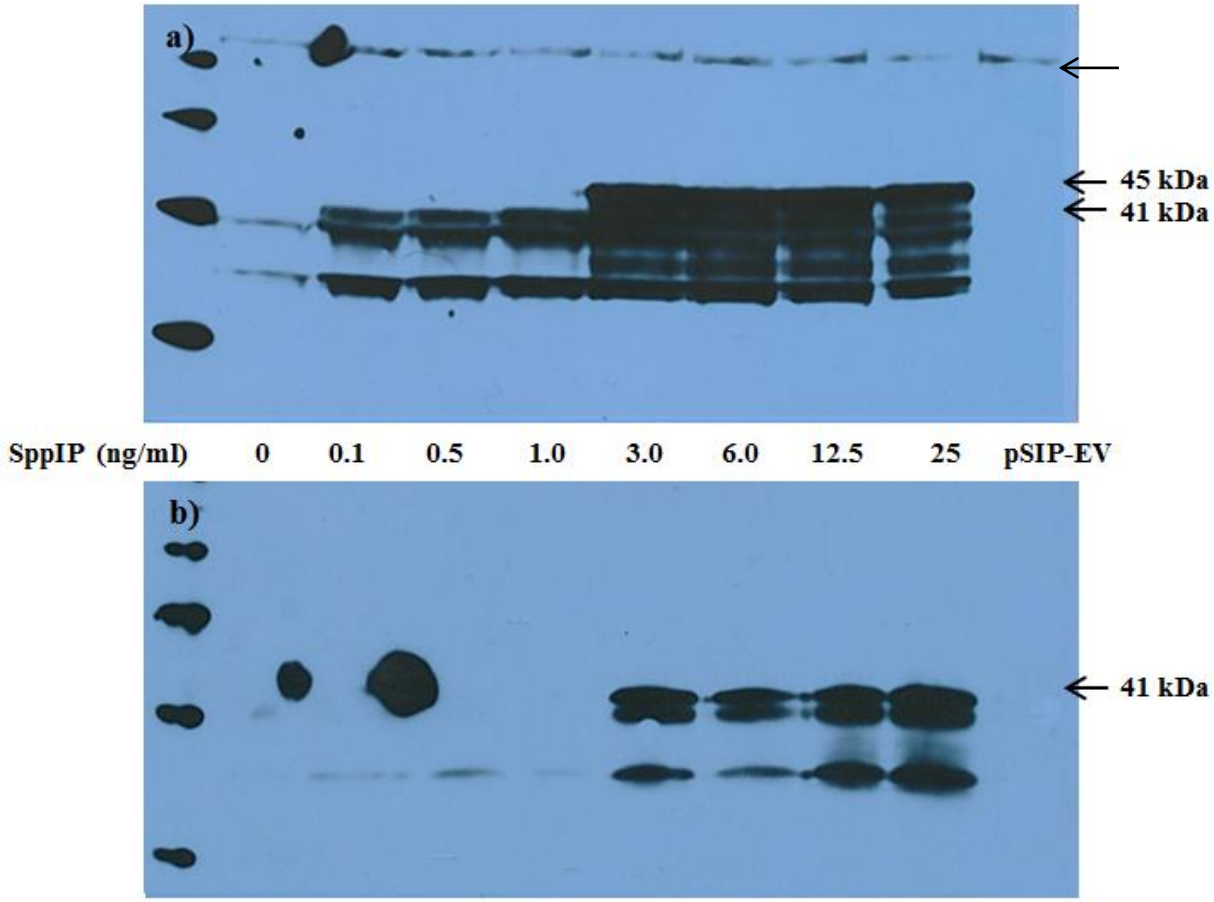

Figure 4. 11: Dose-response effects at 37 ° C. Western blots showing cell extract (a) and methanol/chloroform precipitated protein from the supernatant (b) of *L. plantarum* harbouring the pLp_3050Ag85B-E6 construct for secretion of Ag85B-ESAT6. The following concentrations of inducer peptide (SppIP) were added to the cultures; 0,1 ng/ml, 0,5 ng/ml, 1,0 ng/ml, 3,0 ng/ml, 6,0 ng/ml, 12,5 ng/ml and 25 ng/ml, as indicated, whereas one culture was left non-induced ("0"). The control culture (*L. plantarum* carrying pSIP-EV) was induced with 25 ng/ml SppIP. The amount of cell extract (a) loaded onto the gel corresponds to 1/3 of the amount of supernatant (b) loaded onto the gel.

The growth curves for these experiments are shown in the Appendix, Figure A3 and A4. Induction with dosages higher than 1 ng/ml SppIP, i.e. dosage leading to maximum production levels, resulted in impaired growth at both temperatures. Generally higher growth rates were observed at 37 ºC. Based on these results and the prospects of using these constructs as vaccines for human use, it was decided to use 37 ºC as the incubation temperature for further experiments.

4.2.4 Construction of plasmids for constitutive production and secretion in *L. plantarum*

An effort was made to construct plasmids encoding a promoter for high-level (p11) or lower-level (p27) constitutive production and secretion of Ag85B-ESAT6. The pCyt-p11Ag85B-E6 and The pCyt-p27Ag85B-E6 plasmids were used as templates for the construction. The Lp_3050-Ag85B-ESAT6 fragment was excised from the pLp_3050Ag85B-E6 plasmid by digesting with the restriction enzymes NdeI and HindIII. The resulting fragment was ligated into the NdeI and HindII-digested pCyt-p11Ag85B-E6 plasmid. The resulting *E. coli* TOP 10 transformants were sequenced using primers pSekF and pSipSeqR. It was found that the correct Lp_3050-Ag85B-ESAT6 fragment had been inserted, but that a fragment of varying size, always including the constitutive promoter region, had been removed from all sequenced plasmids, indicating it's toxicity to *E. coli*. This conclusion was further supported by the very few transformants achieved in the process. Direct transformation of the ligated plasmid into *L. plantarum* was attempted, but no transformants were observed. A similar strategy was attempted using the low-level expression p27 promoter; unfortunately the same results were observed for these transformants.

4.3 Cell wall- and membrane -anchoring of Ag85B-ESAT6 in *L. plantarum* WCFS1

Anchoring of heterologous proteins in *L. plantarum* is of great interest for the display of antigens for vaccination purposes. To obtain cell surface display of Ag85B-ESAT6 in *L. plantarum* the antigen sequence was fused to *L. plantarum* (i.e. homologous) proteins with an anchoring motif, as explained below.

4.3.1 Construction of plasmids for cell wall anchoring of Ag85B-ESAT6

Three different C-terminal fragments of the *L. plantarum* protein Lp_2578, each including the motif for LPXTG motif for cell-wall anchoring of this protein were fused to Ag85B-ESAT6. The three length fragments of Lp_2578 are referred to as cell wall-anchor 1, 2 and 3 (cwa1-3) respectively, where cwa1 is the longest and cwa3 the shortest fragment (Fredriksen 2007). Figure 4. 12 outline the cloning cassette originally constructed by Fredriksen (2007) for easy exchange of signal peptide, target protein and cell wall-anchor. In this figure Ag85B-ESAT6 is shown as the target protein.

Figure 4. 12: A schematic outline of the SP-Ag85B-ESAT6-CWA cassette. The precursor protein consists of a signal peptide, the Ag85B-ESAT6 protein, and a cell wall anchor polypeptide. The modular system consisting of the SalI linker, the MluI linker and the multiple cloning site (MCS) allows for easy exchange of the SP, the target protein, and the cell wall anchor. The length of the cell wall-anchor domain determines the target proteins extent of exposure on the cell surface. The longest cell-wall anchor, cwa1, consists of a 674 amino acid long sequence, cwa2 consists of 224 amino acids, and the shortest cell-wall anchor, cwa3, consists of 137 amino acids. The function of the LPXTG (LPQTS) motif is described in section 1.4.3. The figure is based on a figure by Fredriksen (2007)

The pLp_0373sOFAcwa1, 2 and 3 plasmids (Fredriksen 2007)(see section 2.6) was used for the construction of the Ag85B-ESAT6 plasmids. In a first attempt the Lp_3050-Ag85B-ESAT6 fragment was PCR -amplified from pLp_3050Ag85B-E6 using primers pSekF and AgMluI-R. The AgMluI-R primer was designed to introduce a MluI restriction site in the amplified fragment for ligation into the cell wall anchor-encoding vector. Several attempts to clone this particular amplified fragment in the TOPO vector failed, indicating that the insert somehow is toxic for the *E. coli* TOP 10 cells. A new PCR amplification was performed, this time using AgMluI-R and the forward primer AgSalI-F, targeting only the Ag85B-ESAT6 fragment, not the signal peptide. The resulting antigen fragment was successfully TOPO cloned and digested with the restriction enzymes SalI and MluI, prior to ligation into the SalI and MluI digested pLp_0373sOFAcwa1, 2 or 3 plasmids (see Figure 4. 12 for relevant restriction sites). The ligation mix was transformed into *E. coli* TOP 10 and plasmids from transformants were sequenced. Correct sequences was found for the plasmids pLp_0373Ag85B-E6cwa2 and pLp_0373Ag85B-E6cwa3, while no correct transformants were observed for plasmids containing the cwa1-gene fragment (containing the longest anchor sequence). In the next step pLp_3050Ag85B-E6cwa2 and pLp_3050Ag85B-E6cwa3 were constructed by replacing the *Ag85B-ESAT6* gene fragment of pLp_3050Ag85B-ESAT6, with the *Ag85B-ESAT6cwa2* (or 3) fragment. This fragment was excised from pLp_0373Ag85B-E6cwa2 (or 3) by digesting with the restriction enzymes SalI and HindIII, and ligated into the SalI/HindIII digested pLp_3050Ag85B-E6 plasmid. All resulting plasmids were transformed into *E. coli* TOP10, prior to transformation into *L. plantarum* WCFS1.

4.3.2 Construction of a plasmid for membrane-anchoring of Ag85B-ESAT6

A plasmid for anchoring of Ag85B-ESAT6 to the cell membrane, through a lipobox-domain, was constructed using the pLp_1261InvL plasmid (described in section 2.6). The Ag85B-ESAT6 gene fragment was excised from pLp_3050Ag85B-E6 using restriction enzymes SalI and HindIII. The resulting Ag85B-ESAT6 fragment was ligated into the SalI and HindIII digested pLp_1261InvL plasmid, resulting in the pLp_1261Ag85B-E6 plasmid. Figure 4.13 shows an outline of the lipoprotein cassette originally constructed by Nygaard (2011) for easy exchange of target proteins, as it looks in the pLp_1261Ag85B-E6 plasmid constructed in this study.

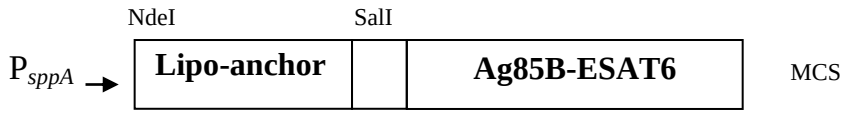

Figure 4.13: A schematic overview of the lipo-anchor-Ag85B-ESAT6 cassette. The cassette consists of a lipoprotein anchor domain, a SalI linker, and a target protein. The SalI linker and the multiple cloning site (MCS) allow for easy exchange of target protein. The Lp_1261 lipoprotein anchor domain consists of a 75 amino acid sequence, including the signal peptide and the lipobox motif (described in section 1.4.2).

4.3.3 Detection of anchoring of Ag85B-ESAT6 to the surface of *L. plantarum* WCFS1

Three different strategies were used to demonstrate membrane- and cell wall anchoring of Ag85B-ESAT6 in *L. plantarum* WCFS1. First, it was attempted to localise the anchored antigen in the total cell extract, the supernatant and a cell wall fraction of induced *L. plantarum* cells, harbouring the anchoring plasmids pLp_3050Ag85B-E6cwa2, pLp_3050Ag85B-E6cwa3, pLp_0373Ag85B-E6cwa2, pLp_0373-Ag85B-E6cwa3 and pLp_1261Ag85B-E6. Overnight cultures of *L. plantarum* strains harbouring the various Ag85B-ESAT6 plasmids were diluted in fresh MRS medium and the cultures grown to OD_{600} ~0.30. After induction by addition 10 ng/ml SppIP the cells were incubated for two hours at 37 ºC. The growth curves from this experiment are shown in the Appendix, Figure A5, and show a decline in growth rate for cells harbouring plasmids encoding cell wall -anchors 2 and 3. The growth of the cell membrane anchoring strain (pLp_1261Ag85B-E6) was comparable to that of the faster growing strain for intracellular production of antigen (pCytAg85B-E6). Western blot analysis of the intracellular fraction (Figure 4. 14) showed the protein bands of the intracellular anchoring precursors. Of the anchoring constructs, the membrane-anchored antigen (Figure 4. 14, lane 6) appears especially abundant in the intracellular fraction. Figure

4. 14 shows that most *L. plantarum* strains supposed to produce anchoring precursors indeed produced proteins of the correct size, with the exception of the two cwa3 precursors (see Figure 4. 14, lanes 3 and 5) which deviates somewhat from the theoretical molecular mass (seen at 60 kDa, not at the expected 68 kDa).

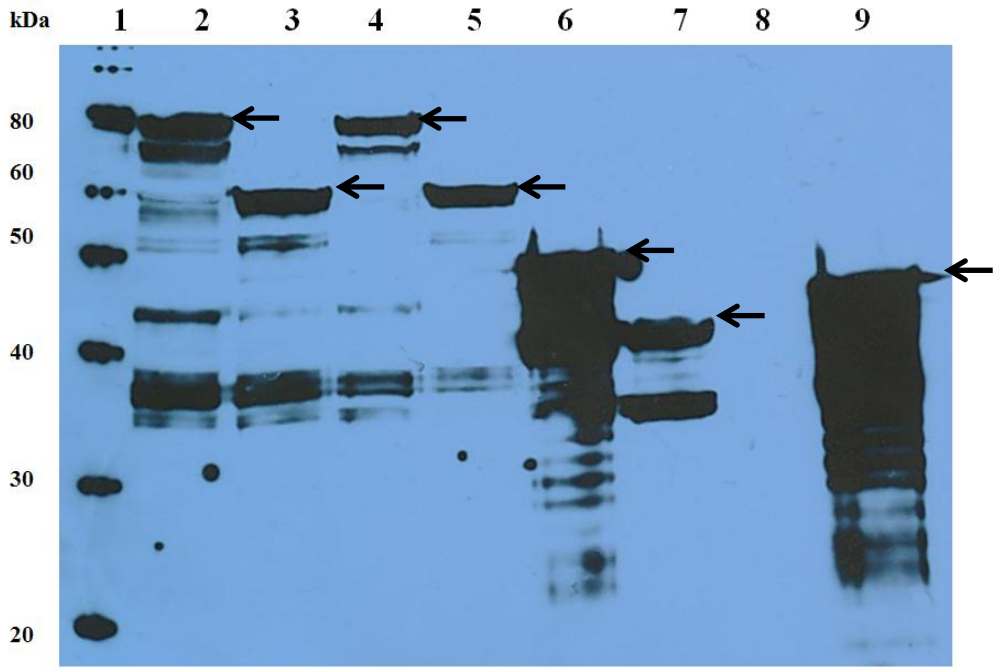

Figure 4. 14: Analysis of intracellular anchoring precursors by Western blotting. The blot shows crude, cell-free protein extracts from induced *L. plantarum* strains harbouring various plasmids for anchoring of Ag85B-ESAT6. The lanes show the following samples (**t**he theoretical molecular masses for unprocessed precursors are given between paranthesis and bands corresponding to these masses are marked by arrows on the blot) (1) MagicMark protein ladder; (2) pLp_3050Ag85B-E6cwa2 (68 kDa); (3) pLp_3050Ag85B-E6cwa3 (59 kDa); (4) pLp_0373Ag85B-E6cwa2 (68 kDa); (5) pLp_0373Ag85B-E6cwa3 (59 kDa); (6) pLp_1261Ag85B-E6 (49 kDa); (7) pCytAg85B-E6 (41 kDa); (8) pSIP-EV; (9) pLp_3050Ag85B-E6 (45 kDa). The volume of sample loaded to gel was calculated according to the absorbance of each individual strain as measured by OD_{600} at the time of harvest, allowing an approximately comparising of protein production in the different strains.

Western blot analysis of precipitated culture supernatants (Figure 4. 15) showed the presence of cwa2 –containing Ag85B-ESAT6 and membrane-anchored containing Ag85B-ESAT6 (Figure 4.15, lanes 2, 4 and 6). The cwa3-containing form of Ag85B-ESAT6 was not detected in the supernatant, even though it is present intracellularly in approximately the same amounts as the cwa2-containg anchored antigen.

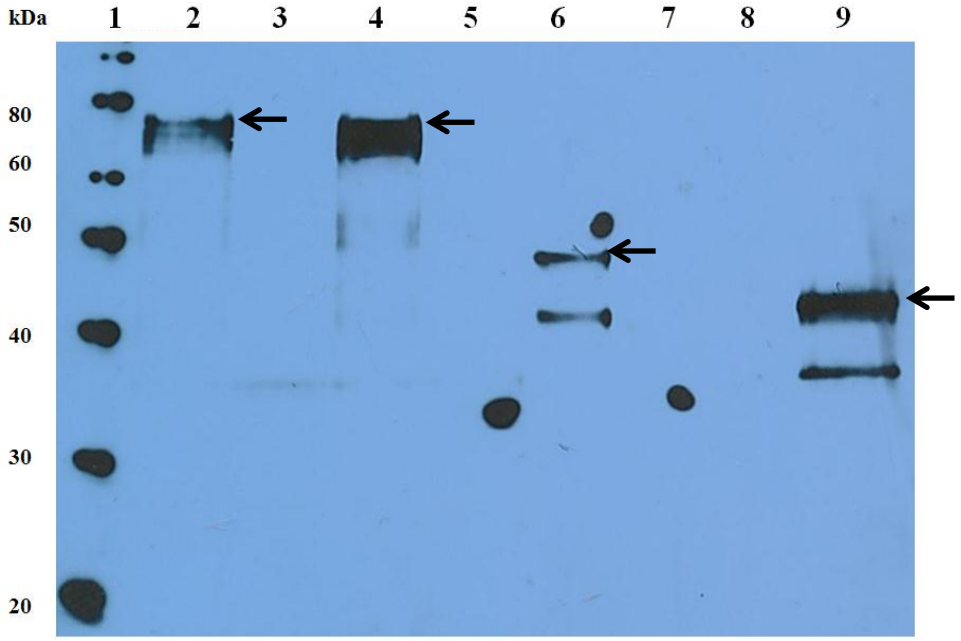

Figure 4. 15: Analysis of extracellular, anchor sequence-containing Ag85B-ESAT6 by Western blotting. Blot showing protein in TCA-precipitated supernatant from induced *L. plantarum* strains harbouring various plasmids for anchoring of Ag85B-ESAT6. The theoretical molecular weight of the correctly processed proteins is given for each of the different Ag85B-ESAT6 constructs (1) MagicMark protein ladder; (2) pLp_3050Ag85B-E6cwa2 (63 kDa); (3) pLp_3050Ag85B-E6cwa3 (55 kDa); (4) pLp_0373Ag85B-E6cwa2 (63 kDa); (5) pLp_0373Ag85B-E6cwa3 (55 kDa); (6) pLp_1261Ag85B-E6 (49 kDa); (7) pCytAg85B-E6 (41 kDa); (8) pSIP-EV; (9) pLp_3050Ag85B-E6 (41 kDa). The volume of sample loaded to gel was calculated according to the absorbance of each individual strain as measured by OD_{600} at the time of harvest, and equaled approximately 4 ml of cell culture.

A Western blot analysis was performed on cell wall fractions obtained by enzymatic digestion of the cell wall of induced *L. plantarum* strains harbouring the various Ag85B-ESAT6 plasmids (see section 3.10.2), and the results are shown in Figure 4. 16. The blot revealed very weak Ag85B-ESAT-6 signals of the expected size in the cell wall fraction of cells expressing the two cell wall anchored versions, containing cwa2 or cwa3 (Figure 4. 16; lanes 3-6). To control for cell lysis during the fractionation procedure, the cell wall fraction of *L. plantarum* cells producing non-secreted antigen (pCytAg85B-E6) was subjected to the same treatment as the cells harbouring the plasmids for anchoring (Figure 4. 16, lane 8). As further controls, strains harbouring pLp_1261Ag85B-E6 and pLp_3050Ag85B-E6 and a strain carrying the empty vector (pSIP-EV) were also analysed. A band of approximately 35 kDa is visible for all strains, except for the strain carrying pSIP-EV (not harbouring the Ag85B-ESAT6 gene) (Figure 4. 16, lane 2).

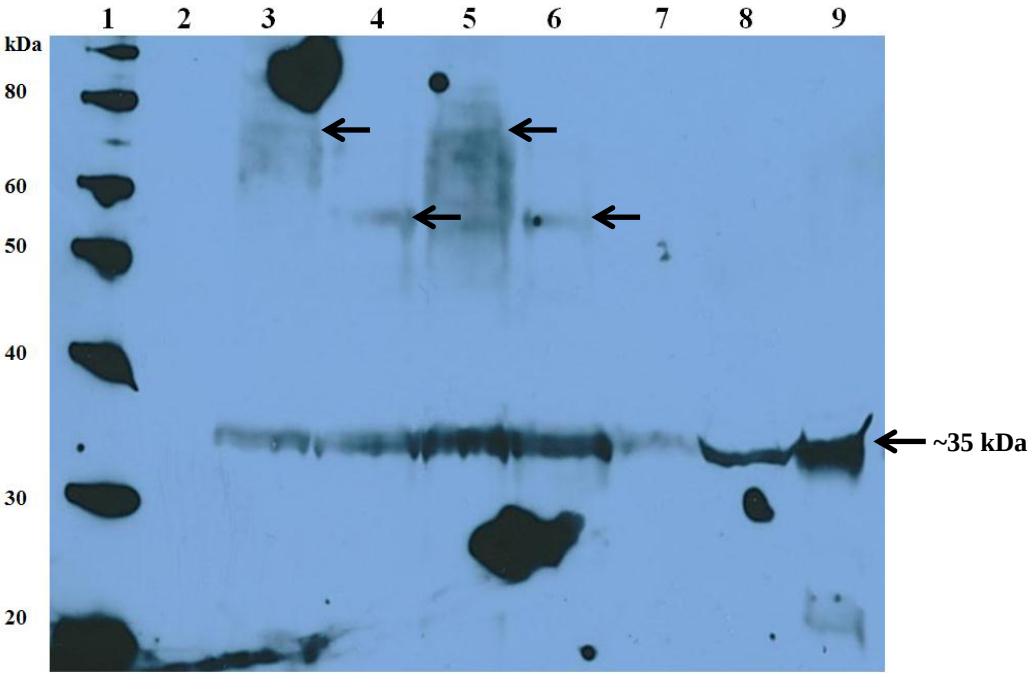

Figure 4. 16: Analysis of cell wall fraction by Western blotting. Blot showing the cell wall fraction of induced *L. plantarum* harbouring different plasmids for anchoring of Ag85B-ESAT6. The lanes show the following samples (**t**he theoretical molecular masses for processed proteins are given between paranthesis and bands corresponding to these masses are marked by arrows on the blot) (1) MagicMark protein ladder; (2) pSIP-EV; (3) pLp_3050Ag85B-E6cwa2 (63 kDa); (4) pLp_3050Ag85B-E6cwa3 (55 kDa); (5) pLp_0373Ag85B-E6cwa2 (63 kDa); (6) pLp_0373Ag85B-E6cwa3 (55 kDa); (7) pLp_1261Ag85B-E6 (49 kDa); (8) pCytAg85B-E6 (41 kDa); (9) pLp_3050Ag85B-E6 (41 kDa). The volume of sample loaded to gel was calculated according to the absorbance of each individual strain as measured by OD_{600} at the time of harvest, and equaled approximately 4 ml of cell culture.

4.3.4 Detection of Ag85B-ESAT6 anchoring by immunostaining

A different approach, based on utilising fluorescent staining to visualise the presence of Ag85B-ESAT6 on the cell surface, was performed using a FITC-conjugated secondary antibody (see section 3.13). The FITC-labelled secondary antibody was bound to cell surface Ag85B-ESAT6 via binding to a monoclonal primary antibody specific for an ESAT6 epitope. The resulting fluorescent staining of cells was evaluated by fluorescent microscopy and flow cytometry. The preparation of samples for fluorescent microscopy and flow cytometry was performed as described in section 3.13.1.

Fluorescent microscopy revealed FITC staining of antigens on the surface of all strains expressing putatively anchored forms of Ag85B-ESAT6, whereas no staining was visible for strains producing the non-secreted antigen (pCytAg85B-E6) or the empty vector (pSIP-EV), as illustrated in Figure 4. 17. For the strains producing the cwa2 and cwa3 versions of Ag85B-ESAT6, the majority of the cells were stained. Cells harbouring the membrane anchored version of the antigen (pLp_1261Ag85B-E6) were generally not stained and only very few stained cells were visible. As a final control, a strain expressing secreted antigen (pLp_3050Ag85B-E6) was also analysed. Also in this case some stained cells were observed.

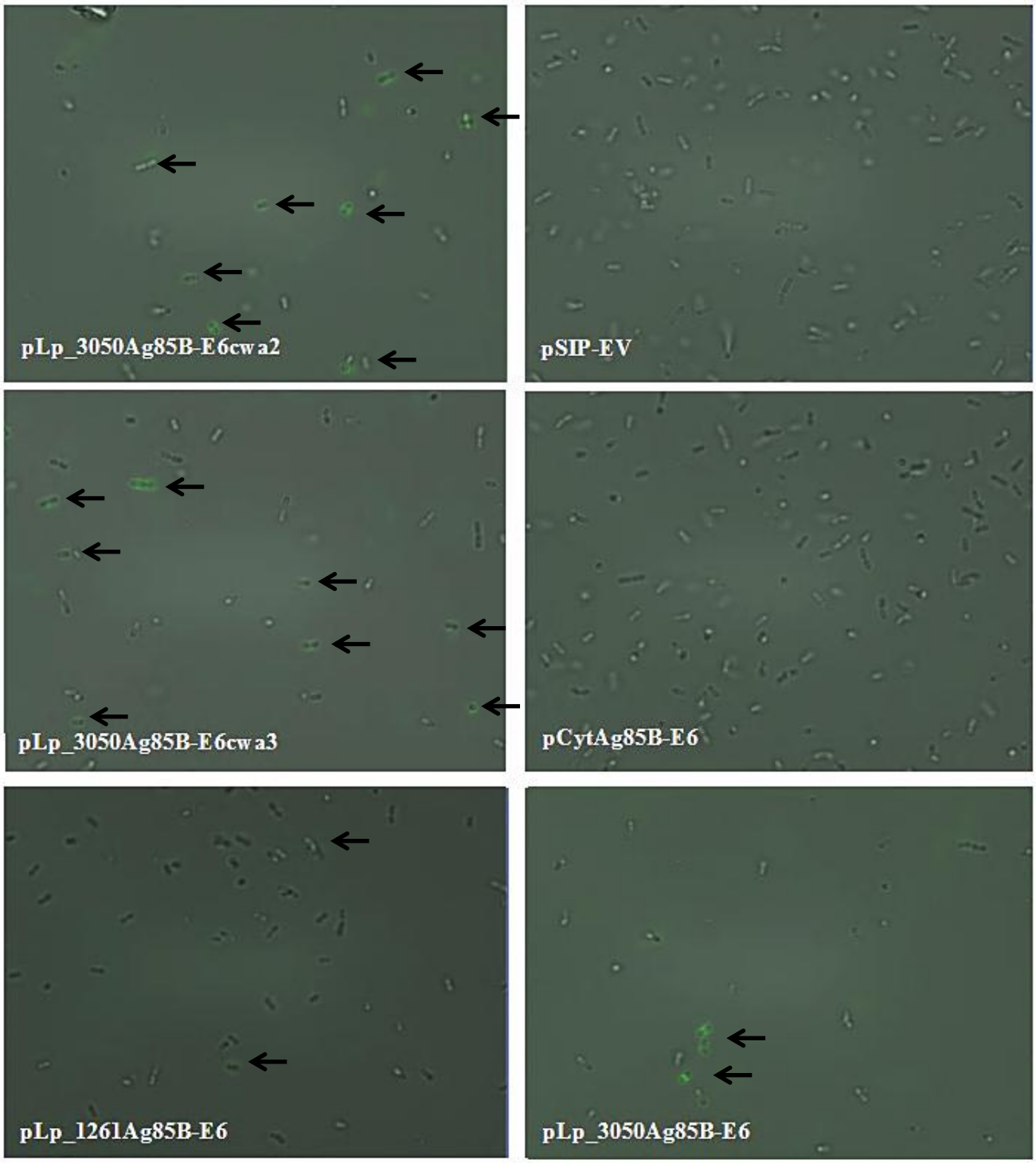

Figure 4. 17: Fluorescent microscopy of induced *L. plantarum* cells harbouring various plasmids for Ag85B-ESAT6 production, secretion or anchoring. Strains were induced by addition of 10 ng/ml SppIP, and harvested 2 hours after induction. The primary antibody ESAT6 Mouse mcAb (ab26246), and the secondary antibody Anti-Mouse IgG FITC was used for the immunostaining. The arrows indicate fluorescent bacterial cells. The saturation of the images have been slightly altered in order to better visualise the green fluorescent signals.

The FITC stained *L. plantarum* cells were also analysed by flow cytometry. A suspension of FITC-stained bacterial cells was run through a flow cytometer and the fluorescent properties of individual cells were measured. The results are depicted in Figure 4. 18 and Figure 4. 19. Generally the flow cytometry results correlates well with the results from the fluorescence microscopy, except perhaps for cells carrying the secreted version of Ag85B-ESAT6 (plp_3050Ag85B-E6). In Figure 4. 18 the results are shown as single dimension histograms, which display the relative fluorescence plotted against the number of events (the number of single particles counted by the flow cytometer). The more the peak shifts towards the right side of the x-axis the more fluorescent signal is measured by the flow cytometer. The histograms for the negative controls, i.e. for cells carrying pSIP-EV or pCytAg85B-E6, clearly show a lack of fluorescent signal, supporting the microscopy results. As observed in the microscopic analysis, cells carrying pLp_1261Ag85B-E6 (membrane anchored) show a lower amount of fluorescent signal than cells carrying the constructs for cell wall anchoring. All three clearly show fluorescent staining of the cells. As observed by fluorescence microscopy (Figure 4. 17), cells producing the secreted antigen (pLp_3050Ag85B-E6) are also stained. In fact, the flow cytometry data indicate a similar degree of staining as for cells producing the cwa2- and cwa3- containing variants of Ag85B-ESAT6. This similarity is also visible in Figure 4. 19, discussed below.

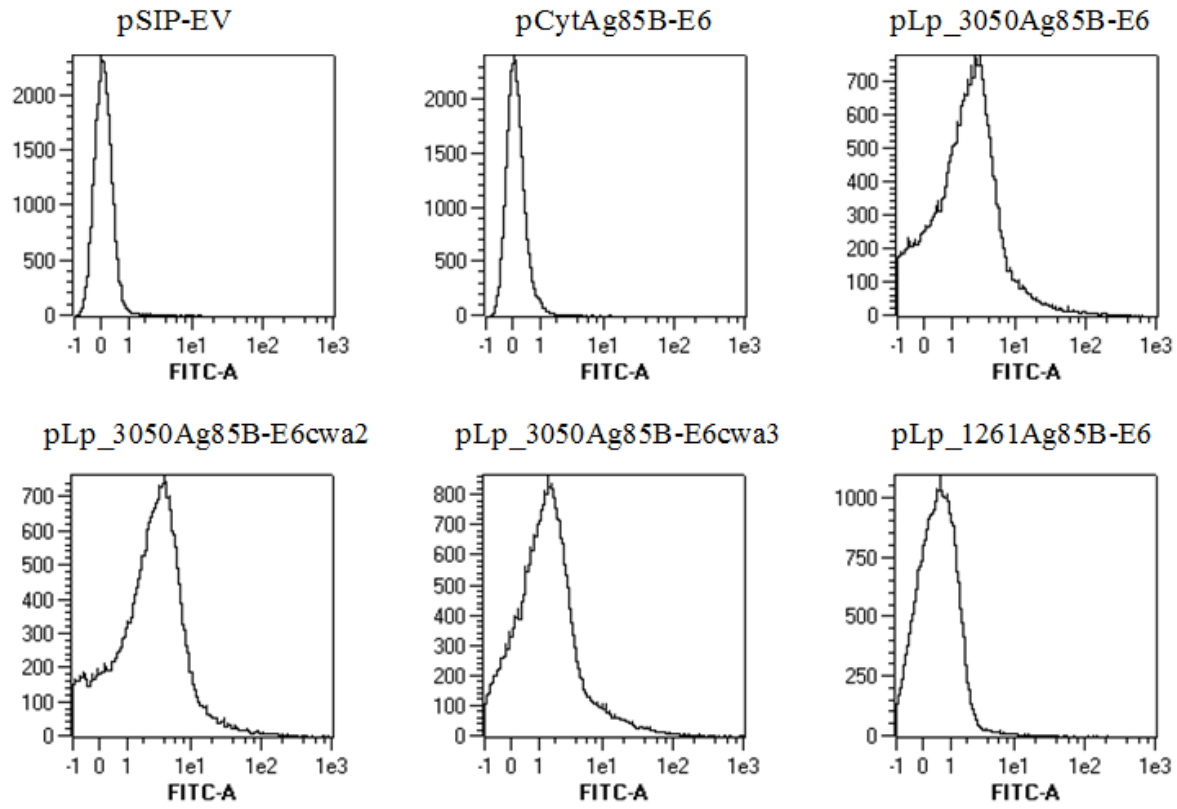

Figure 4. 18: Flow cytometry analysis of FITC stained *L. plantarum* cells harbouring various plasmids for Ag85B-ESAT6 production, secretion or anchoring. The results are shown as single dimension histograms where the relative fluorescence (x-axis) is plotted against the number of events (y-axis), which also indicates the numer of cells analysed. The cells were harvested and stained two hours after induction, using a procedure identical to that used in the microscopy analysis of Figure 4. 17. The results are presented as dot plots in Figure 4. 19. The primary antibody ESAT6 Mouse mcAb (ab26246), and the secondary antibody Anti-Mouse IgG FITC was used for the immunostaining.

In Figure 4. 19 the same flow cytometry data are shown as dot plots which display the relative fluorescence (x-axis) plotted against the sideways light scatter (y-axis). The y-axis is indicative of the granularity of the measured cells (indicative of the cell size and structure). Each dot is the registration of a cell particle in the flow cytometer. Particle counts are shown by dot density, and the density in a given region of the plot is given by the colour. Red indicates high density of particles, while blue indicates low density. The shift of the high density region to the right for cells producing the anchored variants of the antigen is indicative of increased fluorescent signals on the surface of these cells. It should be noted that such a shift is also observed for cells producing secreted Ag85B-ESAT6 (pLp_3050Ag85B-E6), but not for cells producing the cytoplasmic version or carrying the empty vector.

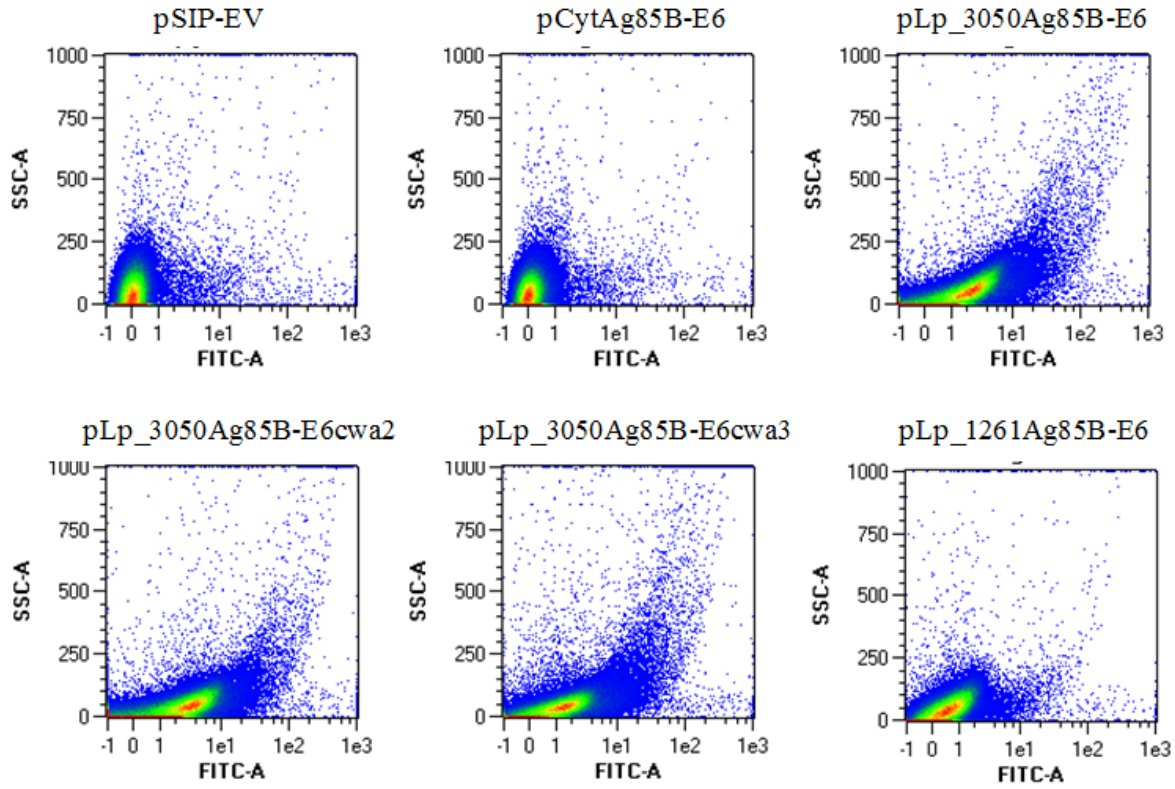

Figure 4. 19: Flow cytometry analysis of FITC stained *L. plantarum* cells harbouring various plasmids for Ag85B-ESAT6 production, secretion or anchoring. The results are shown as dot plots where the relative fluorescence (x-axis) plotted against the sideways light scatter (y-axis). The cells were harvested and stained two hours after induction, using a procedure identical to that used in the microscopy analysis of Figure 4. 17. The results are presented as histograms in Figure 4. 18. The primary antibody ESAT6 Mouse mcAb (ab26246), and the secondary antibody Anti-Mouse IgG FITC was used for the immunostaining.

The flow cytometry results depicted in Figure 4. 18 and Figure 4. 19 clearly show fluorescent staining of cells harbouring the two cell wall anchoring constructs, indicating the presence of Ag85B-ESAT6 on the cell surface. Fluorescent signals on cells producing the membrane-anchored antigen variant (pLp_1261Ag85B-E6) were barely detectable by fluorescent microscopy (Figure 4. 17), but the flow cytometry data shown here strongly indicated the presence of some fluorescent signal on the surface of these cells. The staining of cells carrying the construct for secretion of Ag85B-ESAT6 as observed by fluorescent microscopy was also confirmed in the flow cytometry analysis, indicating that anchoring of Ag85B-ESAT6 cannot be fully confirmed by this method.

4.3.5 Detection of surface-located Ag85B-ESAT6 in strains subjected to conditions met in dendritic cell experiments.

Based on the promising detection of cell surface Ag85B-ESAT6 in strains grown and induced in MRS medium, it was investigated whether antigen could be detected on the surface of cells incubated in RPMI, a medium used for growing of dendritic cells. This was done to see if Ag85B-ESAT6 would be present on the surface of cells subjected to the conditions met in a dendritic cell experiment (see below). This presence is essential if experiments with dendritic cells are to be used to evaluate the immunogenicity of the different antigen producing strains.

The presence of antigen on the cell surfaces of induced *L. plantarum* cells harbouring the different anchoring constructs were explored by incubating the cell cultures in RPMI at 37 ºC, for 24 hours after induction with 10 ng/ml SppIP, according to the protocol described in section 3.14.2. Ag85B-ESAT6 antigen was stained with monoclonal ESAT6 Mouse mcAb (ab26246) and FITC-conjugated secondary antibody (see section 3.13.1). The presence of fluorescent signals was evaluated by flow cytometry and fluorescent microscopy as described above.

After 24 hours of incubation in RPMI, fluorescent signals were detected by both methods, for all strains putatively producing anchored antigen (
Figure 4. 20Figure 4. 21). Due to an experimental error the secreted antigen containing construct was not included in this analysis. Fluorescent signals were not seen in the negative controls, pCytAg85B-E6 and pSIP-EV as seen in
Figure 4. 20 Figure 4. 21. Fluorescent signals were seen for cells carrying the cwa2 and cwa3 anchored versions of the antigen, which is consistent with the previous results (Figure 4. 17Figure 4. 19). Interestingly, an increase in fluorescent signal was seen for the strain expressing the membrane anchored antigen (pLp_1261Ag85B-E6), compared to the previous results shown in Figure 4. 18Figure 4. 19. The single dimension histogram in
Figure 4. 20 (top) show that a subpopulation of the cells producing the membrane anchored antigen (pLp_1261Ag85B-ESAT6) exhibited an increased fluorescent signal, as depicted from the observed shift to the right in the histogram and dot plot. The increased fluorescence for cells carrying the pLp_1261Ag85B-E6 construct is also supported by the fluorescent microscopy image for the membrane anchored construct (pLp_1261Ag85B-E6), where a vague fluorescent signal can be observed on the surface of nearly all cells (Figure 4. 21).

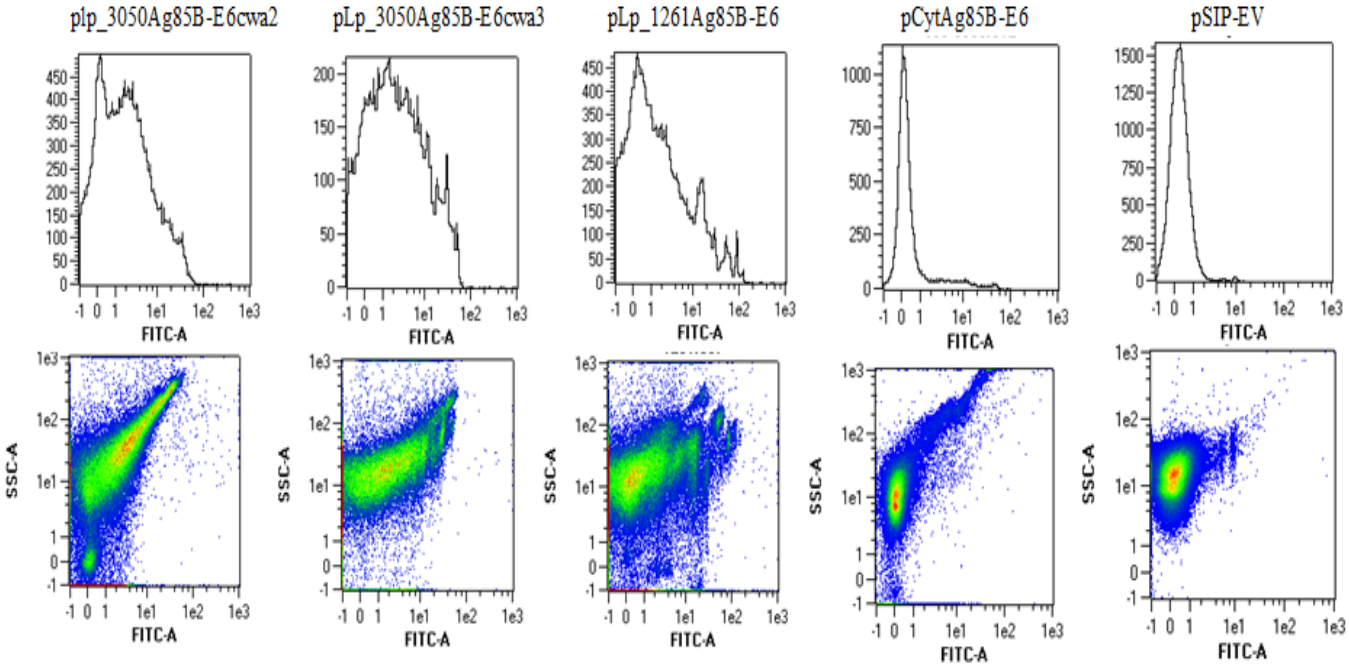

Figure 4. 20: Flow cytometry analysis of FITC stained *L. plantarum* cells harbouring various plasmids for anchoring of Ag85B-ESAT6. The results areshown as; (top) single dimension histograms where the relative fluorescence (x-axis) plotted against the number of events (y-axis); (bottom) dot plots where the relative fluorescence (x-axis) plotted against the sideways light scatter (y-axis). The cells were harvested and stained 24 hours after induction , using a procedure identical to that used in the microscopy analysis of Figure 4. 21. The primary antibody ESAT6 Mouse mcAb (ab26246), and the secondary antibody Anti-Mouse IgG FITC was used for the immunostaining.

Figure 4. 21: Fluorescent microscopy of induced *L. plantarum* cells harbouring various plasmid-constructs for anchoring of Ag85B-ESAT6. Cells were induced with 10 ng/ml SppIP, and harvested 24 hours after induction. The primary antibody ESAT6 Mouse mcAb (ab26246), and the secondary antibody Anti-Mouse IgG FITC was used for the immunostaining. The arrows indicate fluorescent bacterial cells. The saturation of the images have been slightly altered in order to better visualise the green fluorescent signals.

4.4 Effects of the Ag85B-ESAT6 expressing strains on dendritic cells

Based on the immunostaining results described in section 4.3 *in vitro* dendritic cell experiments were performed to check for cytokine responses against induced *L. plantarum* WCFS1 harbouring plasmids for expression of the Ag85B-ESAT6 antigen. The cytokines released by dendritic cells could give information about the immunogenicity of the different antigen producing strains. Dendritic cells were obtained by differentiation of CD14+ cells isolated from human peripheral blood leucocytes as described in section 3.14.1. The same blood donor was used in all experiments. The plasmids tested were the three anchor-constructs pLp_3050Ag85B-E6cwa2, pLp_3050Ag85B-E6cwa3 and pLp_1261Ag85B-E6, in addition to pLp_3050Ag85B-E6 (secretion), pCytAg85B-E6 (intracellular) and pSIP-EV (control). The empty vector pSIP-EV was included to take in account the effect of *L. plantarum* it selves. *L. plantarum* cells harbouring the recombinant plasmids were cultured and induced according to the protocol described in section 3.14.2. The induced cells where co-incubated with dendritic cells for 24 hours according to the protocol (section 3.14.2) and the cytokine profile of the supernatants was evaluated by ELISA. ELISA of TNF-alpha and IL-10 were performed immediately after harvest of supernatant, while the samples for ELISA of IL-12 were stored at -80 ºC prior to analysis. Each of the bacterial strains was represented by four biological replicas and each of these replicas was analysed in duplicates. The ELISAs were performed according to the procedures described in section 3.14.3.

The results from the analysis of TNF-alpha and IL-10 are shown in Figure 4. 22 and Figure 4. 23, respectively. The basal release of TNF-alpha and IL-10 could not be set due to an experimental error concerning the negative control samples (DCs not incubated with lactobacilli). All strains were able to elicit a cytokine response in dendritic cells. Interestingly the strain expressing the membrane-anchored antigen (pLp_1261Ag85B-E6) elicited the strongest release of both cytokines (on average 1800 pg TNF-alpha and 1100 pg IL-10 pr 0.6 million DCs). This strain particularly stands out in the case of IL-10 release (Figure 4. 23), the average release of IL-10 from DCs incubated with the other strains being in the order of 400-600 pg, and thus considerably lower. The cell wall anchoring strains with the other antigen producing strains (intracellular or secreted Ag85B-ESAT6) or the control strain (pSIP-EV) does not reveal significant differences or trends. In particular, it must be noted that *L. plantarum* harbouring pSIP-EV also was able to induce a response by the DCs.

The ELISA of IL-12 did not give any interpretable results even though the standard curve was reasonable. The IL-12 kit was specified to be less sensitive than the two other kits used, which may explain the reason for the lack of results from this analysis.

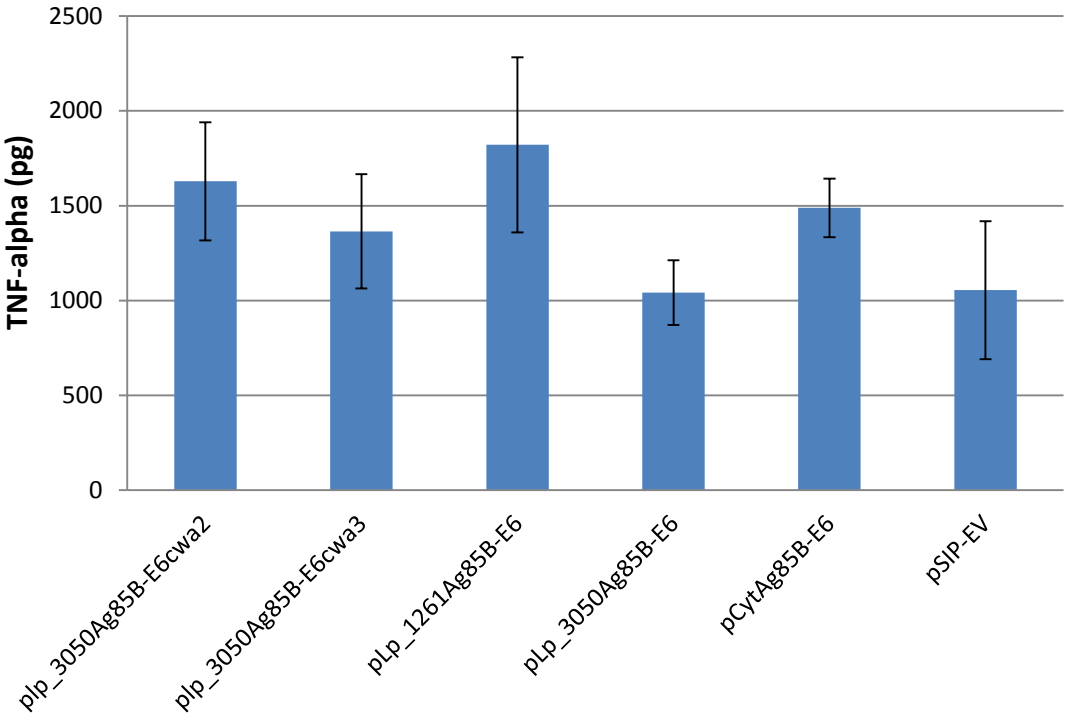

Figure 4. 22: Analysis of TNF-alpha by ELISA. The amount of TNF-alpha (pg) secreted by dendritic cells co-incubated with *L. plantarum* strains harbouring various Ag85B-ESAT6-plasmids. The standard deviation (from four biological replicas, each analysed in duplicates) is indicated as a vertical line at the top of each column.

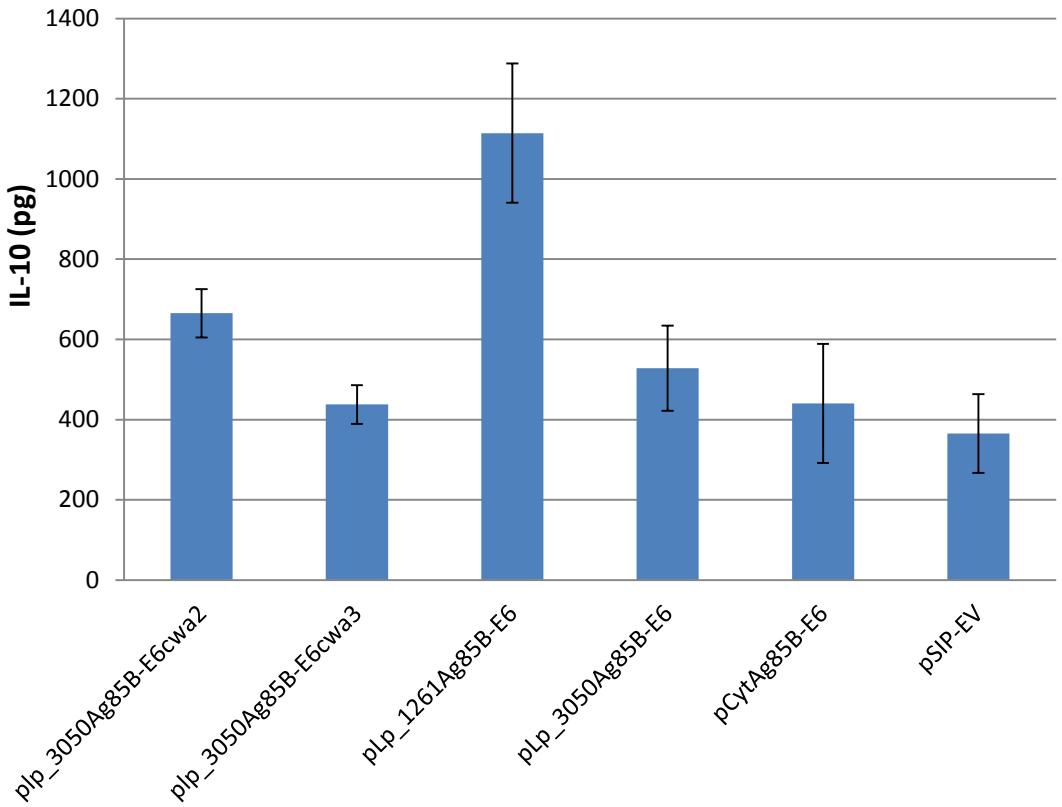

Figure 4. 23: Analysis of IL-10 by ELISA. The amount of IL-10(pg) secreted by dendritic cells co-incubated with *L. plantarum* strains harbouring various Ag85B-ESAT6-plasmids. The standard deviation (from four biological replicas, each analysed in duplicates) is indicated as a vertical line at the top of each column.

5 DISCUSSION

5.1 Intracellular production of a *Mycobacterium tuberculosis* fusion protein in *L. plantarum* WCFS1

The ability of *L. plantarum* to produce the heterologous fusion protein Ag85B-ESAT6 using the pSIP-system was fundamental to the following studies of secretion and anchoring of this antigen. The Ag85B-ESAT6 fusion protein has not previously been expressed in *L. plantarum*.

Ag85B-ESAT6 was successfully produced intracellularly in *L. plantarum* by the use of an inducible pSIP401-vector derivative expressing the *Mycobacterium* fusion protein Ag85B-ESAT6 (Figure 4. 3). The growth rate of induced *L. plantarum* cells harbouring the plasmid for intracellular production of Ag85B-ESAT6 (pCytAg85B-E6) was equal to that of non-induced cells (see Appendix, Figure A1), indicating that intracellular production of Ag85B-ESAT6 does not subject the bacteria to great stress within the time period tested.

A Western blot analysis of protein production over time showed detectable intracellular production of Ag85B-ESAT6 after 30 min (Figure 4. 3). It has previously been shown that the pSIP-system is able to produce detectable amounts of protein 10-15 minutes after the addition of inducer peptide (SppIP) (personal communication Dimitrijevic (2011)), and this is also likely to be the case for production of Ag85B-ESAT6. Ag85B-ESAT6 could not be detected by Coomassie staining or Silver staining of gels (results not shown), indicating that a lower amount of antigen is produced. Several protein bands below the 41 kDa Ag85B-ESAT6 protein band can be seen in the Western blot in Figure 4. 3, two hours after induction. These bands could probably be fragments of Ag85B-ESAT6 resulting from degradation by intracellular proteases. The *L. plantarum* genome is predicted to encode 19 different intracellular peptidases (Kleerebezem et al. 2003). Intracellular degradation of heterologous proteins has previously been identified as a problem in studies on heterologous protein production in *Lc. lactis* (Miyoshi et al. 2002; Miyoshi et al. 2006).

Constitutive production of therapeutic proteins could be advantageous to the use and administration of a vaccine delivery system, by enabling induction-free production of the protein in question. Constitutive expression of Ag85B-ESAT6 was successfully accomplished by replacing the inducible P_{sppA} promoter of the SIP-based vectors with the p11 and p27 promoters (Rud et al. 2006), for high and lower constitutive gene expression, respectively. Western blot analysis of crude cell-free protein extracts from these experiments indicates that the p11 promoter, selected for high-level gene expression, is able to match the protein production of the inducible promoter (Figure 4. 4). Compared to the p27 promoter, (selected for lower gene expression) the p11 promoter provides a larger amount of Ag85B-ESAT6 product (Figure 4. 4Figure 4. 5). The growth rates of *L. plantarum* cultures harbouring plasmids for constitutive expression of the intracellular form of Ag85B-ESAT6 were equal to that of cultures with induced or non-induced production of Ag85B-ESAT6, indicating that the constitutive production of this protein is adequately tolerated by the cells. Despite the success of constitutive intracellular production of Ag85B-ESAT6, several attempts of constructing vectors for constitutive secretion of this antigen failed. Constitutive production and secretion of this and other heterologous proteins in *L. plantarum* probably have a cytotoxic effect to the transformed *E. coli* host cells, as observed in this study and previously described by Fredriksen (2007). Direct transformation into *L. plantarum* was also attempted, but unsuccessful.

All in all, the results of this first part of the study showed that *L. plantarum* is able to produce the heterologous fusion protein Ag85B-ESAT6, both by the use of an induction system and in a constitutive manner.

5.2 Secretion of Ag85B-ESAT6 in *L. plantarum* WCFS1

The selection of signal peptides was based on their previously reported properties and performances when targeting the reporter proteins Nuclease A (NucA) and Amylase A (AmyA) for secretion (Mathiesen et al. 2008; Mathiesen et al. 2009). Data from these studies indicate that efficient secretion and maximized secretion levels in *Lactobacillus* are highly dependent on both the SP and the targeted protein in question and that there is no way to predict optimal combinations of the SP and target protein.

The secretion capacity of 11 different signal peptides was tested in *L. plantarum* using Ag85B-ESAT6 plasmids based on pSIP derivatives previously constructed for secretion of NucA by Sveen (2007). Successful secretion of Ag85B-ESAT6 was obtained with all constructs (Figure 4. 8). Among the best performing signal peptides (Figure 4. 8), were the SPs from proteins Lp_3050, Lp_3676, Lp_0469 and Lp_1448. The secretion efficiency was tested for these constructs by comparing the amount of intracellular and secreted Ag85B-ESAT6 (Figure 4. 9). The Lp_3050 and Lp_0373 proteins of *L. plantarum* were selected for further studies. The selection of Lp_3050 was based on its good performance when targeting Ag85B-ESAT6 for secretion (Figure 4. 8 & 4.9). The Lp_0373 signal peptide was selected out of interest to compare with the better performing SP, and to investigate the possible advantages of cells experiencing less production and secretion pressure. The Lp_3050 signal peptide has previously been shown to be the best NucA secreting SP out of 78 tested SPs in *L. plantarum*, while Lp_0373was ranked as number 8 in the same study by Mathiesen et al.(2009). The secretion efficiency of α-amylase by Lp_3050 was in the same study reported as 7 %, while Lp_0373 had a secretion efficiency of 30 %. Showing that ultimately, secretion levels and secretion efficiency is not determined by the SP alone, but also by the peptide targeted for secretion.

Generally the secretion efficiency of the different signal peptides appeared to be low, as indicated by the build-up of intracellular antigen levels that were high compared to the lower amounts of antigen in the surrounding media (Figure 4. 9). Thus, while secretion was achivied, the secretion levels were certainly not optimal. The data indicate that the Ag85B-ESAT6 fusion protein is susceptible to degradation in the intracellular environment as previously observed when expressing intracellular Ag85B-ESAT6. Heterologous proteins lacking sufficient stability could be prone to degradation by intracellular proteases and might

never reach the translocation apparatus (Li et al. 2004). This may in itself be one reason for the low secretion efficiency. Secretion stress resulting from suboptimal adaption of the level and rate of protein production to the capacity of the translocation apparatus can also result in low processing rates and/or proteolytic degradation of the product (Bolhuis et al. 1999; Lulko et al. 2007). The N-terminal signal peptide is thought to be of great importance in maintaining the precursor protein in a translocation competent state and in promoting its transportation through the secretion machinery (Brockmeier et al. 2006; Li et al. 2004), again underpinning the importance of evaluating several signal peptides when selecting an optimal signal peptide for secretion of a heterologous protein. In this study 11 different signal peptides were tested, which should give a high chance of discovering an optimal one. Since secretion efficiencies were low in all cases, it is likely that the fusion protein Ag85B-ESAT6, derived from *M. tuberculosis*, is the main cause of the low secretion efficiency in *L. plantarum.*

For the signal peptide Lp_600, a protein band similar in size to the intracellular precursor protein was observed in the supernatant fraction (Figure 4. 8, lane 10). Silver staining of proteins in supernatants was performed to see if the apparently unprocessed Ag85B-ESAT6 preprotein could be present as a result of cell lysis. No major intracellular proteins could be detected in the supernatant of any of the signal peptide constructs, indicating that the secreting cells were not prone to cell lysis (Appendix, Figure A2). The Lp_0600Ag85B-ESAT6 precursor protein could possibly have been secreted into the medium unprocessed. Another possibility could be the anchoring of Ag85B-ESAT by this SP (Lp_0600), and that the unexpected protein band in the blot (Figure 4. 8, lane 10) is a result of protein shedding. The Lp_0600 protein is a putative extracellular zinc metalloproteinase thought to be secreted by *L. plantarum* (Boekhorst 2006), indicating that the protein band observed in the blot is not a result of protein shedding.

Several bands below the size of the 41 kDa Ag85B-ESAT6 protein band were observed in the supernatant fractions, as seen in Figure 4. **8**. These bands could be fragments of the secreted Ag85B-ESAT6 protein that could result from extracellular degradation by proteases. It is known that the presence of extracellular proteases may cause problems when using *B. subtilis* for secretion of heterologous proteins (Jensen et al. 2000; Murashima et al. 2002). *Bacillus* species are known to secrete degrading enzymes into the surrounding environment, and several extracellular proteases have been identified in *B. subtilis* (Simonen & Palva 1993).

Extracellular proteases have also been predicted in *L. plantarum* (Kleerebezem et al. 2003) and could possibly contribute to the degradation of secreted heterologous proteins, as observed for extracellular Ag85B-ESAT6.

5.2.1 The effects of inducer peptide on production and secretion of Ag85B-ESAT6 in *L. plantarum*

There was great interest in performing dose-response experiments to evaluate the effect of inducer peptide dosage on the production and secretion of heterologous protein in the inducible pSIP expression system. Optimisation of inducer peptide dosage could result in better bacterial growth, secretion and protein anchoring. In addition the effect of incubation temperature was included in the experiment to see if the pSIP system responded differently when incubated at 30 or 37 ºC. In a previous study evaluating intracellular protein production driven by the same promoter (P_{sppA}) in the same pSIP system, the optimal incubation temperature was found to be 30ºC and a clear dose-response relationship was observed between the amount of product produced and the amount of pheromone added to the culture. The pheromone range tested was 0-100 ng/ml, and peak production was observed after induction by 25 ng/ml inducer pheromone (Mathiesen et al. 2004). Reduced growth rates were observed upon induction and this was attributed to the production of the product and not simply the presence of the peptide pheromone itself.

Seven different concentrations of inducer peptide were evaluated, ranging from 0.1 -25 ng/ml SppIP. Additionally a non-induced culture was included to control for leakage of the P_{sppA} promoter, in other words to control for initiation of transcription in absence of the inducer peptide. Some leakage was observed at 37 ºC, which is consistent with the results obtained by Mathiesen et al. (2004). Western blot analysis performed crude cell-free protein extracts and precipitated supernatants of the harvested cultures are shown in Figure 4. 10 and 4.11. In the cultures grown at 30 ºC, Ag85B-ESAT6 was detected both intracellularly and in the supernatant at an inducer dosage of 1.0 ng/ml SppIP. For cultures grown at 37 ºC, secreted Ag85B-ESAT6 was on the other hand not observed until induction by dosages of 3 ng/ml SppIP or higher. As observed in Figure 4.11 (a) it appears that the protein precursor has been processed (lack of a 45 kDa band), suggesting that the lack of Ag85B-ESAT6 in the supernatant could be arbitrary as a result of loss of protein during the precipitation procedure. The maximal production of Ag85B-ESAT6 appears to be reached at 3 ng/ml SppIP at both

temperatures (Figure 4. 10 & 4.11), and indicates the great sensitivity of the pSIP promoter. A clear dose-response effect is observed for intracellular protein production at induction dosages between 0 and 3 ng/ml SppIP at 30 ºC, and this effect appears to be even more evident at 37 ºC. At inducer concentrations of 3.0 ng/ ml SppIP and higher, the growth rates were reduced, which may indicate stress of the cells when the protein expression reaches a certain level and the pressure on the translocation machinery increases (growth curves in Appendix, Figure A3 and A4).

Interestingly most of the protein seem to remain intracellularly (at both temperatures), even at low –level production, suggesting that optimisation of production levels by optimisation of inducer dosage does not result in more efficient secretion. These results thus indicate that other means of optimising the secretion efficiency, such as selecting the best performing SP for a given target protein, is of greater importance

5.3 Anchoring of Ag85B-ESAT6 in *L. plantarum* WCFS1

Two different versions of the LPXTG-type peptidoglycan anchor sequence of the Lp_2578 protein of *L. plantarum* were used in this study to anchor the Ag85B-ESAT6 protein to the cell wall via its C-terminal end. The only difference between the cell wall anchor sequences was their length, theoretically making it possible to localise Ag85B-ESAT6 at two different distances away from the site of covalent attachment to the cell wall peptidoglycan. Cloning of a third and longer version of this anchor was not successful. Anchoring of heterologous proteins in *L. plantarum* by the use of these cell wall-anchor sequences has been described previously in a study by Fredriksen et al. (2010) where the anchored protein (a human cancer antigen) successfully was detected in the cell wall. In addition to the LPXTG-type anchors, a secretion and anchoring sequence of the lipoprotein Lp_1261 of *L. plantarum,* was used to N-terminally anchor Ag85B-ESAT6 to the lipids of cell membrane by a thioether linkage. The purpose of comparing these different constructs was to find the optimal way of displaying proteins on the cell surface and to find out to which degree the different anchors could provide stable anchoring of Ag85B-ESAT6 and elicit a sufficient immune response in a future host organism.

The two signal peptides, Lp_0373 and Lp_3050 showed similar properties when targeting Ag85B-ESAT6-cwa2 and 3 for translocation. The slow growth rates of these two cell wall-anchoring strains indicate that the cells are subjected to stress due to the introduction of the anchoring sequences (cwa2 or -3) (see Appendix, Figure A5). *L. plantarum* harbouring the plasmid encoding the shortest cell wall-anchor, cwa3, showed an earlier decline in growth rate than cells harbouring the plasmid encoding the longer cell wall-anchor, cwa2. This indicates that production and processing of Ag85B-ESAT6cwa2 was marginally better tolerated than that of Ag85B-ESAT6cwa3. The growth rate of pLp_1261Ag85B-E6 (membrane anchor) was found to be comparable to that of pCytAg85B-E6 (Appendix, Figure A5), and no decline in growth rate could be observed within the experiment's time limit, suggesting that the membrane-anchored construct (Lp_1261Ag85B-ESAT6) is better tolerated by the bacteria.

Western blot analyses were performed on crude cell-free protein extracts, culture supernatants and the cell wall fractions of induced *L. plantarum* strains harbouring the various plasmids. The Western blot of Figure 4. 14 shows the protein bands of the intracellular Ag85B-ESAT6-anchoring precursors. Intracellular degradation of the anchoring constructs was observed, as previously experienced in this study. Such intracellular degradation of heterologous proteins could likely be attributed to intracellular proteases and have been reported in other studies (Miyoshi et al. 2002; Miyoshi et al. 2006) as described in section 5.1.

Ag85B-ESAT6 was observed in the supernatant fractions of *L. plantarum* strains harbouring the Ag85B-ESAT6 construct of cwa2 and the lipoprotein anchor (Lp_1261) (Figure 4. 15, lanes 2, 4 & 6), indicating release of some Ag85B-ESAT6cwa2. The finding of cell wall targeted heterologous proteins in culture supernatants has been reported (Brinster et al. 2007; Dieye et al. 2001; Fredriksen et al. 2010). No Ag85B-ESAT6cwa3 could be detected in the supernatant, indicating that no detectible amount of the antigen was released into the culture medium. The shorter cell wall-anchor 3 (cwa3) could possibly anchor the antigen more successfully in the cell wall than the longer cwa2 anchor, thereby making it less prone for release into the surrounding media. The size of the protein band in the supernatant is comparable to that expected for the Ag85B-ESAT6cwa2 construct lacking the SP, and could indicate that its presence in the supernatant is not due to cell lysis, but rather failure of the sortase to anchor the antigen to the cell wall. A weaker signal representing the cell membrane anchored-Ag85B-ESAT6 construct (pLp_1261Ag85B-E6) was also observed in the

supernatant, and could indicate inadequate processing of the lipobox or shedding of the protein. Shedding of membrane anchored proteins (lipoproteins) to the surrounding media has been observed in several studies (Antelmann et al. 2001; Tjalsma et al. 2008).

Identification of cell wall associated Ag85B-ESAT6 was attempted by Western blot analysis of isolated cell wall fractions. As seen in Figure 4. 16 (lanes 3-6) very weak bands indicate the presence of Ag85B-ESAT6cwa2 and Ag85B-ESAT6cwa3 in the cell wall fraction. The smaller sizes of these bands (Figure 4. 16, lanes 3-6) as compared to the sizes of the precursor proteins seen intracellularly, indicate that the LPXTG anchor have been processed, which would imply that anchoring of Ag85B-ESAT6 is possible. The approximately 35 kDa band visible for all Ag85B-ESAT6 producing strains may indicate that the cells could be prone to lysis during the cell wall isolation procedure. On the other hand it would be expected to observe bands of 41 and 45 kDa in Figure 4.16 if the cells of the two control strains; pCytAg85B-E6 and pLp_3050Ag85B-E6 (lane 8 and 9, respectively) had been lysed during the enzymatic degradation of the cell wall. The 35 kDa protein observed in Figure 4.16 does not exist in the empty vector (pSIP-EV), and is therefore most likely related to Ag85B-ESAT6. Alternative transcription or translation-processes could possibly results in ~35 kDa relatives of Ag85B-ESAT6 that may be located in the cell wall. Thus, some of the potential degradation products observed in cell extracts and supernatants of Ag85B-ESAT6 producing strains in this study could possibly be a result of such alternative processes.

The cell wall isolation procedure used in this study was based on Mujahid et al. (2007), and has been successfully used to detect heterologous proteins (a human cancer antigen) in the cell wall fraction of *L. plantarum* (Fredriksen et al. 2010). Despite several attempts to optimize the method, the results of this approach remained somewhat non-conclusive. Still, anchoring of Ag85B-ESAT6 looks promising.

Fluorescent staining was used to visualise the presence of antigens on the cell surface of *L. plantarum* cells expressing putatively anchored forms of Ag85B-ESAT6. Fluorescent microscopy and flow cytometry revealed FITC staining of antigens on the surface of all strains thought to produce anchored forms of Ag85B-ESAT6, whereas no staining was visible for strains producing the non-secreted antigen (pCytAg85B-E6) or the empty vector (pSIP-

EV), as illustrated in Figure 4.17-4.19. In addition, stained cells were also observed for the strain expressing secreted Ag85B-ESAT6.

FITC staining of antigens on the surface of cells producing the two putative cell wall anchoring Ag85B-ESAT6 constructs support the finding of Ag85B-ESAT6cwa2 and -3 in the cell wall fraction (Figure 4. 16). Fluorescent microscopy indicated that not all cells appeared to be stained, in particular for cells harbouring the shortest cell wall anchor, cwa3 (Figure 4.17). The flow cytometry data regarding cells producing cell wall anchored Ag85B-ESAT6 show a narrow distribution of the fluorescence intensities from each bacterial cell analysed. This type of distribution indicate that the individual cells have about the same amount of stained antigen on the cell surface (Nhan et al. 2011). This suggests that among the cells that have been stained an almost equal amount of antigen is present on their cell surface. The lack of fluorescent signal in some cells could be explained by the presence of to low amounts of target protein for detection. Alternatively, Ag85B-ESAT6 may to different degrees be embedded in the membrane. The specific antibody epitopes could as a result of this become unavailable for binding by the monoclonal primary antibody used in this study. Another possibility is that not all cells were induced, and therefore had no target protein to display on the cell surface. Induction of the P_{sppA} promoter could be investigated by fusing the promoter to a green fluorescent protein. Since cells producing green fluorescent protein is easily visualised by fluorescent microscopy, it would enable the percentage of induced cells to be calculated.

A weak fluorescent signal was detected on a few of the *L. plantarum* cells harbouring the pLp_1261Ag85B-E6 plasmid for cell membrane anchoring of Ag85B-ESAT6. This observation could be attributed to the fact that the antigen is likely to be anchored in the cell membrane, and that this localisation possibly renders the antigen more embedded in the cell envelope. Similar results when utilising lipoproteins to anchor heterologous proteins in *L. plantarum* have been obtained in other studies (Nygaard 2011). Nygaard (2011) found that partial enzymatic degradation of the cell wall led to easier fluorescent detection of cell membrane-anchored heterologous proteins. Such partial enzymatic degradation of the cell wall was not performed in this study. The detection of stronger fluorescent signal on the cell surface of *L. plantarum* cells producing Lp_1261Ag85B-ESAT6, 24 hours after induction (

Figure 4. **20** & Figure 4. 21), could indicate that the cell membrane-anchored antigen takes longer time to pass through the cell wall and protrude on the cell surface. The fact that Ag85B-ESAT6 is still detected on the cell surface of all *L. plantarum* strains producing putatively anchored forms of Ag85B-ESAT6 after 24 hours could further imply that the antigen is in fact anchored to the cell-wall or the cell membrane.

The lack of fluorescent signal in the control strains producing the non-secreted antigen (pCytAg85B-E6) or the empty vector (pSIP-EV) (illustrated in Figures 4.17- 4.19) demonstrates that the presence of fluorescent signals is not an artefact due to unspecific binding of the primary antibody or lysis of cells. However, cells producing secreted Ag85B-ESAT6 (pLp_3050Ag85B-E6) were also stained. This would imply that the experimental setup is unable to discriminate between cells producing putatively anchored Ag85B-ESAT6 from cells secreting Ag85B-ESAT6. Insufficient washing of cells prior to antibody hybridization could leave secreted Ag85B-ESAT6 retained in the cell wall. Alternatively, unprocessed Lp_3050Ag85B-ESAT6 (secreted antigen) might be unintentionally anchored in the cell membrane or cell wall, due to properties of the signal peptide-target protein entity. The Lp_3050 SP is considered an effective signal peptide, and it is not likely that the signal peptide it selves is not processed. The *M. tuberculosis* fusion protein could possibly have some attraction to the cell wall and therefore take longer time to diffuse into the extracellular environment.

Cell wall associated Ag85B-ESAT6 was identified in the cell wall fraction of *L. plantarum* cells harbouring plasmids encoding putative cell wall-anchored versions of Ag85B-ESAT6. Cells of these strains also exhibited antigen specific fluorescent signals on the cell surface when stained with FITC, indicating cell surface localisation of antigen. Weak fluorescent signals were also seen on the cell surface of cells producing putative cell membrane-anchored Ag85B-ESAT6 (pLp_1261Ag85B-E6). However, due to the finding of stained cells of the strain expressing secreted Ag85B-ESAT6, these results could be considered somewhat non-conclusive. With this result in mind it should be noted that the Ag85B-ESAT6cwa3 construct was not detected in the supernatant. Still cells producing this construct were stained by FITC, indicating that there is a great possibility that this cell wall-anchoring variant is able to anchor Ag85B-ESAT6 to the cell wall. Optimisation of the FITC-staining procedure by introduction of tougher washing steps could be an option in order to remove antigens that are non-

covalently associated with the cell wall. Another possible strategy to determine whether the results obtained in this study are a result of actual anchoring of Ag85B-ESAT6 to the cell membrane or the cell wall could be a minimal mutation of the anchoring motive (the lipobox- or LPXTG-motive, respectively) in order to (hopefully) prevent anchoring of Ag85B-ESAT6. If cells producing the altered versions of the anchoring motifs appeared unstained, it would be legitimate to conclude that anchoring of heterologous proteins using these anchoring motifs is possible.

All in all, the results of this part of the study still imply that covalent cell membrane or cell wall -anchoring of Ag85B-ESAT6 in *L. plantarum* is likely.

5.4 Effects of the Ag85B-ESAT6 expressing strains on dendritic cells

On the basis of the results of the Western blot analyses and the fluorescent staining of cells, a selection of Ag85B-ESAT producing strains were subjected to co-incubation with dendritic cells isolated from human peripheral blood leucocytes. Dendritic cells (DCs) are an important part of the immune system and are constantly in communication with other cells in the body, often by means of cell-to-cell contacts based on the interactions of cell-surface proteins. This communication can also occur at a distance by the release of cytokines (Stagg et al. 2003; Wan & Dupasquier 2005). Dendritic cells play an active role in the uptake of bacteria across mucosal surfaces and immature DCs are recognised by their capacity to constantly sample the environment for antigenic material. When in contact with an antigen the DC becomes activated and enters a mature state, it then releases cytokines and migrates to a lymph node to function as an antigen presenting cell (Rimoldi et al. 2004). *In vitro* incubations of DCs with *L. plantarum* cells producing various Ag85B-ESAT6 constructs could give information about the immunogenicity of Ag85B-ESAT6 at different cellular locations, based on the various constructs ability to elicit a cytokine response.

ELISA kits detecting the cytokines IL-10 and TNF-alpha were used to evaluate the cytokine profile of DCs co-incubated with *L. plantarum* harbouring various plasmids for production, secretion or anchoring of Ag85B-ESAT6. Figure 4. 22 and Figure 4. 23 shows the results of the analysis of Il-10 and TNF-alpha release by DCs. Unfortunately basal release of TNF-alpha and IL-10 could not be estimated due to an experimental error concerning the negative control

samples (cytokine release by DCs not incubated with lactobacilli). According to a similar experiment performed with DCs from a different donor (data not shown) the basal release was estimated to be approximately 5 pg (per 0.6 million DCs) for IL-10 and TNF-alpha, values that are consistent with previous reports (Hafsi et al. 2004). TNF-alpha plays an important role in protection against *M. tuberculosis* and is involved in eliciting $T_H1$response, thereby stimulating systemic inflammation, as well as acting as a stimulant for the acute phase reaction in response to infection (Su et al. 2010). The ability to elicit a TNF-alpha response is therefore considered important in a vaccine strategy against tuberculosis. Contradictory TNF-alpha also play a major role in the pathology of tuberculosis and is thought to be a major contributor to the formation of granuloma in the lungs (Mootoo et al. 2009), implying that there is a fine balance between beneficial and harmful effects of TNF-alpha in the fight against tuberculosis. It is not possible to predict the TNF-alpha release (or cytokine release in general) by *in vivo* DCs, in response to an antigen. The *in vitro* experiments described in this study simply show that all *L. plantarum* strains were able to stimulate *in vitro* release of TNF-alpha, and the results cannot conclusively demonstrate that the presence of overexpressed Ag85B-ESAT6 plays a role in this response.

In contrast to TNF-alpha, IL-10 acts as a pro-inflammatory cytokine, stimulating a T_H2 response as well as down-regulating T_H1 response by inhibiting the cytokine production of macrophages (Abbas et al. 1994). The release of IL-10 by DCs in response to the recombinant *L. plantarum* cells is shown in Figure 4. 23. All *L. plantarum* strains, including pSIP-EV (not encoding Ag85B-ESAT6) were able to stimulate *in vitro* release of IL-10. The amount of IL-10 detected in the supernatants was generally lower than for TNF-alpha, with the exception of pLp_1261Ag85B-E6 (membrane anchored). The greater ability of pLp_1261Ag85B-E6 to elicit secretion of IL-10 by DCs is not considered beneficial in the context of developing a tuberculosis vaccine, due to the inhibitive effect of IL-10 on the T_H1 response. None the less, the *in vitro* effect of the Ag85B-ESAT6 producing lactobacilli, does not give a complete picture of their immunogenicity in a live host, but merely indicates a potential ability to elicit an immune response. Similar to the TNF-alpha analysis, these results do not conclusively show that the presence of overexpressed Ag85B-ESAT6 plays a role in the DCs response.

The incubation of *L. plantarum* strains harbouring various Ag85B-ESAT6 plasmids with DCs indicates that the bacteria themselves and possibly the Ag85B-ESAT6 antigen presented by

some of the strains are able to elicit a cytokine response in DCs. The presence of Ag85B-ESAT6 on the cell surface of *L. plantarum* after 24 hours of incubation in RPMI-medium confirmed the presence of cell surface antigen on cells treated similarly to the cells co-incubated with DCs. It is important to note that the results of these experiments only indicate that the bacteria are able to induce a reaction by the DCs, and that several factors can affect the outcome of such an experiment. It is a possibility that DC cultures can contain DCs in different stages of development or contaminant cell types. In addition there can also be a variation in functional capacity of DCs differentiated from different starter cell populations (O'Neill & Wilson 2004). In this study the DCs were generated from the peripheral blood of one human donor to minimize the impact of donor specific differences.

5.5 Concluding remarks and future perspectives

This thesis describes the successful production and secretion of a *Mycobacterium tuberculosis* fusion protein, Ag85B-ESAT6, in *L. plantarum* WCFS1using the pSIP-system and homologous signal peptides. Covalent anchoring of Ag85B-ESAT6 to the cell membrane or the cell wall could not be confirmed with certainty, but the Ag85B-ESAT6 fusion protein was detected on the cell surface by fluorescent staining, and several experiments indicate anchoring, especially the cell wall fractionation experiment. The results of the dendritic cell experiments could not conclusively demonstrate that the presence of overexpressed Ag85B-ESAT6 plays a role in the release of cytokines (TNF-alpha or IL-10) by DCs in response to co-incubation with recombinant *L. plantarum* cells.

The use of *L. plantarum*, its signal peptides and its cell wall anchors offers a promising strategy for *in situ* delivery of antigens and therapeutic proteins to the mucosal surfaces. Mucosal delivery of antigens utilising recombinant lactic acid bacteria has been successfully demonstrated in several studies, and strategies relying on cytoplasmic containment, secretion or anchoring of heterologous proteins have all been shown to elicit correct immune responses (Daniel et al. 2011). The optimal localisation of antigens is still debated, but studies indicate that cell surface display of antigens provides a potent immunogenic presentation of antigens to the immune system (Norton et al. 1996; Scavone et al. 2007).

Despite the presence of a large genetic toolbox several challenges remains to be solved in order to genetically engineer the optimal bacterial delivery vehicle for vaccination purposes. The translocation process appears to be the main bottleneck of Ag85B-ESAT6 secretion in *L. plantarum* in this study, and the degradation of Ag85B-ESAT6 seen in the intracellular environment could be an indirect result of the accumulation of intracellular antigen. Secretion of heterologous proteins could in some cases be improved by investigating several signal peptides as demonstrated in this work. Different anchoring strategies should be investigated to find the optimal presentation of an antigen in order to elicit a sufficient immune response at the mucosal surfaces. Targeting dendritic cells by fusing a DC targeting peptide to an antigen produced by a recombinant bacterial vector is another strategy that has been successfully used to increase immunity(Mohamadzadeh et al. 2009). Further work on constitutive production of heterologous proteins in live bacterial vectors by integrating constitutive promoters in gene expression systems, could be beneficial for vaccination purposes, eliminating the need of an inducer compound. Integration of genes for heterologous protein production in the bacterial genome provides another alternative.

The work in this thesis shows that *L. plantarum* WCFS1 is a promising vector for delivery of heterologous proteins for vaccination purposes. The strain showed the ability to secrete a heterologous fusion protein using homologous signal peptides. Anchoring of the antigen to the cell membrane and the cell wall using homologous anchoring domains was likely, and the antigen was detected on the cell surface by staining with a fluorochrome. The *M. tuberculosis* fusion protein, Ag85B-ESAT6 is considered a potent antigen and *L. plantarum* WCFS1 is believed to have an adjuvant effect at the mucosal surfaces, making this a promising combination for vaccination purposes. In addition the ability of *L. plantarum* WCFS1 to persist in the human gastrointestinal tract represents a major advantage for the use of this LAB as a delivery vehicle. The immunogenicity of *L. plantarum* WCFS1 harbouring the various Ag85B-ESAT6 encoding plasmids and their ability to elicit a proper immune response against *M. tuberculosis* will be evaluated in future a mice trial.

6. REFERENCES

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APPENDIX

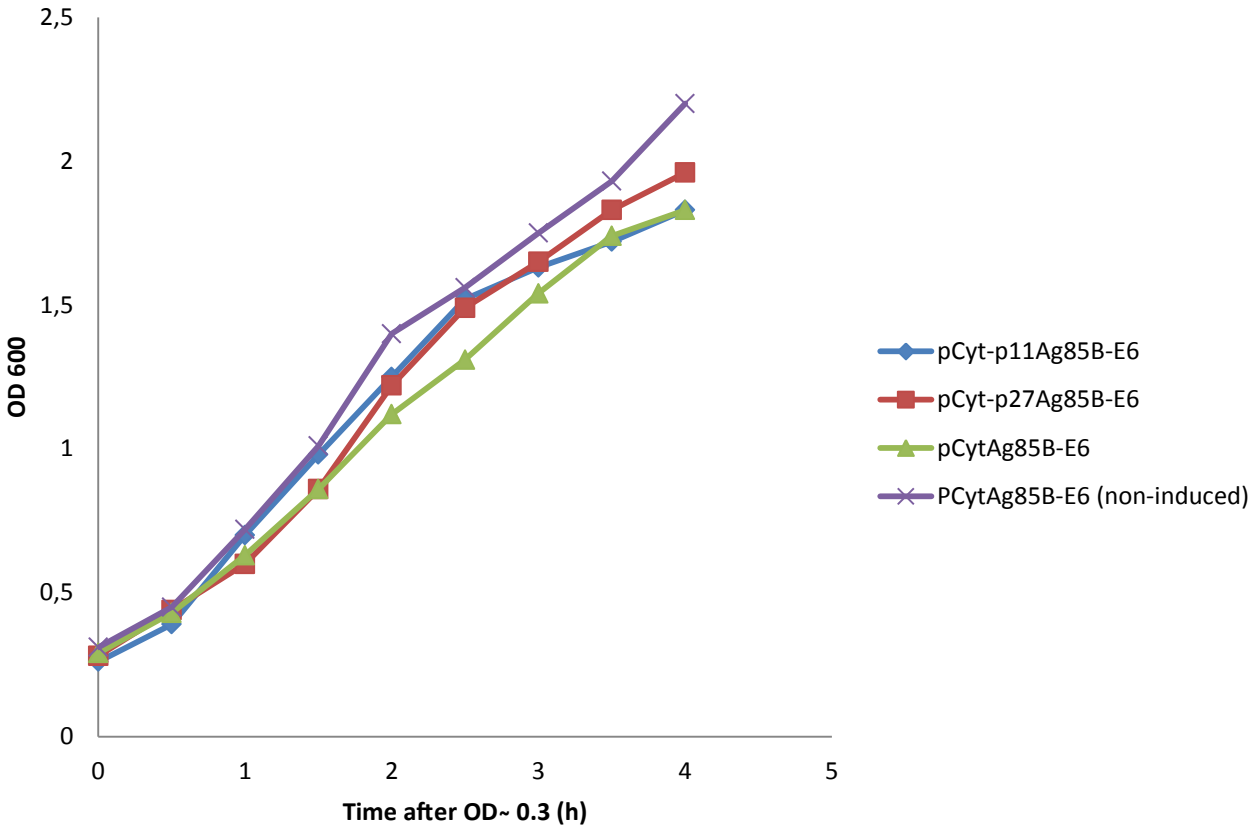

Figure A1: Growth of *L. plantarum* strains harbouring plasmids for induced or constitutive production of Ag85B-ESAT6 at 37ºC. pCytAg85B-E6 was induced with 25 ng/ml SppIP at OD_{600} ~0.30. The OD_{600} was then measured every 30 minutes after induction. The pCyt-p11Ag85B-E6 plasmid was constructed for high-level, and the pCyt-p27Ag85B-E6 plasmid was constructed for lower-level constitutive production of non-secreted Ag85B-ESAT6.

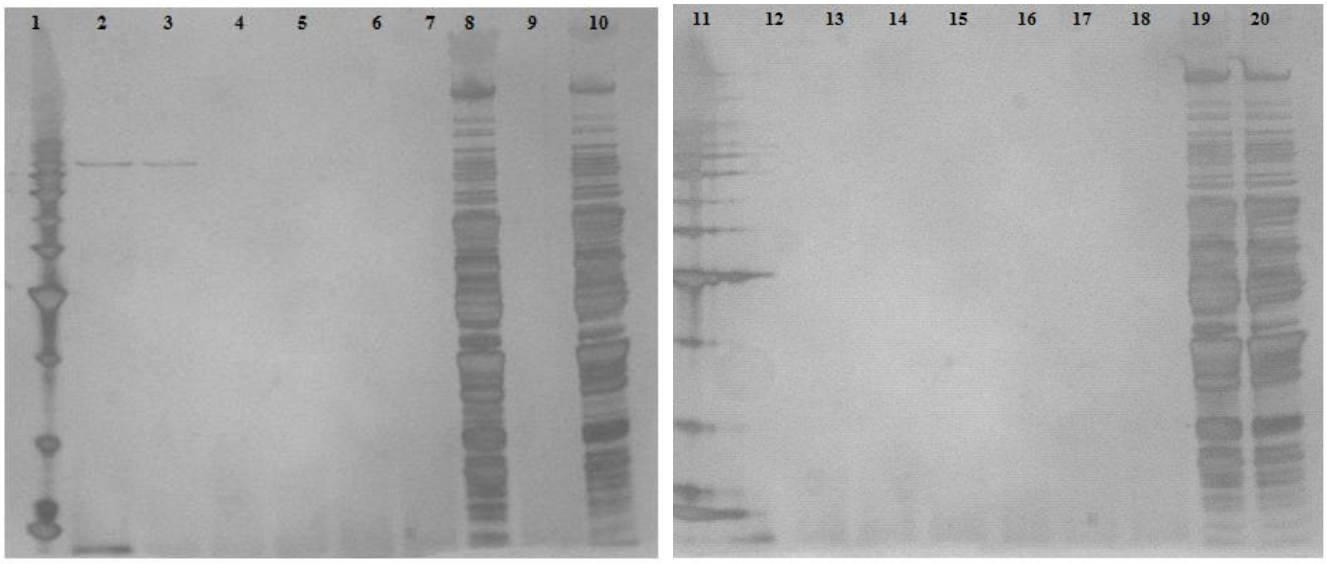

Figure A2: Analysis of supernatants by Silver staining. The gels show the silver staining of proteins in supernatants from induced *L. plantarum* WCFS1 harbouring various plasmids for secretion of Ag85B-ESAT6. Lanes (1) and (11) Benchmarker protein ladder; (8) and (19) cell extract of pLp_0373sNucA; (10) and (19) cell extract of pCytAg85B-E6; (2) pLp_0373sNucA; (3) pCytAg85B-E6; (4) pLp_0373Ag85B-E6; (5) pLp_3189Ag85B-E6; (6) pLp_0297Ag85B-E6; (7) pLp_3117Ag85B-E6; (9) pLp_0600Ag85B-E6; (12) pLp_0373Ag85B-E6; (13) pLp_1448Ag85B-E6; (14) pLp_0469Ag85B-E6; (15) pLp_3676Ag85B-E6; (16) pLp_2174Ag85B-E6; (17) pLp_0141Ag85B-E6: (18) pLp_3050Ag85B-E6. The amount of supernatant loaded to the gel equals that of 0,5 ml cell culture; the amount of cell extract loaded equals 1/3 of the amount of supernatant

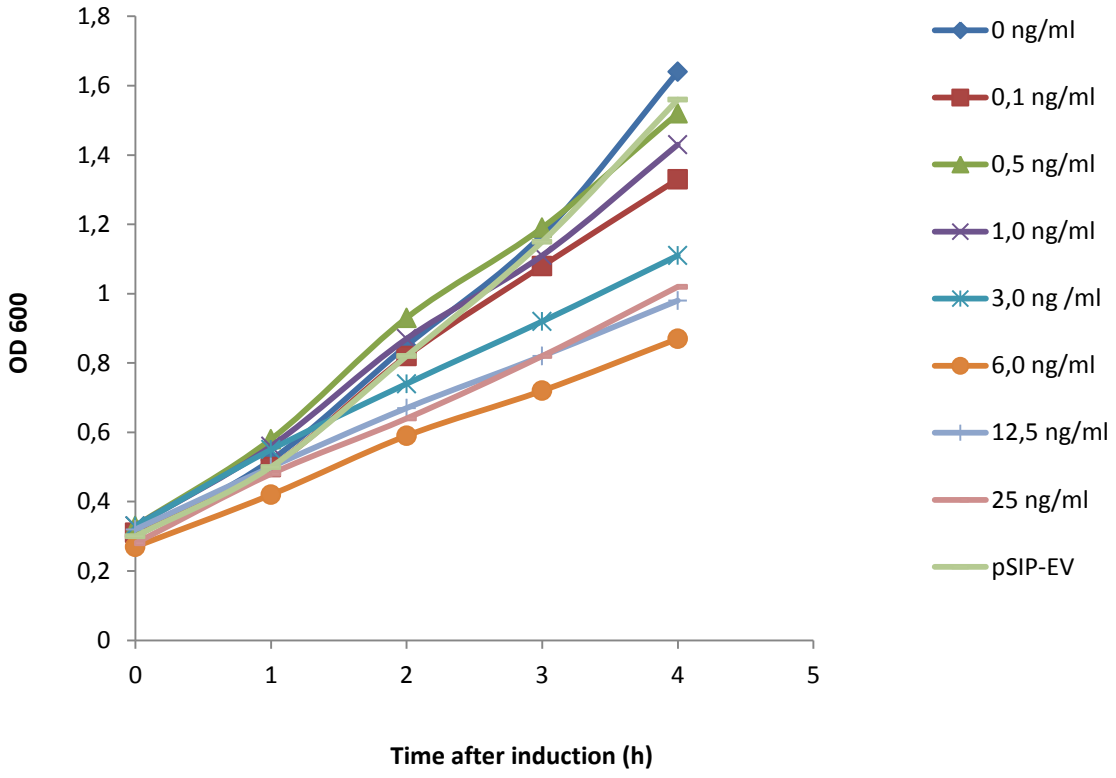

Figure A3: Dosage-response at 30 ºC . Growth of *L. plantarum* WCFS1 harbouring plasmid pLp_3050Ag85B-E6 for secretion of Ag85B-ESAT6 at 30 ºC. Inducer dosages of 0.1 to 25 ng/ml SppIP were used.

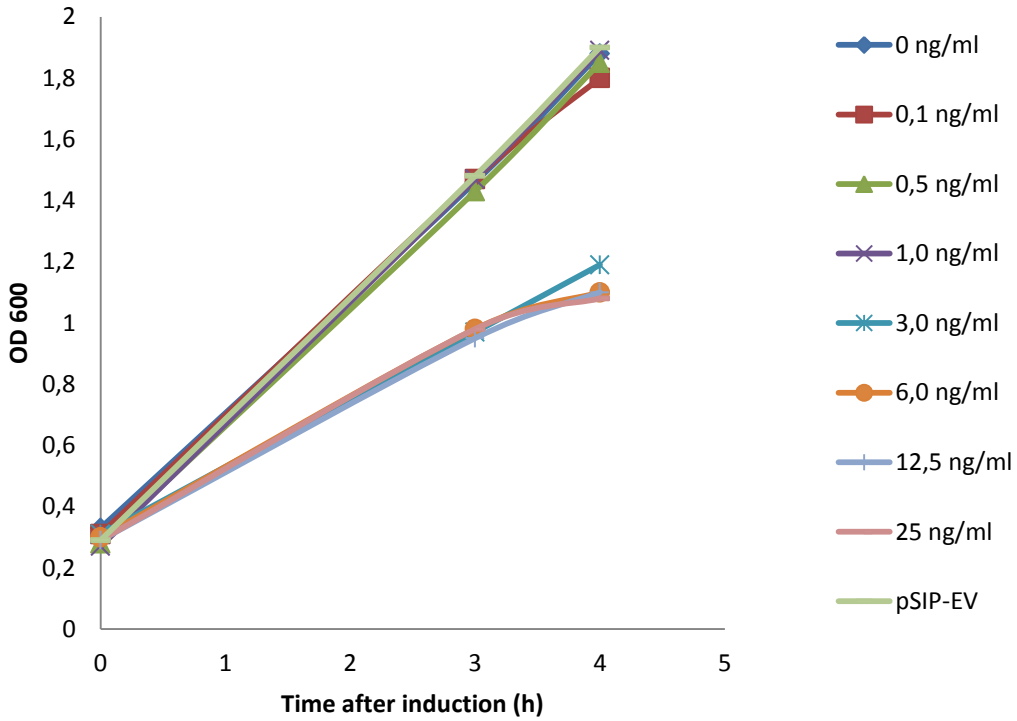

Figure A4: Dosage-response at 37 ºC. Growth of *L. plantarum* WCFS1 harbouring plasmid pLp_3050Ag85B-E6 for production and secretion of Ag85B-ESAT6 at 37 ºC. Inducer dosages of 0.1 to 25 ng/ml SppIP were used.

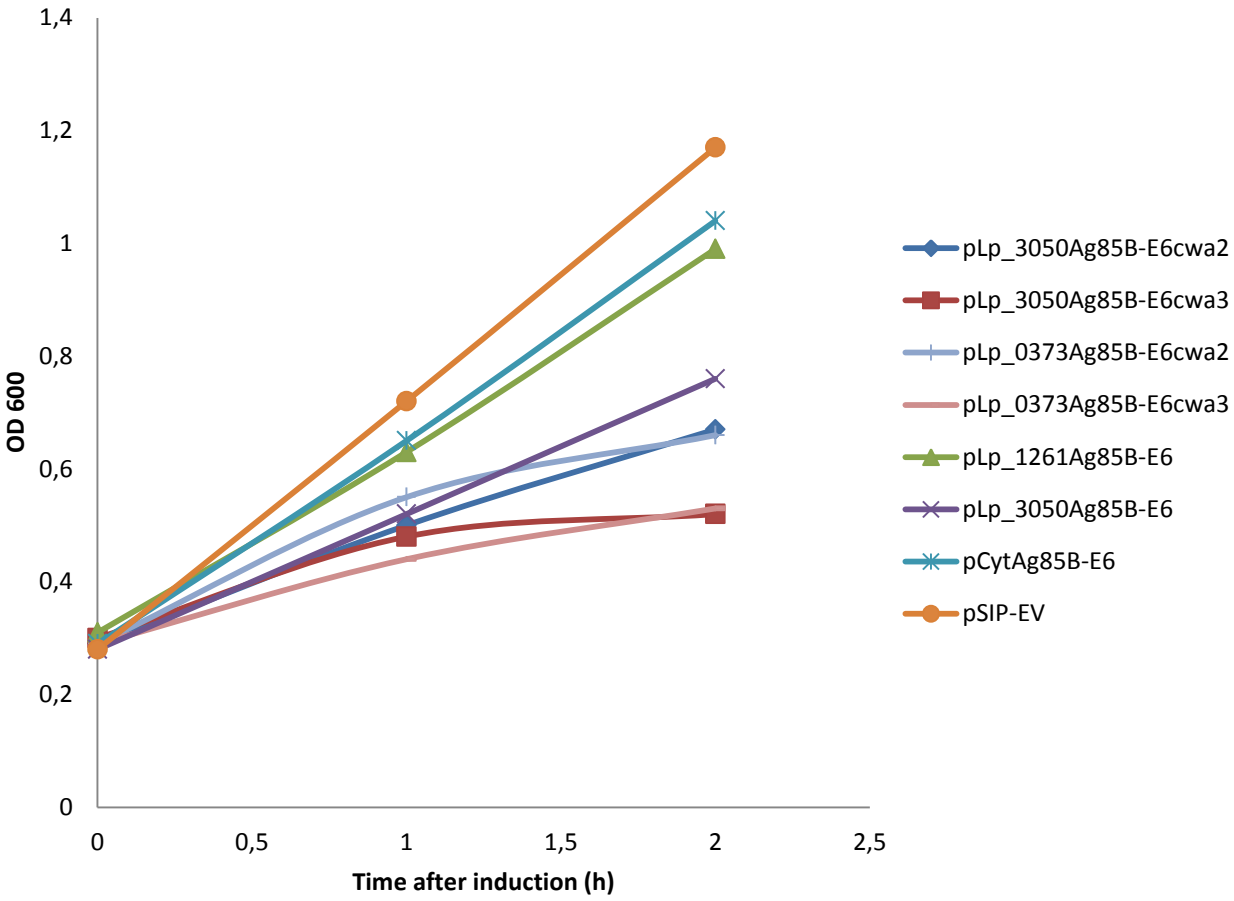

Figure A5: Growth of *L. plantarum* WCFS1 harbouring plasmids for production and anchoring of Ag85B-ESAT6 at 37 ºC. All strains were induced by adding 10 ng/ml SppIP.